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Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA Reductase inhibitory activity

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Abstract

Objective

Bay leaf, one of the plants in Indonesia that has been shown to have activities to reduce cholesterol in the blood. HMG-CoA Reductase inhibition is one of many mechanisms in lowering the level of cholesterol in the blood. Here, we reported the inhibitory activity of HMG-CoA Reductase of bay leaves ethanol extracts that we suspected to be the mechanism of action of bay leaves in reducing cholesterol in the blood. In this research we also investigated the correlation between the inhibitory activities, the total phenol content and antioxidant activities of bay leaves (*Syzygium polianthum*) ethanol extracts.

Methods

The inhibitory activity of HMG-CoA Reductase was determined kinetically at 340 nm using simvastatin as positive control. *In vitro* scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and beta-carotene method were used to determine the antioxidant activities. The total phenolic content was determined by Folin-Ciocalteu's method.

Results

The IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity were 49.50 ± 0.700 µg/mL and 15.50 ± 0.707 µg/mL, respectively, while the IC_{50} of simvastatin was 0.00238 ± 0.00004 µg/mL. The antioxidant activity and total phenolic content of bay leaves ethanolic extract obtained by Soxhlet extraction method was higher compared to the percolation method (DPPH and beta-carotene assay results). The 3D linear analysis showed that there was a high correlation between the inhibition activities of HMG-CoA Reductase pattern of both extract types and the total phenol pattern and also the antioxidant pattern of these extracts.

Conclusion

The result showed that the bay leaves ethanolic extract have a potent activity to reduce the cholesterol serum level by inhibition of HMG-CoA Reductase activity. The activity was due to the phenolic compounds in the extracts as well as the antioxidant activity of the extracts.

Keywords: Biochemistry; Molecular biology; Natural product chemistry

1 Introduction

Cardiovascular disease contributed largely to the high mortality rate worldwide year by year. Based on the research in epidemiology, the risk factor of cardiovascular disease is a combination of two or more risk factors. The risk factors of cardiovascular disease are classified into two groups, which are the modifiable risk factors (dyslipidemia, hypertension, smoking, diabetes mellitus, stress, obesity) and the non-modifiable risk factors (heredity, age, gender). A common risk factor of cardiovascular disease is high serum cholesterol level [1, 2, 3].

Cholesterol is a lipid produced in the liver with a number of important roles, such as a membrane constituent and the parent molecule for steroid hormones [4]. Cholesterol can be synthesized by the body and also can be derived from daily food. The increase of cholesterol level in the bloodstream can cause hypercholesterolemia [1]. Hypercholesterolemia is one of the risk factors for the emergence of atherosclerosis, which is inflammatory disorders in artery walls characterized by the formation of atheroma [5]. Atherosclerosis plaque could clog the heart's blood vessel area. This blockage then leads to cardiovascular disease [6]. The increase in cholesterol level can be caused by excessive cholesterol synthesis, the excess of cholesterol absorption, and high cholesterol intake from daily food. Decreasing the cholesterol level can be done by inhibiting cholesterol synthesis through inhibiting the activity of HMG-CoA Reductase which converts Acetyl-CoA into mevalonate [1, 7]. This enzyme is a pharmacological treatment target for group of drugs called HMG-CoA Reductase inhibitor (statins) [8]. However, anti-cholesterol drugs usually are used in combination, and this may increase the chance of unexpected side effects in long-term use.

Bay leaves (*Syzygium polyanthum*) is one of the plants that can be used to decrease the cholesterol level [9]. Bay leaves contain secondary metabolites, such as saponin, terpenoid, flavonoid, polyphenol, alkaloid, and essential oil. Some previous *in vivo* studies showed that the extract of bay leaves could lower cholesterol levels in the animal blood [10, 11]. It is believed that flavonoid (phenolic compound) as one of the chemical content of the bay leaves plays a role in the decrease of cholesterol levels in the blood. In addition, the research conducted by Lee et al. [12] proved that flavonoids can lower cholesterol levels by inhibiting the action of HMG-CoA Reductase. Several experiment showed that flavonoids and phenolic acids, which are classes of polyphenolic compounds have antioxidant properties, including induction of anti-inflammatory actions, inhibition of oxidative enzymes, and scavenging of free radicals [13].

Based on the researches that have been done to the animals treated with bay leaves, further research about the potency of bay leaves as the anti-hypercholesterolemia *in vitro* is needed with the enzymatic measurement. The extract of bay leaves used was obtained by Soxhlet extraction and percolation method. The measurement of antioxidant activities in each extract was also done to seek the correlation of antioxidant activities and HMG-CoA Reductase inhibition activities. This research covers the taxonomy of Biochemistry and Molecular Biology.

2 Materials and methods

2.1 Equipment and materials

The equipment used during the study were analytical scales (Sartorius, Germany); oven (Binder); infrared moisture balance (Kett, China); 5 µL capillary tubes; microtubes (Mini spin, USA); vortex; micropipettes; blue tips; white tips; membrane filters; glasswares; chamber; soxhlet; water bath; spectrophotometer (Multiscan Go, Thermo Scientific, USA); cuvettes (Bio-Rads Lab, 2000 Alfred Nobel Drive Hercules, Catalog number 9109250).

Dried bay leaves (*Syzygium polyanthum*) obtained from PT. HRL International Indonesia, Pasuruan, East Java, the enzyme used was the HMG-CoA Reductase Assay Kit (Catalog number CS 1090, Sigma, Germany), 96% ethanol, phytochemical screening reagents, water for injection, sodium hydrogen phosphate (NaH_2PO_4) (Merck, Indonesia), sodium dihydrogen phosphate (Na_2HPO_4) (Merck, Indonesia), simvastatin tablet, antioxidant assay reagents.

2.2 Preparation of extract

Standardization was done to the dried bay leaves prior to the extraction. The extraction was done with percolation and Soxhlet extraction method using ethanol 96% as the solvent. The mass of the dried bay leaves used for percolation method was 1 kg in total 3.6 liter of solvent, while the mass used for Soxhlet extraction was 0.5 kg in total 3.03 liter of solvent divided in several steps, which was 20 gram of dried bay leaves in 120 ml solvent for each process. The rendemen of extract obtained from percolation method was 25.05%, while from soxhlet extraction method was 23.62%.

The extract was then evaporated on a water bath then was stored in a sterile bottle. The dried extract was further standardized to determine the organoleptic characteristics, total ash content, water content, and the solubility in ethanol to ensure the quality. Phytochemical screening was also done to the dried extract prior to antioxidant and enzymatic assay.

2.3 HMG-CoA Reductase activity assay

366 µl 1x assay buffer was mixed with 24 µl HMG-CoA substrate, 8 µl NADPH, and 2 µl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes [14].

125 mg of bay leaves ethanol extract was dissolved in 25 ml of sterile water to make the standard solution 5000 ppm. The solution was further made into different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. The solution was centrifuged and filtered using a 0.45 µm filter membrane to remove the residual sediment from the extract. 364 µl 1x assay buffer was mixed with 24 µl HMG-CoA substrate, 8 µl NADPH, 2 µl extract from each concentration and 2 µl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

Standard solution of simvastatin was taken 2 µl from each concentration 0 ppm, 0.0010 ppm, 0.0014 ppm, 0.0018 ppm, 0.0022 and 0.0026 ppm. Each 2 µl solution was mixed with 364 µl 1x assay buffer, 24 µl HMG-CoA substrate, 8 µl NADPH and 2 µl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

The data from spectrophotometric was analyzed to determine the enzyme activity (Sigma-Aldrich, 2013), using this equation:

$$\text{Specific activity} = \frac{(\Delta A\,(\text{sample}/\,\text{min}) \times \text{TV})}{12.44 \times \text{V}\ \ \text{enzyme} \times 0.6 \times \text{LP}}$$

where ΔA: Change of absorbance, TV: Total volume of the reaction in ml, 12.44: coefficient of NADPH, V enzyme: volume of enzyme used in the assay, 0.6: Enzyme concentration in mg-protein, LP: Lightpath in cm.

2.4 Statistical analysis

All test scores were presented as mean values of inhibition ±standard deviation from two replications. The percent of inhibition was obtained from the activity without inhibitor minus activity with inhibitor divided by activity without inhibitor. For statistical data analysis, each group was compared using independent sample T-test with 95% level of confidence.

2.5 Antioxidant assays

Antioxidant activities of the extracts were assayed by three different methods, which were the DPPH method, the FRAP method, and beta-carotene method. The DPPH method states the antioxidant activity as the oxidation inhibition by referring to Chandra and Dave [15] and Shafazila et al. [16]. The antioxidant potency was measured using % Scavenging effect. The antioxidant assay using FRAP reagent refers to Benzie and Strain [17] where the antioxidant capacity stated as µmoles Trolox/g dry powder. The beta-carotene assay was done according to Utami et al. [18]. The antioxidant potency of the sample was expressed as the concentration with exhibit 50% of the antioxidant activity (EC_{50}).

2.6 Total phenolic content

Extract solution of bay leaves was prepared in different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. Each solution of bay leaves extract was pipetted 100 µl and was mixed with 300 µl of 2% sodium carbonate, 1.58 ml of deionized water, and 100 µl of 10% Folin-Ciocalteu reagent. The absorbance of the reaction mixture was observed at 750 nm (Multiscan Go, Thermo Scientific, USA) after 30 min incubation at room temperature. Gallic acid was used as a standard [19]. The data were expressed as ppm gallic acid equivalents.

3 Results & discussion

Choosing the right extraction method is one of the supporting factors in the success of a therapy, including lowering cholesterol level in the blood. This can be caused by the solubility of secondary metabolites in plants depending on the type of solvent and temperature used during extraction. From the phytochemical screening results, both bay leaves ethanol extract (percolation method and soxhletation method) contain alkaloid, flavonoid, saponin, tannin, steroid.

The results of inhibition potency and IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity can be seen in Tables 1 and 2. Simvastatin, the first generation of statins, was used as a reference compound in this research. The inhibition potency of simvastatin toward HMG-CoA Reductase enzyme is shown in Fig. 1. The IC_{50} value of simvastatin measured in this study was 0.00238 ± 0.00004 µg/mL, which is smaller than the values found in the former researches which were about 0.00376–0.00778 µg/mL [7, 20, 21]. These values (49.50 ± 0.700 µg/mL for extract obtained by percolation, and 15.50 ± 0.707 µg/mL for extract obtained by Soxhlet extraction) were significantly different ($p > 0.05$) if compared to the IC_{50} of simvastatin. The potency of ethanolic extract of bay leaves in inhibiting HMG-CoA Reductase is smaller when compared with simvastatin, where the ability of simvastatin in inhibiting HMG-CoA Reductase about six thousand to twenty thousand times greater than the ethanolic extract of bay leaves.

Table 1 The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by percolation method.

alt-text: Table 1

Concentration (µg/ml)	% of Inhibition		Mean	SD	IC50 (µg/ml)
	n1	n2			
0	0	0	0	0	n1 = 50.00

10	28.49	21.03	24.760	5.275	n2 = 49.00
25	47.10	42.59	44.845	3.189	
50	57.10	57.03	57.065	0.049	
150	64.40	67.02	65.710	1.853	
300	66.24	74.66	70.450	5.954	
600	82.24	83.28	82.760	0.735	
Mean ± SD = 49.50 ± 0.700					

Table 2 The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by Soxhlet method.

alt-text: Table 2

Concentration (µg/ml)	% of Inhibition		Mean	SD	IC50 (µg/ml)
	n1	n2			
0	0	0	0	0.000	n1 = 15.00
10	47.17	48.55	47.860	0.976	n2 = 16.00
25	54.72	56.16	55.440	1.018	
50	69.81	66.67	68.240	2.220	
150	79.25	76.09	77.670	2.234	
300	88.68	84.42	86.550	3.012	
600	101.9	97.10	99.500	3.394	
Mean ± SD = 15.50 ± 0.707					

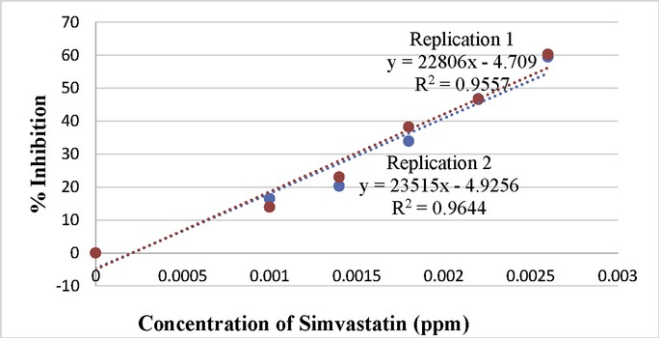

Fig. 1 Graphic of HMG-CoA Reductase inhibition by simvastatin.

alt-text: Fig. 1

Several other reports have also reported the potency of plant extracts in HMG-CoA Reductase inhibition. *Opuntia ficus-indica* (L) Miller extract was reported by Ressaissi et al. [22] to have IC_{50} 20.3 µg/ml and said as to have moderate potency. Ademosun et al. [23] reported that grapefruit peels had an IC_{50} on HMG-CoA Reductase activity 0.11 µg/ml. *Vernonia condensata* extract showed the IC_{50} value of 271.7 µg/ml [24] and *Gnetum gnemon* extract had an IC_{50} value on HMG-CoA Reductase of 400 µg/ml [25]. There are also studies that have assayed the potency of several isolated chemical contents of the plants in HMG-CoA Reductase inhibition, and it was reported that the compounds inhibit the enzyme activity with the IC_{50} value 8.34–149.6 µg/ml [22, 26]. Based on these several studies it can be stated that certain plant extract is said to have HMG-CoA Reductase inhibition potency in the range value of IC_{50}

between 0.1 to 400 µg/ml [22, 23, 25, 27]. Thus, the ethanol extracts of bay leaves are also a potent HMG-CoA Reductase inhibitor.

The potency of ethanol extract of bay leaves obtained by Soxhlet extraction is three times higher than the potency of ethanol extract of bay leaves obtained by percolation. This showed that the Soxhlet process was able to extract more active constituent that responsible for the inhibition of HMG-CoA Reductase and that the active constituents are stable under heating. It is suspected that these active constituents are polyphenolic compounds such as gallic acid, eugenol, kaempferol and quercetin [28]. Some studies have shown that polyphenolic compounds (luteolin, quercetin, and isorhamnetin) contained in many plant extracts play a role in inhibiting HMG-CoA Reductase activity [22, 27]. The phenolic compound of grapefruit peels (genistein and daidzein) showed inhibition of HMG-CoA Reductase activity competitively against HMG-CoA as substrate [23]. Flavonoids, in specific, are stated by Lee et al. [12] to have the ability to inhibit the activity of the HMG-CoA Reductase. The research conducted by Anggraeni [29] which states that at the same concentration (10 µg/ml) quercetin and rutin are able to inhibit the activity of HMG-CoA Reductase respectively 41.10% and 60.17 % also support this hypothesis. However, other studies have not mentioned the inhibition kinetics of other flavonoid groups.

The hypothesis that the inhibition of HMG-CoA Reductase in ethanol extract of bay leaves was due to the polyphenolic content was proved by searching the correlation between the inhibition activity and the total phenolic content in the extract. Besides that, we also measured the antioxidant activity of each extract to study the correlation of it to inhibition activity and types of extract. The total phenolic content and antioxidant activity of each concentration involved in the measurement of HMG-CoA Reductase inhibition activity were reported in Tables 3 and 4. The total phenol in the soxhlet extract is greater than the total phenol in the percolation extract, which in accordance with the inhibition of HMG-CoA Reductase activity pattern. The antioxidant activity of each extract, measured by DPPH, FRAP and beta-carotene method, was compared to gallic acid and quercetin (Table 5). The DPPH and beta-carotene method gave the same pattern results, which showed that the antioxidant activity of Soxhlet extract was higher when compared to the percolation extract. These results also in line with the inhibition of HMG-CoA Reductase activity pattern. The FRAP method in the other way gave a different result, which showed that the antioxidant activity of the percolation method is higher than that of the Soxhlet method. This could be caused by the difference in the mechanism of the assay. FRAP method assay was based on the reduction of ferric ion to ferrous ion. Not all of the Fe^{3+} reductants are antioxidant, and some antioxidants are not able to reduce Fe^{3+} [30].

Table 3 Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by percolation method.

alt-text: Table 3

Concentration (µg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	1.9960A	0A	0.0000A
10	53.6B	3.5532B	0A	10.2513B
25	56.0B	3.8627B	0A	8.0186C
50	61.4C	5.1647C	0.9625A	13.1217D
150	99.0D	8.5790D	8.3633B	7.9707E
300	150.4E	24.9729E	21.3933C	12.5075F
600	193.7F	43.3887F	22.2472C	21.2928G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.

Values with the same letter are not significantly different ($P < 0.05$).

[a]FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4. (Please change the position of this sentence with the sentence of 'b' (the next sentence).)

[b]IC_{50} was the concentration of substance that provides 50% inhibition. (Please change the position of this sentence with the sentence of "a" (the previous sentence))

[c]EC_{50} represents the effective concentration at 50% of total antioxidant activity.

Table 4 Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by Soxhlet method.

alt-text: Table 4

Concentration (µg/ml)	Total phenol content (ppm)	Antioxidant activity		

		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	2.2224A	0A	0A
10	35.4B	4.1808B	0A	14.9736B
25	90.8C	5.1574C	0A	15.2237C
50	92.4C	10.0685D	0A	18.4625D
150	139.0D	20.1246E	0A	20.6429E
300	187.9E	46.5714F	4.2877B	27.6990F
600	201.8F	66.9863G	19.1348C	29.0379G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.

Values with the same letter are not significantly different (P < 0.05).

[a] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.(Please change the position of this sentence with the sentence of 'b' (the next sentence). Thank you)

[b] IC_{50} was the concentration of substance that provides 50% inhibition.(Please change the position of this sentence with the sentence of 'a' (the previous sentence). Thank you.)

[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

Table 5 Antioxidant activity value of ethanol extract of bay leaves obtained by Soxhlet Method.

alt-text: Table 5

Samples	Antioxidant activity		
	DPPH method (IC_{50} – ppm)[a]	FRAP method (FRAP value – ppm)[b]	Beta-Carotene method (EC_{50} – ppm)[c]
Gallic Acid	23.87 ± 0.00A	10.60 ± 0.01A	24.87 ± 0.24A
Quercetin	48.87 ± 0.00B	21.94 ± 0.00B	98.44 ± 0.39B
Bay leaves ethanolic extract - percolation	888.08 ± 0.05C	295.00 ± 0.02C	2965.62 ± 0.65C
Bay leaves ethanolic extract - soxhlet	437.89 ± 0.03D	684.00 ± 0.03D	2230.35 ± 1.20D

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.

Values with the same letter are not significantly different (P < 0.05).

[a] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4(Please change the position of this sentence with the sentence of 'b' (the next sentence). Thank you.).

[b] IC_{50} was the concentration of substance that provides 50% inhibition.(Please change the position of this sentence with the sentence of 'a' (the previous sentence). Thank you.)

[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

The correlation analysis between each factor in this research was done by 3D linear analysis using SigmaPlot 12.5. The results of the analysis were shown in Tables 6, 7, and 8. Table 6 showed the correlation between extraction method (expressed in concentration, x-axis) and total phenolic content (y-axis) towards antioxidant activity. The level of correlation was shown by the R^2 value. The results showed that there is a high correlation between the extraction method and total phenolic content towards antioxidant activity. The higher to total phenolic content in both extracts will cause the increase in the antioxidant activity.

Table 6 Correlation between extraction method and total phenolic content towards antioxidant activity.

alt-text: Table 6

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 0.8310 + 0.445x + 0.0742y	0.9890
	FRAP	f = 0.7649 + 0.0419x+0.0196y	0.9663
	Beta – Carotene Bleaching	f = 3.7012 + 0.0068x + 0.0652y	0.8511
Soxhlet	DPPH	f = 5.2176 + 0.0288x + 0.20083y	0.9137
	FRAP	f = 1.2690 + 0.0465x−0.0501y	0.9949
	Beta – Carotene Bleaching	f = 5.1409 + 0.0032x + 0.1196y	0.9156

Table 7 Correlation between extraction method and total phenolic content towards percent of HMG-CoA Reductase inhibition.

alt-text: Table 7

Extraction method	Function	R^2
Percolation	f = 3.9241−0.0955x + 0.6945y	0.8688
Soxhlet	f = 15.4733−0.0299x + 0.4829y	0.8871

Table 8 Correlation between extraction method and antioxidant activity towards percent of HMG-CoA Reductase inhibition.

alt-text: Table 8

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 38.8052−0.3180x−3.1319y	0.6154
	FRAP	f = 32.6035 + 0.0486x + 1.1740y	0.6006
	Beta – Carotene Bleaching	f = 15.5054 + 0.0362x + 2.6778y	0.7075
Soxhlet	DPPH	f = 43.3496 + 0.2689x−2.2742y	0.5670
	FRAP	f = 43.5523 + 0.0533x + 1.3197y	0.5750
	Beta – Carotene Bleaching	f = 1.4981−0.0057x + 3.4218y	0.9759

Table 7 showed the correlation between extraction method (concentration, x-axis) and total phenolic content (y-axis) towards percent of HMG-CoA Reductase inhibition. There was also a strong correlation between each factor towards the inhibition of HMG-CoA Reductase activity, but the concentration of extract gave a different effect against the inhibition of HMG-CoA Reductase activity when compared to the total phenolic content. It can be explained that the increase of the concentration of extract will cause the increase also in the total phenolic content, but not all of the phenolic compounds in the extract act as an inhibitor of HMG-CoA Reductase. Thus, some of the phenolic compounds in the extract may act as an activator of the HMG-CoA Reductase.

Correlation between extraction method (concentration, x-axis) and antioxidant activity (y-axis) towards percent of HMG-CoA Reductase inhibition was shown in Table 8. The results of the 3D linear analysis showed a poor correlation between the concentration of extract and antioxidant activity towards the inhibition of HMG-CoA Reductase activity. Thus, though the HMG-CoA Reductase catalyze the reduction-oxidation activity, its inhibition mechanism was not related to the antioxidant mechanism. We conclude that antioxidant compounds might be contributes to inhibit HMG-CoA Reductase but does not go through in the reduction-oxidation mechanisms.

Based on these results, it can be concluded that the inhibition of HMG-CoA Reductase activity by the percolation and soxhlet extracts are caused by the phenolic compounds in the extracts, and it was suspected due to the flavonoids compounds. Further research needs to be done to confirm this report. The relationship between the flavonoid structure (Fig. 2B) with its activity as an enzyme inhibitor of HMG-CoA Reductase is due to the presence of -OH groups in C3 ′, C4′, and C5. It is also caused by the C=O group at C4. These groups play a role in forming hydrogen bonds with amino acids from HMG-CoA Reductase through hydrophobic interaction [26]. It is suspected that these groups play a role in their activity inhibiting the HMG-CoA Reductase enzyme because they have similarities in the pharmacophores group of the simvastatin. In the simvastatin structure (Fig. 2A) there is an -OH group and a C=O

group (a pharmacophore group) that will form a bond with the enzyme, so that the enzyme work becomes inhibited. The C=O group in lactone ring of simvastatin will be hydrolyzed to become an active form (acid). The hydrolyzed simvastatin will then bind to the HMG-CoA Reductase by hydrogen bonding with the amino acids located on the active site of the enzyme. The structure of the hydrolyzed simvastatin in the lactone ring corresponds to the structure of the HMG-CoA substrate (Fig. 2C) so that the enzyme is able to bind with simvastatin and form the complex of enzymes.

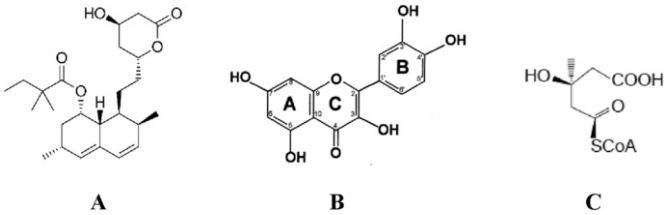

Fig. 2 Structure of simvastatin (A), flavonoid (B), and HMG-CoA (C) [31, 32, 33].

alt-text: Fig. 2

Declarations

Author contribution statement

Lanny Hartanti, Sumi Wijaya: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu: Performed the experiments; Wrote the paper.

Henry Kurnia Setiawan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Lisa Soegianto: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the Ministry of Research and Technology Higher Educati (Please change the term into: "the Ministry of Research, Technology and Higher Education of the Republic of Indonesia")on Republic of Indonesia.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Heliyon

Received:
18 October 2018
Revised:
16 March 2019
Accepted:
3 April 2019

Cite as: Lanny Hartanti, Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu, Sumi Wijaya, Henry Kurnia Setiawan, Lisa Soegianto. Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA Reductase inhibitory activity.
Heliyon 4 (2019) e01485.
https://doi.org/10.1016/j.heliyon.2019.e01485

Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA Reductase inhibitory activity

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Abstract

Objective

Bay leaf, one of the plants in Indonesia that has been shown to have activities to reduce cholesterol in the blood. HMG-CoA Reductase inhibition is one of many mechanisms in lowering the level of cholesterol in the blood. Here, we reported the inhibitory activity of HMG-CoA Reductase of bay leaves ethanol extracts that we suspected to be the mechanism of action of bay leaves in reducing cholesterol in the blood. In this research we also investigated the correlation between the inhibitory activities, the total phenol content and antioxidant activities of bay leaves (*Syzygium polianthum*) ethanol extracts.

Methods

The inhibitory activity of HMG-CoA Reductase was determined kinetically at 340 nm using simvastatin as positive control. *In vitro* scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP),

https://doi.org/10.1016/j.heliyon.2019.e01485

and beta-carotene method were used to determine the antioxidant activities. The total phenolic content was determined by Folin-Ciocalteu's method.

Results

The IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity were 49.50 ± 0.700 μg/mL and 15.50 ± 0.707 μg/mL, respectively, while the IC_{50} of simvastatin was 0.00238 ± 0.00004 μg/mL. The antioxidant activity and total phenolic content of bay leaves ethanolic extract obtained by Soxhlet extraction method was higher compared to the percolation method (DPPH and beta-carotene assay results). The 3D linear analysis showed that there was a high correlation between the inhibition activities of HMG-CoA Reductase pattern of both extract types and the total phenol pattern and also the antioxidant pattern of these extracts.

Conclusion

The result showed that the bay leaves ethanolic extract have a potent activity to reduce the cholesterol serum level by inhibition of HMG-CoA Reductase activity. The activity was due to the phenolic compounds in the extracts as well as the antioxidant activity of the extracts.

Keywords: Biochemistry; Molecular biology; Natural product chemistry

1. Introduction

Cardiovascular disease contributed largely to the high mortality rate worldwide year by year. Based on the research in epidemiology, the risk factor of cardiovascular disease is a combination of two or more risk factors. The risk factors of cardiovascular disease are classified into two groups, which are the modifiable risk factors (dyslipidemia, hypertension, smoking, diabetes mellitus, stress, obesity) and the non-modifiable risk factors (heredity, age, gender). A common risk factor of cardiovascular disease is high serum cholesterol level [1, 2, 3].

Cholesterol is a lipid produced in the liver with a number of important roles, such as a membrane constituent and the parent molecule for steroid hormones [4]. Cholesterol can be synthesized by the body and also can be derived from daily food. The increase of cholesterol level in the bloodstream can cause hypercholesterolemia [1]. Hypercholesterolemia is one of the risk factors for the emergence of atherosclerosis, which is inflammatory disorders in artery walls characterized by the formation of atheroma [5]. Atherosclerosis plaque could clog the heart's blood vessel area. This blockage then leads to cardiovascular disease [6]. The increase in cholesterol level can be caused by excessive cholesterol synthesis, the excess of cholesterol absorption, and high cholesterol intake from daily food. Decreasing the cholesterol level can be done by inhibiting cholesterol synthesis through inhibiting

https://doi.org/10.1016/j.heliyon.2019.e01485

the activity of HMG-CoA Reductase which converts Acetyl-CoA into mevalonate [1, 7]. This enzyme is a pharmacological treatment target for group of drugs called HMG-CoA Reductase inhibitor (statins) [8]. However, anti-cholesterol drugs usually are used in combination, and this may increase the chance of unexpected side effects in long-term use.

Bay leaves (*Syzygium polyanthum*) is one of the plants that can be used to decrease the cholesterol level [9]. Bay leaves contain secondary metabolites, such as saponin, terpenoid, flavonoid, polyphenol, alkaloid, and essential oil. Some previous *in vivo* studies showed that the extract of bay leaves could lower cholesterol levels in the animal blood [10, 11]. It is believed that flavonoid (phenolic compound) as one of the chemical content of the bay leaves plays a role in the decrease of cholesterol levels in the blood. In addition, the research conducted by Lee et al. [12] proved that flavonoids can lower cholesterol levels by inhibiting the action of HMG-CoA Reductase. Several experiment showed that flavonoids and phenolic acids, which are classes of polyphenolic compounds have antioxidant properties, including induction of anti-inflammatory actions, inhibition of oxidative enzymes, and scavenging of free radicals [13].

Based on the researches that have been done to the animals treated with bay leaves, further research about the potency of bay leaves as the anti-hypercholesterolemia *in vitro* is needed with the enzymatic measurement. The extract of bay leaves used was obtained by Soxhlet extraction and percolation method. The measurement of antioxidant activities in each extract was also done to seek the correlation of antioxidant activities and HMG-CoA Reductase inhibition activities. This research covers the taxonomy of Biochemistry and Molecular Biology.

2. Materials and methods

2.1. Equipment and materials

The equipment used during the study were analytical scales (Sartorius, Germany); oven (Binder); infrared moisture balance (Kett, China); 5 μL capillary tubes; microtubes (Mini spin, USA); vortex; micropipettes; blue tips; white tips; membrane filters; glasswares; chamber; soxhlet; water bath; spectrophotometer (Multiscan Go, Thermo Scientific, USA); cuvettes (Bio-Rads Lab, 2000 Alfred Nobel Drive Hercules, Catalog number 9109250).

Dried bay leaves (*Syzygium polyanthum*) obtained from PT. HRL International Indonesia, Pasuruan, East Java, the enzyme used was the HMG-CoA Reductase Assay Kit (Catalog number CS 1090, Sigma, Germany), 96% ethanol, phytochemical screening reagents, water for injection, sodium hydrogen phosphate (NaH_2PO_4)

https://doi.org/10.1016/j.heliyon.2019.e01485

(Merck, Indonesia), sodium dihydrogen phosphate (Na_2HPO_4) (Merck, Indonesia), simvastatin tablet, antioxidant assay reagents.

2.2. Preparation of extract

Standardization was done to the dried bay leaves prior to the extraction. The extraction was done with percolation and Soxhlet extraction method using ethanol 96% as the solvent. The mass of the dried bay leaves used for percolation method was 1 kg in total 3.6 liter of solvent, while the mass used for Soxhlet extraction was 0.5 kg in total 3.03 liter of solvent divided in several steps, which was 20 gram of dried bay leaves in 120 ml solvent for each process. The rendemen of extract obtained from percolation method was 25.05%, while from soxhlet extraction method was 23.62%.

The extract was then evaporated on a water bath then was stored in a sterile bottle. The dried extract was further standardized to determine the organoleptic characteristics, total ash content, water content, and the solubility in ethanol to ensure the quality. Phytochemical screening was also done to the dried extract prior to antioxidant and enzymatic assay.

2.3. HMG-CoA Reductase activity assay

366 μl 1x assay buffer was mixed with 24 μl HMG-CoA substrate, 8 μl NADPH, and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes [14].

125 mg of bay leaves ethanol extract was dissolved in 25 ml of sterile water to make the standard solution 5000 ppm. The solution was further made into different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. The solution was centrifuged and filtered using a 0.45 μm filter membrane to remove the residual sediment from the extract. 364 μl 1x assay buffer was mixed with 24 μl HMG-CoA substrate, 8 μl NADPH, 2 μl extract from each concentration and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

Standard solution of simvastatin was taken 2 μl from each concentration 0 ppm, 0.0010 ppm, 0.0014 ppm, 0.0018 ppm, 0.0022 and 0.0026 ppm. Each 2 μl solution was mixed with 364 μl 1x assay buffer, 24 μl HMG-CoA substrate, 8 μl NADPH and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

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The data from spectrophotometric was analyzed to determine the enzyme activity (Sigma-Aldrich, 2013), using this equation:

$$\text{Specific activity} = \frac{(\Delta A\,(\text{sample}/\,\text{min}) \times \text{TV})}{12.44 \times \text{V}\ \ \text{enzyme} \times 0.6 \times \text{LP}}$$

where ΔA: Change of absorbance, TV: Total volume of the reaction in ml, 12.44: coefficient of NADPH, V enzyme: volume of enzyme used in the assay, 0.6: Enzyme concentration in mg-protein, LP: Lightpath in cm.

2.4. Statistical analysis

All test scores were presented as mean values of inhibition ±standard deviation from two replications. The percent of inhibition was obtained from the activity without inhibitor minus activity with inhibitor divided by activity without inhibitor. For statistical data analysis, each group was compared using independent sample T-test with 95% level of confidence.

2.5. Antioxidant assays

Antioxidant activities of the extracts were assayed by three different methods, which were the DPPH method, the FRAP method, and beta-carotene method. The DPPH method states the antioxidant activity as the oxidation inhibition by referring to Chandra and Dave [15] and Shafazila et al. [16]. The antioxidant potency was measured using % Scavenging effect. The antioxidant assay using FRAP reagent refers to Benzie and Strain [17] where the antioxidant capacity stated as μmoles Trolox/g dry powder. The beta-carotene assay was done according to Utami et al. [18]. The antioxidant potency of the sample was expressed as the concentration with exhibit 50% of the antioxidant activity (EC_{50}).

2.6. Total phenolic content

Extract solution of bay leaves was prepared in different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. Each solution of bay leaves extract was pipetted 100 μl and was mixed with 300 μl of 2% sodium carbonate, 1.58 ml of deionized water, and 100 μl of 10% Folin-Ciocalteu reagent. The absorbance of the reaction mixture was observed at 750 nm (Multiscan Go, Thermo Scientific, USA) after 30 min incubation at room temperature. Gallic acid was used as a standard [19]. The data were expressed as ppm gallic acid equivalents.

https://doi.org/10.1016/j.heliyon.2019.e01485

3. Results & discussion

Choosing the right extraction method is one of the supporting factors in the success of a therapy, including lowering cholesterol level in the blood. This can be caused by the solubility of secondary metabolites in plants depending on the type of solvent and temperature used during extraction. From the phytochemical screening results, both bay leaves ethanol extract (percolation method and soxhletation method) contain alkaloid, flavonoid, saponin, tannin, steroid.

The results of inhibition potency and IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity can be seen in Tables 1 and 2. Simvastatin, the first generation of statins, was used as a reference compound in this research. The inhibition potency

Table 1 The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by percolation method.

Concentration (μg/ml)	% of Inhibition		Mean	SD	IC50 (μg/ml)
	n1	n2			
0	0	0	0	0	n1 = 50.00
10	28.49	21.03	24.760	5.275	n2 = 49.00
25	47.10	42.59	44.845	3.189	
50	57.10	57.03	57.065	0.049	
150	64.40	67.02	65.710	1.853	
300	66.24	74.66	70.450	5.954	
600	82.24	83.28	82.760	0.735	
Mean ± SD = 49.50 ± 0.700					

Table 2 The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by Soxhlet method.

Concentration (μg/ml)	% of Inhibition		Mean	SD	IC50 (μg/ml)
	n1	n2			
0	0	0	0	0.000	n1 = 15.00
10	47.17	48.55	47.860	0.976	n2 = 16.00
25	54.72	56.16	55.440	1.018	
50	69.81	66.67	68.240	2.220	
150	79.25	76.09	77.670	2.234	
300	88.68	84.42	86.550	3.012	
600	101.9	97.10	99.500	3.394	
Mean ± SD = 15.50 ± 0.707					

https://doi.org/10.1016/j.heliyon.2019.e01485

of simvastatin toward HMG-CoA Reductase enzyme is shown in Fig. 1. The IC_{50} value of simvastatin measured in this study was 0.00238 ± 0.00004 μg/mL, which is smaller than the values found in the former researches which were about 0.00376–0.00778 μg/mL [7, 20, 21]. These values (49.50 ± 0.700 μg/mL for extract obtained by percolation, and 15.50 ± 0.707 μg/mL for extract obtained by Soxhlet extraction) were significantly different ($p > 0.05$) if compared to the IC_{50} of simvastatin. The potency of ethanolic extract of bay leaves in inhibiting HMG-CoA Reductase is smaller when compared with simvastatin, where the ability of simvastatin in inhibiting HMG-CoA Reductase about six thousand to twenty thousand times greater than the ethanolic extract of bay leaves.

Several other reports have also reported the potency of plant extracts in HMG-CoA Reductase inhibition. *Opuntia ficus-indica* (L) Miller extract was reported by Ressaissi et al. [22] to have IC_{50} 20.3 μg/ml and said as to have moderate potency. Ademosun et al. [23] reported that grapefruit peels had an IC_{50} on HMG-CoA Reductase activity 0.11 μg/ml. *Vernonia condensata* extract showed the IC_{50} value of 271.7 μg/ml [24] and *Gnetum gnemon* extract had an IC_{50} value on HMG-CoA Reductase of 400 μg/ml [25]. There are also studies that have assayed the potency of several isolated chemical contents of the plants in HMG-CoA Reductase inhibition, and it was reported that the compounds inhibit the enzyme activity with the IC_{50} value 8.34–149.6 μg/ml [22, 26]. Based on these several studies it can be stated that certain plant extract is said to have HMG-CoA Reductase inhibition potency in the range value of IC_{50} between 0.1 to 400 μg/ml [22, 23, 25, 27]. Thus, the ethanol extracts of bay leaves are also a potent HMG-CoA Reductase inhibitor.

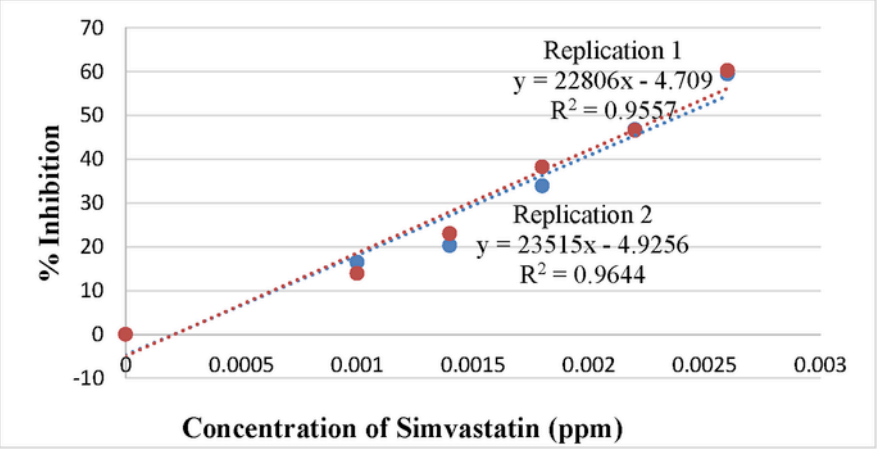

Fig. 1. Graphic of HMG-CoA Reductase inhibition by simvastatin.

https://doi.org/10.1016/j.heliyon.2019.e01485

The potency of ethanol extract of bay leaves obtained by Soxhlet extraction is three times higher than the potency of ethanol extract of bay leaves obtained by percolation. This showed that the Soxhlet process was able to extract more active constituent that responsible for the inhibition of HMG-CoA Reductase and that the active constituents are stable under heating. It is suspected that these active constituents are polyphenolic compounds such as gallic acid, eugenol, kaempferol and quercetin [28]. Some studies have shown that polyphenolic compounds (luteolin, quercetin, and isorhamnetin) contained in many plant extracts play a role in inhibiting HMG-CoA Reductase activity [22, 27]. The phenolic compound of grapefruit peels (genistein and daidzein) showed inhibition of HMG-CoA Reductase activity competitively against HMG-CoA as substrate [23]. Flavonoids, in specific, are stated by Lee et al. [12] to have the ability to inhibit the activity of the HMG-CoA Reductase. The research conducted by Anggraeni [29] which states that at the same concentration (10 μg/ml) quercetin and rutin are able to inhibit the activity of HMG-CoA Reductase respectively 41.10% and 60.17 % also support this hypothesis. However, other studies have not mentioned the inhibition kinetics of other flavonoid groups.

The hypothesis that the inhibition of HMG-CoA Reductase in ethanol extract of bay leaves was due to the polyphenolic content was proved by searching the correlation between the inhibition activity and the total phenolic content in the extract. Besides that, we also measured the antioxidant activity of each extract to study the correlation of it to inhibition activity and types of extract. The total phenolic content and antioxidant activity of each concentration involved in the measurement of HMG-CoA Reductase inhibition activity were reported in Tables 3 and 4. The total phenol in the soxhlet extract is greater than the total phenol in the percolation extract, which in accordance with the inhibition of HMG-CoA Reductase activity pattern. The antioxidant activity of each extract, measured by DPPH, FRAP and beta-carotene method, was compared to gallic acid and quercetin (Table 5). The DPPH and beta-carotene method gave the same pattern results, which showed that the antioxidant activity of Soxhlet extract was higher when compared to the percolation extract. These results also in line with the inhibition of HMG-CoA Reductase activity pattern. The FRAP method in the other way gave a different result, which showed that the antioxidant activity of the percolation method is higher than that of the Soxhlet method. This could be caused by the difference in the mechanism of the assay. FRAP method assay was based on the reduction of ferric ion to ferrous ion. Not all of the Fe^{3+} reductants are antioxidant, and some antioxidants are not able to reduce Fe^{3+} [30].

https://doi.org/10.1016/j.heliyon.2019.e01485

Table 3 Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by percolation method.

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	1.9960A	0A	0.0000A
10	53.6B	3.5532B	0A	10.2513B
25	56.0B	3.8627B	0A	8.0186C
50	61.4C	5.1647C	0.9625A	13.1217D
150	99.0D	8.5790D	8.3633B	7.9707E
300	150.4E	24.9729E	21.3933C	12.5075F
600	193.7F	43.3887F	22.2472C	21.2928G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.
Values with the same letter are not significantly different (P < 0.05).

[a]FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.

[b]IC_{50} was the concentration of substance that provides 50% inhibition.

[c]EC_{50} represents the effective concentration at 50% of total antioxidant activity.

Table 4 Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by Soxhlet method.

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	2.2224A	0A	0A
10	35.4B	4.1808B	0A	14.9736B
25	90.8C	5.1574C	0A	15.2237C
50	92.4C	10.0685D	0A	18.4625D
150	139.0D	20.1246E	0A	20.6429E
300	187.9E	46.5714F	4.2877B	27.6990F
600	201.8F	66.9863G	19.1348C	29.0379G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.
Values with the same letter are not significantly different (P < 0.05).

[a] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.

[b] IC_{50} was the concentration of substance that provides 50% inhibition.

[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

https://doi.org/10.1016/j.heliyon.2019.e01485

Table 5 Antioxidant activity value of ethanol extract of bay leaves obtained by Soxhlet Method.

Samples	Antioxidant activity		
	DPPH method (IC_{50} – ppm)[a]	FRAP method (FRAP value – ppm)[b]	Beta-Carotene method (EC_{50} – ppm)[c]
Gallic Acid	23.87 ± 0.00A	10.60 ± 0.01A	24.87 ± 0.24A
Quercetin	48.87 ± 0.00B	21.94 ± 0.00B	98.44 ± 0.39B
Bay leaves ethanolic extract - percolation	888.08 ± 0.05C	295.00 ± 0.02C	2965.62 ± 0.65C
Bay leaves ethanolic extract - soxhlet	437.89 ± 0.03D	684.00 ± 0.03D	2230.35 ± 1.20D

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD. Values with the same letter are not significantly different ($P < 0.05$).

[a] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.

[b] IC_{50} was the concentration of substance that provides 50% inhibition.

[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

The correlation analysis between each factor in this research was done by 3D linear analysis using SigmaPlot 12.5. The results of the analysis were shown in Tables 6, 7, and 8. Table 6 showed the correlation between extraction method (expressed in concentration, x-axis) and total phenolic content (y-axis) towards antioxidant activity. The level of correlation was shown by the R^2 value. The results showed that there is a high correlation between the extraction method and total

Table 6 Correlation between extraction method and total phenolic content towards antioxidant activity.

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 0.8310 + 0.445x + 0.0742y	0.9890
	FRAP	f = 0.7649 + 0.0419x+0.0196y	0.9663
	Beta – Carotene Bleaching	f = 3.7012 + 0.0068x + 0.0652y	0.8511
Soxhlet	DPPH	f = 5.2176 + 0.0288x + 0.20083y	0.9137
	FRAP	f = 1.2690 + 0.0465x−0.0501y	0.9949
	Beta – Carotene Bleaching	f = 5.1409 + 0.0032x + 0.1196y	0.9156

Table 7 Correlation between extraction method and total phenolic content towards percent of HMG-CoA Reductase inhibition.

Extraction method	Function	R^2
Percolation	f = 3.9241−0.0955x + 0.6945y	0.8688
Soxhlet	f = 15.4733−0.0299x + 0.4829y	0.8871

https://doi.org/10.1016/j.heliyon.2019.e01485

Table 8 Correlation between extraction method and antioxidant activity towards percent of HMG-CoA Reductase inhibition.

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 38.8052−0.3180x−3.1319y	0.6154
	FRAP	f = 32.6035 + 0.0486x + 1.1740y	0.6006
	Beta – Carotene Bleaching	f = 15.5054 + 0.0362x + 2.6778y	0.7075
Soxhlet	DPPH	f = 43.3496 + 0.2689x−2.2742y	0.5670
	FRAP	f = 43.5523 + 0.0533x + 1.3197y	0.5750
	Beta – Carotene Bleaching	f = 1.4981−0.0057x + 3.4218y	0.9759

phenolic content towards antioxidant activity. The higher to total phenolic content in both extracts will cause the increase in the antioxidant activity.

Table 7 showed the correlation between extraction method (concentration, x-axis) and total phenolic content (y-axis) towards percent of HMG-CoA Reductase inhibition. There was also a strong correlation between each factor towards the inhibition of HMG-CoA Reductase activity, but the concentration of extract gave a different effect against the inhibition of HMG-CoA Reductase activity when compared to the total phenolic content. It can be explained that the increase of the concentration of extract will cause the increase also in the total phenolic content, but not all of the phenolic compounds in the extract act as an inhibitor of HMG-CoA Reductase. Thus, some of the phenolic compounds in the extract may act as an activator of the HMG-CoA Reductase.

Correlation between extraction method (concentration, x-axis) and antioxidant activity (y-axis) towards percent of HMG-CoA Reductase inhibition was shown in Table 8. The results of the 3D linear analysis showed a poor correlation between the concentration of extract and antioxidant activity towards the inhibition of HMG-CoA Reductase activity. Thus, though the HMG-CoA Reductase catalyze the reduction-oxidation activity, its inhibition mechanism was not related to the antioxidant mechanism. We conclude that antioxidant compounds might be contributes to inhibit HMG-CoA Reductase but does not go through in the reduction-oxidation mechanisms.

Based on these results, it can be concluded that the inhibition of HMG-CoA Reductase activity by the percolation and soxhlet extracts are caused by the phenolic compounds in the extracts, and it was suspected due to the flavonoids compounds. Further research needs to be done to confirm this report. The relationship between the flavonoid structure (Fig. 2B) with its activity as an enzyme inhibitor of HMG-CoA Reductase is due to the presence of -OH groups in C3 ′, C4′, and C5. It is also caused by the C=O group at C4. These groups play a role in forming hydro-

https://doi.org/10.1016/j.heliyon.2019.e01485

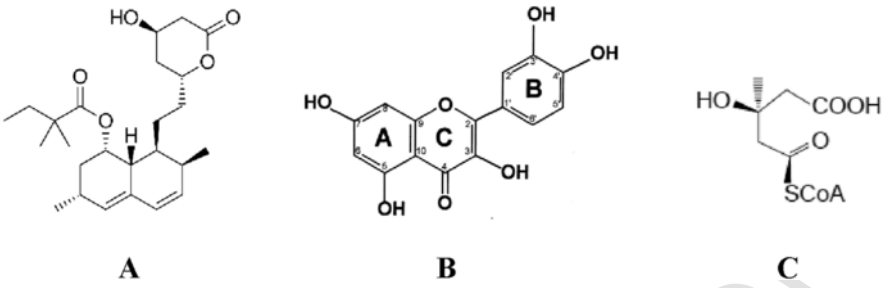

Fig. 2. Structure of simvastatin (A), flavonoid (B), and HMG-CoA (C) [31, 32, 33].

gen bonds with amino acids from HMG-CoA Reductase through hydrophobic interaction [26]. It is suspected that these groups play a role in their activity inhibiting the HMG-CoA Reductase enzyme because they have similarities in the pharmacophores group of the simvastatin. In the simvastatin structure (Fig. 2A) there is an -OH group and a C=O group (a pharmacophore group) that will form a bond with the enzyme, so that the enzyme work becomes inhibited. The C=O group in lactone ring of simvastatin will be hydrolyzed to become an active form (acid). The hydrolyzed simvastatin will then bind to the HMG-CoA Reductase by hydrogen bonding with the amino acids located on the active site of the enzyme. The structure of the hydrolyzed simvastatin in the lactone ring corresponds to the structure of the HMG-CoA substrate (Fig. 2C) so that the enzyme is able to bind with simvastatin and form the complex of enzymes.

Declarations

Author contribution statement

Lanny Hartanti, Sumi Wijaya: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu: Performed the experiments; Wrote the paper.

Henry Kurnia Setiawan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Lisa Soegianto: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the Ministry of Research and Technology Higher Education Republic of Indonesia.

https://doi.org/10.1016/j.heliyon.2019.e01485

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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BUKTI KORESPONDENSI
DENGAN PENERBIT ELSEVIER

4. **Revisi artikel sesuai masukan dari para reviewer yang dikirim kembali kepada penerbit**

(Revision of the article according to feedback from reviewers which is sent back to the publisher)

Reviewer 1	
The manuscript entitled "Influence of extraction methods of bay leaves (*Syzygium polyanthum*) on antioxidant and HMG-CoA reductase inhibitory activity" presented interesting results the inhibitory activity of HMG-CoA reductase by bay leaves (*Syzygium polianthum*) ethanol extracts, but several clarification are required for contribute with the manuscript, as follow:	Thanks for the reviewer's support for our manuscript to be published in Heliyon.
Major compulsory Revisions: Page 2, line: 13 to 14 and in introduction section: The authors affirmed that the inhibition of HMG-CoA reductase is one of many mechanisms in lowering the level cholesterol. In fact, they need to point out that this is the main mechanism yet investigated and, used in treatment.	Thank you for the suggestion. We already did the correction in the article
Page 2, line: 38 to 68 (Introduction section): In the section, at no point was the subject explored on phenolic compounds, antioxidant activity, and especially the relationship between it and the inhibitory activity of HMG-CoA reductase.	Thank you for the suggestion. We already did the correction in the article
Page 5, line: 86 to 88: Add more specific information about the procedure performed, such as: mass used, material: solvent ratio. Time/temperature used. Yield of extract.	The mass of the dried bay leaves used for percolation method was 1 kg in total 3.6 liter of solvent, while the mass used for Soxhlet extraction was 0.5 kg in total 3.03 liter of solvent divided in several steps, which was 20 gram of dried bay leaves in 120 ml solvent for each process. The rendemen of extract obtained from percolation method was 25.05%, while from soxhlet extraction method was 23.62%.
Page 5, line: 88: The paragraph mention a performed of a phytochemical screening, but these results are not present.	Thank you for the correction. We already did the correction in the article simplisia: alkaloid, flavonoid, saponin, tannin, steroid Extract ~~perkolasi~~percolation: alkaloid, flavonoid, saponin, tannin, steroid Extract soxhlet: alkaloid, flavonoid, saponin, tannin, steroid
Page 5, line: 89 to 91, page 6, line 92 to 113, and page 7, line 114 to 120: The HMG-CoA Reductase activity assay should only be present as one subitem. Remove the items "2.4. Inhibition assay of bay leaves ethanol extract towards HMG-CoA Reductase" and "2.5. Inhibition assay of simvastatin towards HMG-CoA Reductase". The description of "statistical analysis" should be separate, and correctly defined.	Thank you for the correction. We already did the correction in the article
Page 8, **Results and discussion** Section: The quality of study (scientific novelty) could be	**Thank you for the suggestion** **For this research, we did the preliminary**

improve if the major polyphenolic compounds (quercetin, gallic acid) is will quantified in the bay leaves were presented (correlation between extraction method and total phenolic content towards percent of HMG-CoA reductase inhibition)	**research to prove the correlation between total phenolic content and the inhibitory effect. Later on, we will continue the research and do the quantitative of the major polyphenolic compounds.**
Page 5, line: 74 - The volume of the micropipettes used is unnecessary.	Thank you for the correction. We have now removed the volume of the micropipettes used. Please refer to Line 75.
Page 5, line: 80 - Adjust in the sentence the term "…ingredients…"	Thank you for the correction. We already did the correction in the article
Page 6, line: 109 - Remove the term "Measurement of ***enzyme activity and***".	We have now removed the term "Measurement of ***enzyme activity and***". Please refer to Line 116.
Page 7, line: 117 - Include the number of repetitions.	We have now included the number of repetition. Please refer to Line 117.
Page 7, line: 123 to 127 - Include how DPPH and beta-carotene assays was expressed.	**Thank you for the correction.** **We already did the correction in the article**
Page 7, line: 128 - Remove the term "Determination of".	Thank you for the correction. We have now removed the term. Please refer to Line 128.
Page 7, line: 129 to 134 - Indicate the concentration range (standard curve) was used in the assay.	Thank you for the suggestion. We have now included the concentration rage of the used standard curve. Please refer to Line 129.
Page 17, Table 3 and 4 - Include how was expressed the DPPH, FRAP, and beta-carotene methods in the tables. Include the standard deviation and statistical analysis.	**Thank you for the correction.** **We already did the correction in the article**
Page 18, Table 5 - Include the standard deviation and statistical analysis.	**Thank you for the correction.** **We already did the correction in the article**
Reviewer 2	
This is an interesting paper where it highlights the differences in the extraction techniques which could lead to obtaining different secondary metabolites.	Thanks for the reviewer's support for our manuscript to be published in Heliyon.
Line 42. "… risk factors (heredity…..)". **Reference required**.	**Thank you for the correction.** **The reference already in the article**
Line 45. "steroids hormone". **Steroid hormones**	Thanks for the correction, now we have changed it according to reviewer suggestion.
Line 46-47. " the increase of cholesterol level in….??? ". Hypercholesterolemia definition is incomplete. **The author needs to include the accurate definition.**	Now we have complete the definition according to the reviewer suggestion. Please refer to Line "The increase of cholesterol level in the bloodstream"
Line 54. This enzyme…. target *with* group…". **for**	We have changed 'with' to 'for' according to the reviewer suggestion, please refer to line …. This enzyme is a pharmacological treatment target for group of drugs called HMG-CoA Reductase inhibitor (statins)
Line 57-58. "Bay leaves…. cholesterol level". **Reference required**.	**Thank you for the correction.** **The reference already in the article**
Line 59. "in vivo". **Italic**	Thanks for the correction, now we have changed it according to reviewer suggestion.
Line 65. "in vitro". **Italic**	Thanks for the correction, now we have

	changed it according to reviewer suggestion.
Line 70. **Captilise** "methods" and "discussion"	**Thank you for the correction.**
Line 75-76. **Plural for** "cuvette" etc.	Thanks for the correction, now we have corrected it according to reviewer suggestion.
Line 84 and 89. The **line spacings are not consistent.**	Thanks for the correction, now we have corrected it according to reviewer suggestion.
Line 85. What do you meant by "standardization was done"? **Could the author further clarify?**	Thank you for the clarification question. What do we meant by "standardization was done" is that "The dried extract that was obtained from previous step (extraction and evaporation to dry) was further standardized to determine the organoleptic characteristics, total ash content, water content, and the solubility in ethanol. We have now edited the explanation in the Line 85, please refer to Line …..
Line 104, Table 2 and within the document. **Inconsistent**. 0,0010 ppm. Some of the numbers are written with "," and ".". **Need to be changed to decimal point**.	Now we have corrected it according to reviewer suggestion. Thank you for the correction.
Line 208. **It would be great if the author could provide some examples with reference of phenolic compounds that acts as an activator of HMG-CoA Reductase.**	**Thank you for the correction.** **We already did the correction in the article**
Line 129. **Could the author specifically include the concentration rather than "a certain concentration"?**	We have specified the concentration according to the reviewer suggestion. Please refer to Line …..
Line 138. **Could the author briefly explain what therapy is being referred to?**	**Thank you for the correction.** **We already did the correction in the article**
Line 219. "....this reports". **Report**	We have changed it according to the reviewer suggestion. Please refer to Line …..
Line 242. "85(1)…? **Incomplete**	We have complete the reference form with the page of the article. Please refer to Line …..
Line 290. **Extra spacing**	Thanks for the correction, now we have corrected it according to reviewer suggestion.
Through the text. **Some of the journals are written in abbreviation and some written in full. Inconsistent.**	Now we have corrected all the journals writing consistently according to reviewer suggestion. Thank you for the correction.
Table 2. Typo error. **The value for the mean column in the first row of 0 mg/ml is missing.**	We have now added the missing value in Table 2. Thank you for the correction.
Figure 2. **The format of the structures is inconsistent**	We have changed the format of the structure to be consistent as the reviewer suggestion.
Reviewer 3	
The article was well discussed about the effect of soxhlet and percolation method to extract bay leaves on the inhibition of HMG-CoA reductase. The idea is novel and give contribution toward the research of dietary human consumption. The article can be accepted after minor revision.	Thanks for the reviewer's support for our manuscript to be published in Heliyon.
In table 3, why the beta carotene method has	The principal of β-Carotene bleaching method

fluctuation antioxidant activity?	is based on the fact that β-Carotene will act as a scavenger for a free radical which produces by linoleic acid which makes β-Carotene becomes colorless. This assay is usually employed to test for lipophilic antioxidants. In this assay, we take the reading every 20 minutes. The fluctuation phenomenon occurred because lipid oxidation occurs at the water/oil interface, where lipophilic antioxidants are located. This may be due by the fact that ethanol was universal solvent which dissolve almost all secondary metabolites with low molecular weight, with diverse polarity level. The percolation method was the extraction method that can extracted all metabolite compounds, including metabolite coumpound that sensitive to the heat. Because of the diversity of the compound in the sample, we suspect several compounds gave absorbance in the wavelength that we used, eventough we already used blangko.

Original Research Article

Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA reductase inhibitory activity

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ABSTRACT

OBJECTIVE: **Bay leaf, one of the plants in Indonesia that has been shown to have activities to reduce cholesterol in the blood.** HMG-CoA Reductase inhibition is one of many mechanisms in lowering the level of cholesterol in the blood. ~~Besides statins, several plant extracts had shown the inhibitory activity of HMG-CoA Reductase~~. Here, we reported the inhibitory activity of HMG-CoA Reductase of bay leaves ethanol extracts that we suspected to be the mechanism of action bay leaves to reduce cholesterol in the blood. ~~,~~ In this research we also investigated the correlation between the inhibitory activities, the total phenol content and antioxidant activities of bay leaves (*Syzygium polianthum*) ethanol extracts.

METHODS: The inhibitory activity of HMG-CoA Reductase was determined kinetically at 340 nm using simvastatin as positive control. *In vitro* scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and beta-carotene method

were used to determine the antioxidant activities. The total phenolic content was determined by Folin-Ciocalteu's method.

RESULTS: The IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity were 49.50 ± 0.700 μg/mL and 15.50 ± 0.707 μg/mL, respectively, while the IC_{50} of simvastatin was 0.00238 ± 0.00004 μg/mL. The antioxidant activity and total phenolic content of bay leaves ethanolic extract obtained by Soxhlet extraction method was higher compared to the percolation method (DPPH and beta-carotene assay results). The 3D linear analysis showed that there was a high correlation between the inhibition activities of HMG-CoA Reductase pattern of both extract types and the total phenol pattern and also the antioxidant pattern of these extracts.

CONCLUSION: The result showed that the bay leaves ethanolic extract have a potent activity to reduce the cholesterol serum level by inhibition of HMG-CoA Reductase activity. The activity was due to the phenolic compounds in the extracts as well as the antioxidant activity of the extracts.

Keywords: *Syzygium polianthum,* HMG-CoA Reductase, inhibitory activity, antioxidant activity, polyphenolic content

1. Introduction

Cardiovascular disease contributed largely to the high mortality rate worldwide year by year. Based on the research in epidemiology, the risk factor of cardiovascular disease is a combination of two or more risk factors. The risk factors of cardiovascular disease are classified into two groups, which are the modifiable risk factors (dyslipidemia, hypertension, smoking, diabetes

mellitus, stress, obesity) and the non-modifiable risk factors (heredity, age, gender). A common risk factor of cardiovascular disease is high serum cholesterol level [1-3].

Cholesterol is a lipid produced in the liver with a number of important roles, such as a membrane constituent and the parent molecule for steroid hormones [4]. Cholesterol can be synthesized by the body and also can be derived from daily food. The increase of cholesterol level in the bloodstream can cause hypercholesterolemia [1]. Hypercholesterolemia is one of the risk factors for the emergence of atherosclerosis, which is inflammatory disorders in artery walls characterized by the formation of atheroma [5]. Atherosclerosis plaque could clog the heart's blood vessel area. This blockage then leads to cardiovascular disease [6]. The increase in cholesterol level can be caused by excessive cholesterol synthesis, the excess of cholesterol absorption, and high cholesterol intake from daily food. Decreasing the cholesterol level can be done by inhibiting cholesterol synthesis through inhibiting the activity of HMG-CoA Reductase which converts Acetyl-CoA into mevalonate [1, 7]. This enzyme is a pharmacological treatment target for group of drugs called HMG-CoA Reductase inhibitor (statins) [8]. However, anti-cholesterol drugs usually are used in combination, and this may increase the chance of unexpected side effects in long-term use.

Bay leaves (*Syzygium polyanthum*) is one of the plants that can be used to decrease the cholesterol level (Abdulrahim Aljamal. 2010. Effects of Bay Leaves on Blood Glucose and Lipid Profiles on the Patients with Type 1 Diabetes. World Academy of Science, Engineering and TechnologyInternational Journal of Medical and Health Sciences. Vol:4, No:9) . Bay leaves contain secondary metabolites, such as saponin, terpenoid, flavonoid, polyphenol, alkaloid, and essential oil. Some previous *in vivo* studies showed that the extract of bay leaves could lower cholesterol levels in the animal blood [9-10]. It is believed that flavonoid

(phenolic coumpound) as one of the chemical content of the bay leaves plays a role in the decrease of cholesterol levels in the blood. In addition, the research conducted by Lee et al. [11] proved that flavonoids can lower cholesterol levels by inhibiting the action of HMG-CoA Reductase. Several experiment showed that flavonoids and phenolic acids, which are classes of polyphenolic compounds have antioxidant properties, including induction of anti-inflammatory actions, inhibition of oxidative enzymes, and scavenging of free radicals (P. F. Moundipa, N. S. E. Beboy, F. Zelefack et al., "Effects of Basella alba and Hibiscus macranthus extracts on testosterone production of adult rat and bull Leydig cells," Asian Journal of Andrology, vol. 7, no. 4, pp. 411–417, 2005).

Based on the researches that have been done to the animals treated with bay leaves, further research about the potency of bay leaves as the anti-hypercholesterolemia *in vitro* is needed with the enzymatic measurement. The extract of bay leaves used was obtained by Soxhlet extraction and percolation method. The measurement of antioxidant activities in each extract was also done to seek the correlation of antioxidant activities and HMG-CoA Reductase inhibition activities.

2. Materials and methods

2.1. Equipment and materials

The equipment used during the study were analytical scales (Sartorius, Germany); oven (Binder); infrared moisture balance (Kett, China); 5 µL capillary tubes; microtubes (Mini spin, USA); vortex; micropipettes; blue tips; white tips; membrane filters; glasswares; chamber; soxhlet; water bath; spectrophotometer (Multiscan Go, Thermo Scientific, USA); cuvettes (Bio-Rads Lab, 2000 Alfred Nobel Drive Hercules, Catalog number 9109250).

Dried bay leaves (*Syzygium polyanthum*) obtained from PT. HRL International Indonesia, Pasuruan, East Java, ~~.~~ ~~t~~The enzyme used was the HMG-CoA Reductase Assay Kit (Catalog number CS 1090, Sigma, Germany~~). ~~), ~~Other ingredients used during the study were~~ 96% ethanol, phytochemical screening reagents, water for injection, sodium hydrogen phosphate (NaH_2PO_4) (Merck, Indonesia), sodium dihydrogen phosphate (Na_2HPO_4) (Merck, Indonesia), simvastatin tablet, antioxidant assay reagents.

2.2. Preparation of extract

Standardization was done to the dried bay leaves prior to the extraction. The extraction was done with percolation and Soxhlet extraction method using ethanol 96% as the solvent.

The extract was then evaporated on a water bath then was stored in a sterile bottle. The dried extract was further standardized to determine the organoleptic characteristics, total ash content, water content, and the solubility in ethanol to ensure the quality. Phytochemical screening was also done to the dried extract prior to antioxidant and enzymatic assay.

2.3.HMG-CoA Reductase activity assay

366 µl 1x assay buffer was mixed with 24 µl HMG-CoA substrate, 8 µl NADPH, and 2 µl enzyme. The mixture was then measured at 37°C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes [12].

125 mg of bay leaves ethanol extract was dissolved in 25 ml of sterile water to make the standard solution 5000 ppm. The solution was further made into different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. The solution was centrifuged and filtered using a 0.45 µm filter membrane to remove the residual sediment from the extract. 364 µl 1x assay buffer was mixed with 24 µl HMG-CoA substrate, 8 µl NADPH, 2 µl

extract from each concentration and 2 µl enzyme. The mixture was then measured at 37°C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

Standard solution of simvastatin was taken 2 µl from each concentration 0 ppm, 0.0010 ppm, 0.0014 ppm, 0.0018 ppm, 0.0022 and 0.0026 ppm. Each 2 µl solution was mixed with 364 µl 1x assay buffer, 24 µl HMG-CoA substrate, 8 µl NADPH and 2 µl enzyme. The mixture was then measured at 37°C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

The data from spectrophotometric was analyzed to determine the enzyme activity (Sigma-Aldrich, 2013), using this equation:

$$\text{Specific activity} = \frac{(\Delta A(\text{sample})/\text{min}) \times TV}{12.44 \times V_{\text{enzyme}} \times 0.6 \times}$$

Where ΔA: Change of absorbance, TV: Total volume of the reaction in ml, 12.44: coefficient of NADPH, V enzyme: volume of enzyme used in the assay, 0.6: Enzyme concentration in mg-protein, LP: Lightpath in cm.

2.4. Statistical analysis

All test scores were presented as mean values of inhibition ± standard deviation from two replications. The percent of inhibition was obtained from the activity without inhibitor minus activity with inhibitor divided by activity without inhibitor. For statistical data analysis, each group was compared using independent sample T-test with 95% level of confidence.

2.5. Antioxidant assays

Antioxidant activities of the extracts were assayed by three different methods, which were the DPPH method, the FRAP method, and beta-carotene method. The DPPH method states the

antioxidant activity as the oxidation inhibition by referring to Chandra and Dave [13] and Shafazila et al. [14]. The antioxidant potency was measured using % Scavenging effect. The antioxidant assay using FRAP reagent refers to Benzie and Strain [15] where the antioxidant capacity stated as µmoles Trolox / g dry powder. The beta-carotene assay was done according to Utami et al. [16]. The antioxidant potency of the sample was expressed as the concentration with exhibit 50% of the antioxidant activity (EC50).

2.6. Total phenolic content

Extract solution of bay leaves was prepared in different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. Each solution of bay leaves extract was pipetted 100 µl and was mixed with 300 µl of 2% sodium carbonate, 1.58 ml of deionized water, and 100 µl of 10% Folin-Ciocalteu reagent. The absorbance of the reaction mixture was observed at 750 nm (Multiscan Go, Thermo Scientific, USA) after 30 min incubation at room temperature. Gallic acid was used as a standard [17]. The data were expressed as ppm gallic acid equivalents.

3. Results and discussion

Choosing the right extraction method is one of the supporting factors in the success of a therapy, including lowering cholesterol level in the blood. This can be caused by the solubility of secondary metabolites in plants depending on the type of solvent and temperature used during extraction. From the phytochemical screening results, both bay leaves ethanol extract (percolation method and soxhletation method) contain alkaloid, flavonoid, saponin, tannin, steroid.

The results of inhibition potency and IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity can be seen in Table 1 and Table 2. Simvastatin, the first generation of statins, was used as a reference compound in this research. The inhibition potency of simvastatin toward HMG-CoA Reductase enzyme is shown in Figure 1. The IC_{50} value of simvastatin measured in this study was 0.00238 ± 0.00004 μg/mL, which is smaller than the values found in the former researches which were about 0.00376 – 0.00778 μg/mL [18-20]. These values (49.50 ± 0.700 μg/mL for extract obtained by percolation, and 15.50 ± 0.707 μg/mL for extract obtained by Soxhlet extraction) were significantly different ($p > 0.05$) if compared to the IC_{50} of simvastatin. The potency of ethanolic extract of bay leaves in inhibiting HMG-CoA Reductase is smaller when compared with simvastatin, where the ability of simvastatin in inhibiting HMG-CoA Reductase about six thousand to twenty thousand times greater than the ethanolic extract of bay leaves.

Several other reports have also reported the potency of plant extracts in HMG-CoA Reductase inhibition. *Opuntia ficus-indica* (L) Miller extract was reported by Ressaissi et al. [21] to have IC_{50} 20.3 μg/ml and said as to have moderate potency. Ademosun et al. [22] reported that grapefruit peels had an IC_{50} on HMG-CoA Reductase activity 0.11 μg/ml. *Vernonia condensata* extract showed the IC_{50} value of 271.7 μg/ml [23] and *Gnetum gnemon* extract had an IC_{50} value on HMG-CoA Reductase of 400 μg/ml [24]. There are also studies that have assayed the potency of several isolated chemical contents of the plants in HMG-CoA Reductase inhibition, and it was reported that the compounds inhibit the enzyme activity with the IC_{50} value 8.34 - 149.6 μg/ml [21, 25]. Based on these several studies it can be stated that certain plant extract is said to have HMG-CoA Reductase inhibition potency in the range value of IC_{50} between 0.1 to 400 μg/ml

[21, 22, 24, 26]. Thus, the ethanol extracts of bay leaves are also a potent HMG-CoA Reductase inhibitor.

The potency of ethanol extract of bay leaves obtained by Soxhlet extraction is three times higher than the potency of ethanol extract of bay leaves obtained by percolation. This showed that the Soxhlet process was able to extract more active constituent that responsible for the inhibition of HMG-CoA Reductase and that the active constituents are stable under heating. It is suspected that these active constituents are polyphenolic compounds such as gallic acid, eugenol, kaempferol and quercetin [27]. Some studies have shown that polyphenolic compounds (luteolin, quercetin, and isorhamnetin) contained in many plant extracts play a role in inhibiting HMG-CoA Reductase activity [21, 26]. The phenolic compound of grapefruit peels (genistein and daidzein) showed inhibition of HMG-CoA Reductase activity competitively against HMG-CoA as substrate [22]. Flavonoids, in specific, are stated by Lee et al. [11] to have the ability to inhibit the activity of the HMG-CoA Reductase. The research conducted by Anggraeni [28] which states that at the same concentration (10 μg/ml) quercetin and rutin are able to inhibit the activity of HMG-CoA Reductase respectively 41.10% and 60.17 % also support this hypothesis. However, other studies have not mentioned the inhibition kinetics of other flavonoid groups.

The hypothesis that the inhibition of HMG-CoA Reductase in ethanol extract of bay leaves was due to the polyphenolic content was proved by searching the correlation between the inhibition activity and the total phenolic content in the extract. Besides that, we also measured the antioxidant activity of each extract to study the correlation of it to inhibition activity and types of extract. The total phenolic content and antioxidant activity of each concentration involved in the measurement of HMG-CoA reductase inhibition activity were reported in Table 3 and 4. The total phenol in the soxhlet extract is greater than the total phenol in the percolation

extract, which in accordance with the inhibition of HMG-CoA reductase activity pattern. The antioxidant activity of each extract, measured by DPPH, FRAP and beta-carotene method, was compared to gallic acid and quercetin (Table 5). The DPPH and beta-carotene method gave the same pattern results, which showed that the antioxidant activity of Soxhlet extract was higher when compared to the percolation extract. These results also in line with the inhibition of HMG-CoA reductase activity pattern. The FRAP method in the other way gave a different result, which showed that the antioxidant activity of the percolation method is higher than that of the Soxhlet method. This could be caused by the difference in the mechanism of the assay. FRAP method assay was based on the reduction of ferric ion to ferrous ion. Not all of the Fe^{3+} reductants are antioxidant, and some antioxidants are not able to reduce Fe^{3+} [29].

The correlation analysis between each factor in this research was done by 3D linear analysis using SigmaPlot 12.5. The results of the analysis were shown in Table 6, Table 7 and Table 8. Table 6 showed the correlation between extraction method (expressed in concentration, x-axis) and total phenolic content (y-axis) towards antioxidant activity. The level of correlation was shown by the R^2 value. The results showed that there is a high correlation between the extraction method and total phenolic content towards antioxidant activity. The higher to total phenolic content in both extracts will cause the increase in the antioxidant activity.

Table 7 showed the correlation between extraction method (concentration, x-axis) and total phenolic content (y-axis) towards percent of HMG-CoA reductase inhibition. There was also a strong correlation between each factor towards the inhibition of HMG-CoA reductase activity, but the concentration of extract gave a different effect against the inhibition of HMG-CoA reductase activity when compared to the total phenolic content. It can be explained that the increase of the concentration of extract will cause the increase also in the total phenolic content,

but not all of the phenolic compounds in the extract act as an inhibitor of HMG-CoA reductase. Thus, some of the phenolic compounds in the extract can act as an activator of the HMG-CoA reductase.

Correlation between extraction method (concentration, x-axis) and antioxidant activity (y-axis) towards percent of HMG-CoA reductase inhibition was shown in Table 8. The results of the 3D linear analysis showed a poor correlation between the concentration of extract and antioxidant activity towards the inhibition of HMG-CoA reductase activity. Thus, though the HMG-CoA reductase catalyze the reduction-oxidation activity, its inhibition mechanism was not related to the antioxidant mechanism. We conclude that antioxidant compounds might be contributes to inhibit HMG-CoA reductase but does not go through in the reduction-oxidation mechanisms.

Based on these results, it can be concluded that the inhibition of HMG-CoA Reductase activity by the percolation and soxhlet extracts are caused by the phenolic compounds in the extracts, and it was suspected due to the flavonoids compounds. Further research needs to be done to confirm this report. The relationship between the flavonoid structure (Fig. 2 (B)) with its activity as an enzyme inhibitor of HMG-CoA Reductase is due to the presence of -OH groups in C3 ', C4', and C5. It is also caused by the C=O group at C4. These groups play a role in forming hydrogen bonds with amino acids from HMG-CoA Reductase through hydrophobic interaction [25]. It is suspected that these groups play a role in their activity inhibiting the HMG-CoA Reductase enzyme because they have similarities in the pharmacophores group of the simvastatin. In the simvastatin structure (Fig. 2 (A)) there is an -OH group and a C=O group (a pharmacophore group) that will form a bond with the enzyme, so that the enzyme work becomes inhibited. The C=O group in lactone ring of simvastatin will be hydrolyzed to become an active

form (acid). The hydrolyzed simvastatin will then bind to the HMG-CoA Reductase by hydrogen bonding with the amino acids located on the active site of the enzyme. The structure of the hydrolyzed simvastatin in the lactone ring corresponds to the structure of the HMG-CoA substrate (Fig. 2 (C)) so that the enzyme is able to bind with simvastatin and form the complex of enzymes.

ACKNOWLEDGMENT

This research work has been supported by the Ministry of Research and Technology Higher Education Republic of Indonesia and PT. HRL International, East Java, Indonesia.

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Table 1. The Inhibition of HMG-CoA Reductase of Ethanol Extract of Bay Leaves Obtained by Percolation Method

Concentration (µg/ml)	% of Inhibition		Mean	SD	IC_{50} (µg/ml)
	n1	n2			
0	0	0	0	0	n1 = 50.00
10	28.49	21.03	24.760	5.275	n2 = 49.00
25	47.10	42.59	44.845	3.189	
50	57.10	57.03	57.065	0.049	
150	64.40	67.02	65.710	1.853	
300	66.24	74.66	70.450	5.954	
600	82.24	83.28	82.760	0.735	
				Mean ± SD = 49.50 ± 0.700	

Table 2. The Inhibition of HMG-CoA Reductase of Ethanol Extract of Bay Leaves Obtained by Soxhlet Method

Concentration (µg/ml)	% of Inhibition		Mean	SD	IC_{50} (µg/ml)
	n1	n2			
0	0	0	0	0.000	n1 = 15.00
10	47.17	48.55	47.860	0.976	n2 = 16.00
25	54.72	56.16	55.440	1.018	
50	69.81	66.67	68.240	2.220	
150	79.25	76.09	77.670	2.234	
300	88.68	84.42	86.550	3.012	
600	101.9	97.10	99.500	3.394	
				Mean ± SD = 15.50 ± 0.707	

Table 3. Total Phenolic Content and Antioxidant Activity of Ethanol Extract of Bay Leaves Obtained by Percolation Method

Concentration (µg/ml)	Total Phenol Content (ppm)	Antioxidant Activity		
		DPPH method	FRAP method	Beta-Carotene method
0	0.0	1.9960	0	0.0000
10	53.6	3.5532	0	10.2513
25	56.0	3.8627	0	8.0186
50	61.4	5.1647	0.9625	13.1217
150	99.0	8.5790	8.3633	7.9707
300	150.4	24.9729	21.3933	12.5075
600	193.7	43.3887	22.2472	21.2928

Table 4. Total Phenolic Content and Antioxidant Activity of Ethanol Extract of Bay Leaves Obtained by Soxhlet Method

Concentration (µg/ml)	Total Phenol Content (ppm)	Antioxidant Activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	2.2224A	0A	0A
10	35.4B	4.1808B	0A	14.9736B
25	90.8C	5.1574C	0A	15.2237C
50	92.4C	10.0685D	0A	18.4625D
150	139.0D	20.1246E	0A	20.6429E
300	187.9E	46.5714F	4.2877B	27.6990F
600	201.8F	66.9863G	19.1348C	29.0379G

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.
Values with the same letter are not significantly different (P<0.05).
[a]FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.
[b]IC_{50} was the concentration of substance that provides 50% inhibition
[c]EC_{50} represents the effective concentration at 50% of total antioxidant activity.

Table 5. Antioxidant activity value of Ethanol Extract of Bay Leaves Obtained by Soxhlet Method

Samples	Antioxidant activity		
	DPPH method (IC_{50} - ppm)[a]	FRAP method (FRAP value – ppm)[b]	Beta-carotene method (EC_{50} – ppm)[c]
Gallic Acid	23.87±0.00A	10.60±0.01A	24.87±0.24A
Quercetin	48.87±0.00B	21.94±0.00B	98.44±0.39B
Bay leaves ethanolic extract - percolation	888.08±0.05C	295.00±0.02C	2965.62±0.65C
Bay leaves ethanolic extract - soxhlet	437.89±0.03D	684.00±0.03D	2230.35±1.20D

Data were obtained from three independent experiments, each performed in triplicates (n=9) and represented as mean ± SD.
Values with the same letter are not significantly different (P<0.05).
[a]FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.
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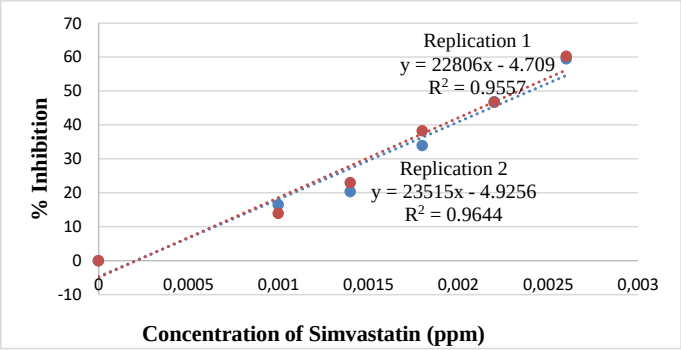

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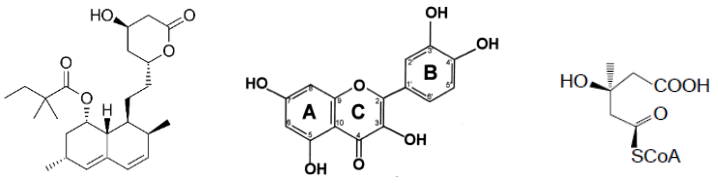
Figure 1. Graphic of HMG-CoA Reductase Inhibition by Simvastatin.



A **B** **C**

Figure 2. Structure of Simvastatin (A), Flavonoid (B), and HMG-CoA (C) [30-32]

Table 6. Correlation between extraction method and total phenolic content towards antioxidant activity

Extraction Method	Antioxidant Method	Function	R^2
Percolation	DPPH	f=0.8310+0.445x+0.0742y	0.9890
	FRAP	f=0.7649+0.0419x+0.0196y	0.9663
	Beta – Carotene Bleaching	f=3.7012+0.0068x+0.0652y	0.8511
Soxhlet	DPPH	f=5.2176+0.0288x+0.20083y	0.9137
	FRAP	f=1.2690+0.0465x-0.0501y	0.9949
	Beta – Carotene Bleaching	f=5.1409+0.0032x+0.1196y	0.9156

Table 7. Correlation between extraction method and total phenolic content towards percent of HMG-CoA reductase inhibition

Extraction Method	Function	R^2
Percolation	f=3.9241-0.0955x+0.6945y	0.8688
Soxhlet	f=15.4733-0.0299x+0.4829y	0.8871

394 Table 8. Correlation between extraction method and antioxidant activity towards percent of
395 HMG-CoA reductase inhibition

Extraction Method	Antioxidant Method	Function	R^2
Percolation	DPPH	f=38.8052-0.3180x-3.1319y	0.6154
	FRAP	f=32.6035+0.0486x+1.1740y	0.6006
	Beta – Carotene Bleaching	f=15.5054+0.0362x+2.6778y	0.7075
Soxhlet	DPPH	f=43.3496+0.2689x-2.2742y	0.5670
	FRAP	f=43.5523+0.0533x+1.3197y	0.5750
	Beta – Carotene Bleaching	f=1.4981-0.0057x+3.4218y	0.9759

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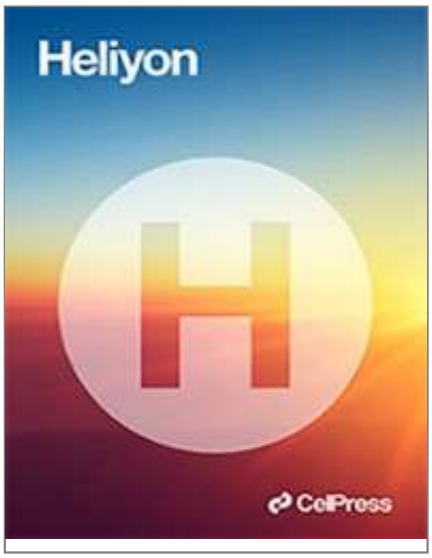

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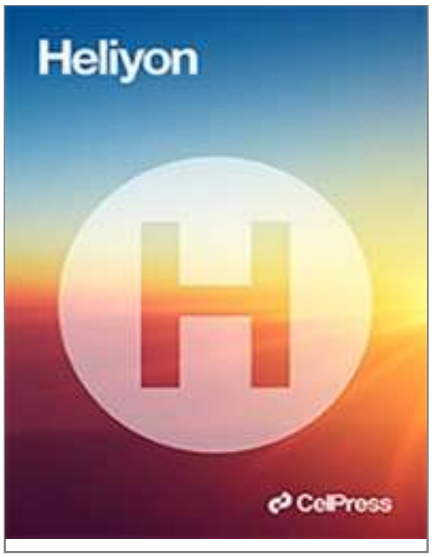

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Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA Reductase inhibitory activity

Lanny Hartanti, Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu, Sumi Wijaya, Henry Kurnia Setiawan, Lisa Soegianto

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Abstract

Objective

Bay leaf, one of the plants in Indonesia that has been shown to have activities to reduce cholesterol in the blood. HMG-CoA Reductase inhibition is one of many mechanisms in lowering the level of cholesterol in the blood. Here, we reported the inhibitory activity of HMG-CoA Reductase of bay leaves ethanol extracts that we suspected to be the mechanism of action of bay leaves in reducing cholesterol in the blood. In this research we also investigated the correlation between the inhibitory activities, the total phenol content and antioxidant activities of bay leaves (*Syzygium polianthum*) ethanol extracts.

Methods

The inhibitory activity of HMG-CoA Reductase was determined kinetically at 340 nm using simvastatin as positive control. *In vitro* scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and beta-carotene method were used to determine the antioxidant activities. The total phenolic content was determined by Folin-Ciocalteu's method.

Results

The IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity were 49.50 ± 0.700 μg/mL and 15.50 ± 0.707 μg/mL,

respectively, while the IC_{50} of simvastatin was 0.00238 ± 0.00004 μg/mL. The antioxidant activity and total phenolic content of bay leaves ethanolic extract obtained by Soxhlet extraction method was higher compared to the percolation method (DPPH and beta-carotene assay results). The 3D linear analysis showed that there was a high correlation between the inhibition activities of HMG-CoA Reductase pattern of both extract types and the total phenol pattern and also the antioxidant pattern of these extracts.

Conclusion

The result showed that the bay leaves ethanolic extract have a potent activity to reduce the cholesterol serum level by inhibition of HMG-CoA Reductase activity. The activity was due to the phenolic compounds in the extracts as well as the antioxidant activity of the extracts.

Previous Next

Keywords

Biochemistry; Molecular biology; Natural product chemistry

1. Introduction

Cardiovascular disease contributed largely to the high mortality rate worldwide year by year. Based on the research in epidemiology, the risk factor of cardiovascular disease is a combination of two or more risk factors. The risk factors of cardiovascular disease are classified into two groups, which are the modifiable risk factors (dyslipidemia, hypertension, smoking, diabetes mellitus, stress, obesity) and the non-modifiable risk factors (heredity, age, gender). A common risk factor of cardiovascular disease is high serum cholesterol level [1, 2, 3].

Cholesterol is a lipid produced in the liver with a number of important roles, such as a membrane constituent and the parent molecule for steroid hormones [4]. Cholesterol can be synthesized by the body and also can be derived from daily food. The increase of cholesterol level in the bloodstream can cause hypercholesterolemia [1]. Hypercholesterolemia is one of the risk factors for the emergence of atherosclerosis, which is inflammatory disorders in artery walls characterized by the formation of atheroma [5]. Atherosclerosis plaque could clog the heart's blood vessel area. This blockage then leads to cardiovascular disease [6]. The increase in cholesterol level can be caused by excessive cholesterol synthesis, the excess of cholesterol absorption, and high cholesterol intake from daily food. Decreasing the cholesterol level can be done by inhibiting cholesterol synthesis through inhibiting the activity of HMG-CoA Reductase which converts Acetyl-CoA into mevalonate [1, 7]. This enzyme is a pharmacological treatment target for group of drugs called HMG-CoA Reductase inhibitor (statins) [8]. However, anti-cholesterol drugs usually are used in combination, and this may increase the chance of unexpected side effects in long-term use.

Bay leaves (*Syzygium polyanthum*) is one of the plants that can be used to decrease the cholesterol level [9]. Bay leaves contain secondary metabolites, such as saponin, terpenoid, flavonoid, polyphenol, alkaloid, and essential oil. Some previous *in vivo* studies showed that the extract of bay

leaves could lower cholesterol levels in the animal blood [10, 11]. It is believed that flavonoid (phenolic compound) as one of the chemical content of the bay leaves plays a role in the decrease of cholesterol levels in the blood. In addition, the research conducted by Lee etal. [12] proved that flavonoids can lower cholesterol levels by inhibiting the action of HMG-CoA Reductase. Several experiment showed that flavonoids and phenolic acids, which are classes of polyphenolic compounds have antioxidant properties, including induction of anti-inflammatory actions, inhibition of oxidative enzymes, and scavenging of free radicals [13].

Based on the researches that have been done to the animals treated with bay leaves, further research about the potency of bay leaves as the anti-hypercholesterolemia *invitro* is needed with the enzymatic measurement. The extract of bay leaves used was obtained by Soxhlet extraction and percolation method. The measurement of antioxidant activities in each extract was also done to seek the correlation of antioxidant activities and HMG-CoA Reductase inhibition activities. This research covers the taxonomy of Biochemistry and Molecular Biology.

2. Materials and methods

2.1. Equipment and materials

The equipment used during the study were analytical scales (Sartorius, Germany); oven (Binder); infrared moisture balance (Kett, China); 5 μL capillary tubes; microtubes (Mini spin, USA); vortex; micropipettes; blue tips; white tips; membrane filters; glasswares; chamber; soxhlet; water bath; spectrophotometer (Multiscan Go, Thermo Scientific, USA); cuvettes (Bio-Rads Lab, 2000 Alfred Nobel Drive Hercules, Catalog number 9109250).

Dried bay leaves (*Syzygium polyanthum*) obtained from PT. HRL International Indonesia, Pasuruan, East Java, the enzyme used was the HMG-CoA Reductase Assay Kit (Catalog number CS 1090, Sigma, Germany), 96% ethanol, phytochemical screening reagents, water for injection, sodium hydrogen phosphate (NaH_2PO_4) (Merck, Indonesia), sodium dihydrogen phosphate (Na_2HPO_4) (Merck, Indonesia), simvastatin tablet, antioxidant assay reagents.

2.2. Preparation of extract

Standardization was done to the dried bay leaves prior to the extraction. The extraction was done with percolation and Soxhlet extraction method using ethanol 96% as the solvent. The mass of the dried bay leaves used for percolation method was 1 kg in total 3.6 liter of solvent, while the mass used for Soxhlet extraction was 0.5 kg in total 3.03 liter of solvent divided in several steps, which was 20 gram of dried bay leaves in 120 ml solvent for each process. The rendemen of extract obtained from percolation method was 25.05%, while from soxhlet extraction method was 23.62%.

The extract was then evaporated on a water bath then was stored in a sterile bottle. The dried extract was further standardized to determine the organoleptic characteristics, total ash content, water content, and the solubility in ethanol to ensure the quality. Phytochemical screening was also done to the dried extract prior to antioxidant and enzymatic assay.

2.3. HMG-CoA Reductase activity assay

366 μl 1x assay buffer was mixed with 24 μl HMG-CoA substrate, 8 μl NADPH, and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes [14].

125 mg of bay leaves ethanol extract was dissolved in 25 ml of sterile water to make the standard solution 5000 ppm. The solution was further made into different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. The solution was centrifuged and filtered using a 0.45 μm filter membrane to remove the residual sediment from the extract. 364 μl 1x assay buffer was mixed with 24 μl HMG-CoA substrate, 8 μl NADPH, 2 μl extract from each concentration and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

Standard solution of simvastatin was taken 2 μl from each concentration 0 ppm, 0.0010 ppm, 0.0014 ppm, 0.0018 ppm, 0.0022 and 0.0026 ppm. Each 2 μl solution was mixed with 364 μl 1x assay buffer, 24 μl HMG-CoA substrate, 8 μl NADPH and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

The data from spectrophotometric was analyzed to determine the enzyme activity (Sigma-Aldrich, 2013), using this equation:

$$\textbf{Specific activity} = \frac{(\Delta \mathbf{A(sample/\ min)} \times \mathbf{TV})}{\mathbf{12.44} \times \mathbf{V}\ \ \mathbf{enzyme} \times \mathbf{0.6} \times \mathbf{LP}}$$

where ΔA: Change of absorbance, TV: Total volume of the reaction in ml, 12.44: coefficient of NADPH, V enzyme: volume of enzyme used in the assay, 0.6: Enzyme concentration in mg-protein, LP: Lightpath in cm.

2.4. Statistical analysis

All test scores were presented as mean values of inhibition ±standard deviation from two replications. The percent of inhibition was obtained from the activity without inhibitor minus activity with inhibitor divided by activity without inhibitor. For statistical data analysis, each group was compared using independent sample T-test with 95% level of confidence.

2.5. Antioxidant assays

Antioxidant activities of the extracts were assayed by three different methods, which were the DPPH method, the FRAP method, and beta-carotene method. The DPPH method states the antioxidant activity as the oxidation inhibition by referring to Chandra and Dave [15] and Shafazila et al. [16]. The antioxidant potency was measured using % Scavenging effect. The antioxidant assay using FRAP reagent refers to Benzie and Strain [17] where the antioxidant capacity stated as μmoles Trolox/g dry powder. The beta-carotene assay was done according to Utami et al. [18]. The antioxidant potency of the sample was expressed as the concentration with exhibit 50% of the antioxidant activity (EC_{50}).

2.6. Total phenolic content

Extract solution of bay leaves was prepared in different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. Each solution of bay leaves extract was pipetted 100 μl and was mixed with 300 μl of 2% sodium carbonate, 1.58 ml of deionized water, and 100 μl of 10% Folin-Ciocalteu reagent. The absorbance of the reaction mixture was observed at 750 nm (Multiscan Go, Thermo Scientific, USA) after 30 min incubation at room temperature. Gallic acid was used as a standard [19]. The data were expressed as ppm gallic acid equivalents.

3. Results & discussion

Choosing the right extraction method is one of the supporting factors in the success of a therapy, including lowering cholesterol level in the blood. This can be caused by the solubility of secondary metabolites in plants depending on the type of solvent and temperature used during extraction. From the phytochemical screening results, both bay leaves ethanol extract (percolation method and soxhletation method) contain alkaloid, flavonoid, saponin, tannin, steroid.

The results of inhibition potency and IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity can be seen in Tables 1 and 2. Simvastatin, the first generation of statins, was used as a reference compound in this research. The inhibition potency of simvastatin toward HMG-CoA Reductase enzyme is shown in Fig. 1. The IC_{50} value of simvastatin measured in this study was 0.00238 ± 0.00004 μg/mL, which is smaller than the values found in the former researches which were about 0.00376–0.00778 μg/mL [7, 20, 21]. These values (49.50 ± 0.700 μg/mL for extract obtained by percolation, and 15.50 ± 0.707 μg/mL for extract obtained by Soxhlet extraction) were significantly different ($p > 0.05$) if compared to the IC_{50} of simvastatin. The potency of ethanolic extract of bay leaves in inhibiting HMG-CoA Reductase is smaller when compared with simvastatin, where the ability of simvastatin in inhibiting HMG-CoA Reductase about six thousand to twenty thousand times greater than the ethanolic extract of bay leaves.

Table 1. The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by percolation method.

Concentration (μg/ml)	% of Inhibition		Mean	SD	IC50 (μg/ml)
	n1	n2			
0	0	0	0	0	n1 = 50.00
10	28.49	21.03	24.760	5.275	n2 = 49.00
25	47.10	42.59	44.845	3.189	
50	57.10	57.03	57.065	0.049	
150	64.40	67.02	65.710	1.853	
300	66.24	74.66	70.450	5.954	
600	82.24	83.28	82.760	0.735	
Mean ± SD = 49.50 ± 0.700					

Table 2. The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by Soxhlet method.

Concentration (μg/ml)	% of Inhibition		Mean	SD	IC50 (μg/ml)
	n1	n2			
0	0	0	0	0.000	n1 = 15.00
10	47.17	48.55	47.860	0.976	n2 = 16.00
25	54.72	56.16	55.440	1.018	
50	69.81	66.67	68.240	2.220	
150	79.25	76.09	77.670	2.234	
300	88.68	84.42	86.550	3.012	
600	101.9	97.10	99.500	3.394	
Mean ± SD = 15.50 ± 0.707					

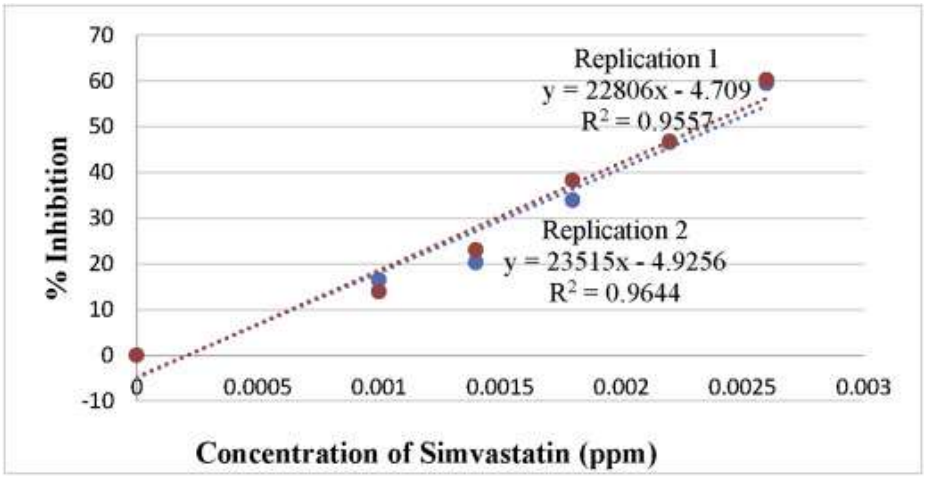

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Fig. 1. Graphic of HMG-CoA Reductase inhibition by simvastatin.

Several other reports have also reported the potency of plant extracts in HMG-CoA Reductase inhibition. *Opuntia ficus-indica* (L) Miller extract was reported by Ressaissi et al. [22] to have IC_{50} 20.3 μg/ml and said as to have moderate potency. Ademosun et al. [23] reported that grapefruit peels had an IC_{50} on HMG-CoA Reductase activity 0.11 μg/ml. *Vernonia condensata* extract showed the IC_{50} value of 271.7 μg/ml [24] and *Gnetum gnemon* extract had an IC_{50} value on HMG-CoA Reductase of 400 μg/ml [25]. There are also studies that have assayed the potency of several isolated chemical contents of the plants in HMG-CoA Reductase inhibition, and it was reported that the compounds inhibit the enzyme activity with the IC_{50} value 8.34–149.6 μg/ml [22, 26]. Based on these several studies it can be stated that certain plant extract is said to have HMG-CoA Reductase inhibition potency in the range value of IC_{50} between 0.1 to 400 μg/ml [22, 23, 25, 27]. Thus, the ethanol extracts of bay leaves are also a potent HMG-CoA Reductase inhibitor.

The potency of ethanol extract of bay leaves obtained by Soxhlet extraction is three times higher than the potency of ethanol extract of bay leaves obtained by percolation. This showed that the Soxhlet process was able to extract more active constituent that responsible for the inhibition of HMG-CoA Reductase and that the active constituents are stable under heating. It is suspected that these active constituents are polyphenolic compounds such as gallic acid, eugenol, kaempferol and quercetin [28]. Some studies have shown that polyphenolic compounds (luteolin, quercetin, and isorhamnetin) contained in many plant extracts play a role in inhibiting HMG-CoA Reductase activity [22, 27]. The phenolic compound of grapefruit peels (genistein and daidzein) showed inhibition of HMG-CoA Reductase activity competitively against HMG-CoA as substrate [23]. Flavonoids, in specific, are stated by Lee etal. [12] to have the ability to inhibit the activity of the HMG-CoA Reductase. The research conducted by Anggraeni [29] which states that at the same concentration (10 μg/ml) quercetin and rutin are able to inhibit the activity of HMG-CoA Reductase respectively 41.10% and 60.17 % also support this hypothesis. However, other studies have not mentioned the inhibition kinetics of other flavonoid groups.

The hypothesis that the inhibition of HMG-CoA Reductase in ethanol extract of bay leaves was due to the polyphenolic content was proved by searching the correlation between the inhibition activity and the total phenolic content in the extract. Besides that, we also measured the antioxidant activity of each extract to study the correlation of it to inhibition activity and types of extract. The total phenolic content and antioxidant activity of each concentration involved in the measurement of HMG-CoA Reductase inhibition activity were reported in Tables 3 and 4. The total phenol in the soxhlet extract is greater than the total phenol in the percolation extract, which in accordance with the inhibition of HMG-CoA Reductase activity pattern. The antioxidant activity of each extract, measured by DPPH, FRAP and beta-carotene method, was compared to gallic acid and quercetin (Table 5). The DPPH and beta-carotene method gave the same pattern results, which showed that the antioxidant activity of Soxhlet extract was higher when compared to the percolation extract. These results also in line with the inhibition of HMG-CoA Reductase activity pattern. The FRAP method in the other way gave a different result, which showed that the antioxidant activity of the percolation method is higher than that of the Soxhlet method. This could be caused by the difference in the mechanism of the assay. FRAP method assay was based on the reduction of ferric ion to ferrous ion. Not all of the Fe^{3+} reductants are antioxidant, and some antioxidants are not able to reduce Fe^{3+} [30].

Table 3. Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by percolation method.

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	1.9960A	0A	0.0000A
10	53.6B	3.5532B	0A	10.2513B
25	56.0B	3.8627B	0A	8.0186C
50	61.4C	5.1647C	0.9625A	13.1217D
150	99.0D	8.5790D	8.3633B	7.9707E
300	150.4E	24.9729E	21.3933C	12.5075F

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
600	193.7F	43.3887F	22.2472C	21.2928G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.

Values with the same letter are not significantly different ($P < 0.05$).

a

IC50 was the concentration of substance that provides 50% inhibition.

b

FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.

c

EC_{50} represents the effective concentration at 50% of total antioxidant activity.

Table 4. Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by Soxhlet method.

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	2.2224A	0A	0A
10	35.4B	4.1808B	0A	14.9736B
25	90.8C	5.1574C	0A	15.2237C
50	92.4C	10.0685D	0A	18.4625D
150	139.0D	20.1246E	0A	20.6429E
300	187.9E	46.5714F	4.2877B	27.6990F
600	201.8F	66.9863G	19.1348C	29.0379G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.

Values with the same letter are not significantly different ($P < 0.05$).

a

IC_{50} was the concentration of substance that provides 50% inhibition.

b

FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.

c

EC_{50} represents the effective concentration at 50% of total antioxidant activity.

Table 5. Antioxidant activity value of ethanol extract of bay leaves obtained by Soxhlet Method.

Samples	Antioxidant activity		
	DPPH method (IC_{50} – ppm)[a]	FRAP method (FRAP value – ppm)[b]	Beta-Carotene method (EC_{50} – ppm)[c]
Gallic Acid	23.87 ± 0.00A	10.60 ± 0.01A	24.87 ± 0.24A
Quercetin	48.87 ± 0.00B	21.94 ± 0.00B	98.44 ± 0.39B
Bay leaves ethanolic extract - percolation	888.08 ± 0.05C	295.00 ± 0.02C	2965.62 ± 0.65C
Bay leaves ethanolic extract - soxhlet	437.89 ± 0.03D	684.00 ± 0.03D	2230.35 ± 1.20D

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.

Values with the same letter are not significantly different ($P < 0.05$).

a

IC_{50} was the concentration of substance that provides 50% inhibition.

b

FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.

c

EC_{50} represents the effective concentration at 50% of total antioxidant activity.

The correlation analysis between each factor in this research was done by 3D linear analysis using SigmaPlot 12.5. The results of the analysis were shown in Tables 6, 7, and 8. Table 6 showed the correlation between extraction method (expressed in concentration, x-axis) and total phenolic content (y-axis) towards antioxidant activity. The level of correlation was shown by the R^2 value. The results showed that there is a high correlation between the extraction method and total phenolic content towards antioxidant activity. The higher to total phenolic content in both extracts will cause the increase in the antioxidant activity.

Table 6. Correlation between extraction method and total phenolic content towards antioxidant activity.

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 0.8310 + 0.445x + 0.0742y	0.9890
	FRAP	f = 0.7649 + 0.0419x+0.0196y	0.9663

Extraction method	Antioxidant method	Function	R^2
	Beta – Carotene Bleaching	f = 3.7012 + 0.0068x + 0.0652y	0.8511
Soxhlet	DPPH	f = 5.2176 + 0.0288x + 0.20083y	0.9137
	FRAP	f = 1.2690 + 0.0465x−0.0501y	0.9949
	Beta – Carotene Bleaching	f = 5.1409 + 0.0032x + 0.1196y	0.9156

Table 7. Correlation between extraction method and total phenolic content towards percent of HMG-CoA Reductase inhibition.

Extraction method	Function	R^2
Percolation	f = 3.9241−0.0955x + 0.6945y	0.8688
Soxhlet	f = 15.4733−0.0299x + 0.4829y	0.8871

Table 8. Correlation between extraction method and antioxidant activity towards percent of HMG-CoA Reductase inhibition.

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 38.8052−0.3180x−3.1319y	0.6154
	FRAP	f = 32.6035 + 0.0486x + 1.1740y	0.6006
	Beta – Carotene Bleaching	f = 15.5054 + 0.0362x + 2.6778y	0.7075
Soxhlet	DPPH	f = 43.3496 + 0.2689x−2.2742y	0.5670
	FRAP	f = 43.5523 + 0.0533x + 1.3197y	0.5750
	Beta – Carotene Bleaching	f = 1.4981−0.0057x + 3.4218y	0.9759

Table 7 showed the correlation between extraction method (concentration, x-axis) and total phenolic content (y-axis) towards percent of HMG-CoA Reductase inhibition. There was also a strong correlation between each factor towards the inhibition of HMG-CoA Reductase activity, but the concentration of extract gave a different effect against the inhibition of HMG-CoA Reductase activity when compared to the total phenolic content. It can be explained that the increase of the concentration of extract will cause the increase also in the total phenolic content, but not all of the phenolic compounds in the extract act as an inhibitor of HMG-CoA Reductase. Thus, some of the phenolic compounds in the extract may act as an activator of the HMG-CoA Reductase.

Correlation between extraction method (concentration, x-axis) and antioxidant activity (y-axis) towards percent of HMG-CoA Reductase inhibition was shown in Table 8. The results of the 3D linear analysis showed a poor correlation between the concentration of extract and antioxidant activity towards the inhibition of HMG-CoA Reductase activity. Thus, though the HMG-CoA Reductase catalyze the reduction-oxidation activity, its inhibition mechanism was not related to the antioxidant

mechanism. We conclude that antioxidant compounds might be contributes to inhibit HMG-CoA Reductase but does not go through in the reduction-oxidation mechanisms.

Based on these results, it can be concluded that the inhibition of HMG-CoA Reductase activity by the percolation and soxhlet extracts are caused by the phenolic compounds in the extracts, and it was suspected due to the flavonoids compounds. Further research needs to be done to confirm this report. The relationship between the flavonoid structure (Fig.2B) with its activity as an enzyme inhibitor of HMG-CoA Reductase is due to the presence of -OH groups in C3 ′, C4′, and C5. It is also caused by the C=O group at C4. These groups play a role in forming hydrogen bonds with amino acids from HMG-CoA Reductase through hydrophobic interaction [26]. It is suspected that these groups play a role in their activity inhibiting the HMG-CoA Reductase enzyme because they have similarities in the pharmacophores group of the simvastatin. In the simvastatin structure (Fig.2A) there is an -OH group and a C=O group (a pharmacophore group) that will form a bond with the enzyme, so that the enzyme work becomes inhibited. The C=O group in lactone ring of simvastatin will be hydrolyzed to become an active form (acid). The hydrolyzed simvastatin will then bind to the HMG-CoA Reductase by hydrogen bonding with the amino acids located on the active site of the enzyme. The structure of the hydrolyzed simvastatin in the lactone ring corresponds to the structure of the HMG-CoA substrate (Fig.2C) so that the enzyme is able to bind with simvastatin and form the complex of enzymes.

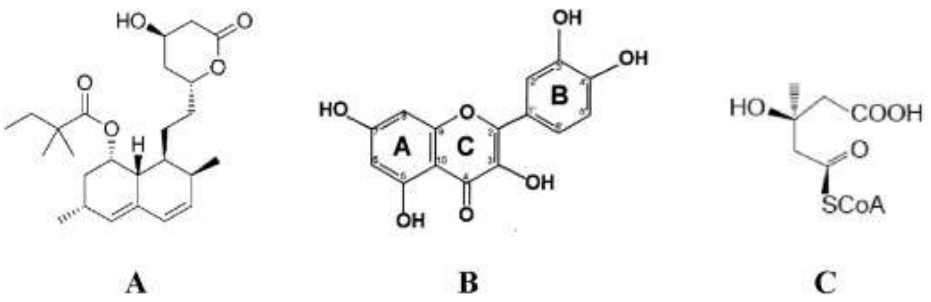

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Fig.2. Structure of simvastatin (A), flavonoid (B), and HMG-CoA (C) [31, 32, 33].

Declarations

Author contribution statement

Lanny Hartanti, Sumi Wijaya: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu: Performed the experiments; Wrote the paper.

Henry Kurnia Setiawan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Lisa Soegianto: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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Heliyon

Received:
18 October 2018
Revised:
16 March 2019
Accepted:
3 April 2019

Cite as: Lanny Hartanti, Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu, Sumi Wijaya, Henry Kurnia Setiawan, Lisa Soegianto. Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA Reductase inhibitory activity. Heliyon 5 (2019) e01485. doi: 10.1016/j.heliyon.2019.e01485

Influence of extraction methods of bay leaves (*Syzygium polyanthum)* on antioxidant and HMG-CoA Reductase inhibitory activity

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Abstract

Objective: Bay leaf, one of the plants in Indonesia that has been shown to have activities to reduce cholesterol in the blood. HMG-CoA Reductase inhibition is one of many mechanisms in lowering the level of cholesterol in the blood. Here, we reported the inhibitory activity of HMG-CoA Reductase of bay leaves ethanol extracts that we suspected to be the mechanism of action of bay leaves in reducing cholesterol in the blood. In this research we also investigated the correlation between the inhibitory activities, the total phenol content and antioxidant activities of bay leaves (*Syzygium polianthum*) ethanol extracts.

Methods: The inhibitory activity of HMG-CoA Reductase was determined kinetically at 340 nm using simvastatin as positive control. *In vitro* scavenging assays of 2,2-diphenyl-1-picrylhydrazyl (DPPH), ferric ion reducing antioxidant power (FRAP), and beta-carotene method were used to determine the antioxidant activities. The total phenolic content was determined by Folin-Ciocalteu's method.

Results: The IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity were

https://doi.org/10.1016/j.heliyon.2019.e01485

49.50 ± 0.700 μg/mL and 15.50 ± 0.707 μg/mL, respectively, while the IC_{50} of simvastatin was 0.00238 ± 0.00004 μg/mL. The antioxidant activity and total phenolic content of bay leaves ethanolic extract obtained by Soxhlet extraction method was higher compared to the percolation method (DPPH and beta-carotene assay results). The 3D linear analysis showed that there was a high correlation between the inhibition activities of HMG-CoA Reductase pattern of both extract types and the total phenol pattern and also the antioxidant pattern of these extracts.

Conclusion: The result showed that the bay leaves ethanolic extract have a potent activity to reduce the cholesterol serum level by inhibition of HMG-CoA Reductase activity. The activity was due to the phenolic compounds in the extracts as well as the antioxidant activity of the extracts.

Keywords: Biochemistry, Molecular biology, Natural product chemistry

1. Introduction

Cardiovascular disease contributed largely to the high mortality rate worldwide year by year. Based on the research in epidemiology, the risk factor of cardiovascular disease is a combination of two or more risk factors. The risk factors of cardiovascular disease are classified into two groups, which are the modifiable risk factors (dyslipidemia, hypertension, smoking, diabetes mellitus, stress, obesity) and the non-modifiable risk factors (heredity, age, gender). A common risk factor of cardiovascular disease is high serum cholesterol level [1, 2, 3].

Cholesterol is a lipid produced in the liver with a number of important roles, such as a membrane constituent and the parent molecule for steroid hormones [4]. Cholesterol can be synthesized by the body and also can be derived from daily food. The increase of cholesterol level in the bloodstream can cause hypercholesterolemia [1]. Hypercholesterolemia is one of the risk factors for the emergence of atherosclerosis, which is inflammatory disorders in artery walls characterized by the formation of atheroma [5]. Atherosclerosis plaque could clog the heart's blood vessel area. This blockage then leads to cardiovascular disease [6]. The increase in cholesterol level can be caused by excessive cholesterol synthesis, the excess of cholesterol absorption, and high cholesterol intake from daily food. Decreasing the cholesterol level can be done by inhibiting cholesterol synthesis through inhibiting the activity of HMG-CoA Reductase which converts Acetyl-CoA into mevalonate [1, 7]. This enzyme is a pharmacological treatment target for group of drugs called HMG-CoA Reductase inhibitor (statins) [8]. However, anti-cholesterol drugs usually are used in combination, and this may increase the chance of unexpected side effects in long-term use.

https://doi.org/10.1016/j.heliyon.2019.e01485

Bay leaves (*Syzygium polyanthum*) is one of the plants that can be used to decrease the cholesterol level [9]. Bay leaves contain secondary metabolites, such as saponin, terpenoid, flavonoid, polyphenol, alkaloid, and essential oil. Some previous *in vivo* studies showed that the extract of bay leaves could lower cholesterol levels in the animal blood [10, 11]. It is believed that flavonoid (phenolic compound) as one of the chemical content of the bay leaves plays a role in the decrease of cholesterol levels in the blood. In addition, the research conducted by Lee et al. [12] proved that flavonoids can lower cholesterol levels by inhibiting the action of HMG-CoA Reductase. Several experiment showed that flavonoids and phenolic acids, which are classes of polyphenolic compounds have antioxidant properties, including induction of anti-inflammatory actions, inhibition of oxidative enzymes, and scavenging of free radicals [13].

Based on the researches that have been done to the animals treated with bay leaves, further research about the potency of bay leaves as the anti-hypercholesterolemia *in vitro* is needed with the enzymatic measurement. The extract of bay leaves used was obtained by Soxhlet extraction and percolation method. The measurement of antioxidant activities in each extract was also done to seek the correlation of antioxidant activities and HMG-CoA Reductase inhibition activities. This research covers the taxonomy of Biochemistry and Molecular Biology.

2. Materials and methods

2.1. Equipment and materials

The equipment used during the study were analytical scales (Sartorius, Germany); oven (Binder); infrared moisture balance (Kett, China); 5 μL capillary tubes; microtubes (Mini spin, USA); vortex; micropipettes; blue tips; white tips; membrane filters; glasswares; chamber; soxhlet; water bath; spectrophotometer (Multiscan Go, Thermo Scientific, USA); cuvettes (Bio-Rads Lab, 2000 Alfred Nobel Drive Hercules, Catalog number 9109250).

Dried bay leaves (*Syzygium polyanthum*) obtained from PT. HRL International Indonesia, Pasuruan, East Java, the enzyme used was the HMG-CoA Reductase Assay Kit (Catalog number CS 1090, Sigma, Germany), 96% ethanol, phytochemical screening reagents, water for injection, sodium hydrogen phosphate (NaH_2PO_4) (Merck, Indonesia), sodium dihydrogen phosphate (Na_2HPO_4) (Merck, Indonesia), simvastatin tablet, antioxidant assay reagents.

2.2. Preparation of extract

Standardization was done to the dried bay leaves prior to the extraction. The extraction was done with percolation and Soxhlet extraction method using ethanol 96% as

https://doi.org/10.1016/j.heliyon.2019.e01485

the solvent. The mass of the dried bay leaves used for percolation method was 1 kg in total 3.6 liter of solvent, while the mass used for Soxhlet extraction was 0.5 kg in total 3.03 liter of solvent divided in several steps, which was 20 gram of dried bay leaves in 120 ml solvent for each process. The rendemen of extract obtained from percolation method was 25.05%, while from soxhlet extraction method was 23.62%.

The extract was then evaporated on a water bath then was stored in a sterile bottle. The dried extract was further standardized to determine the organoleptic characteristics, total ash content, water content, and the solubility in ethanol to ensure the quality. Phytochemical screening was also done to the dried extract prior to antioxidant and enzymatic assay.

2.3. HMG-CoA Reductase activity assay

366 μl 1x assay buffer was mixed with 24 μl HMG-CoA substrate, 8 μl NADPH, and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes [14].

125 mg of bay leaves ethanol extract was dissolved in 25 ml of sterile water to make the standard solution 5000 ppm. The solution was further made into different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. The solution was centrifuged and filtered using a 0.45 μm filter membrane to remove the residual sediment from the extract. 364 μl 1x assay buffer was mixed with 24 μl HMG-CoA substrate, 8 μl NADPH, 2 μl extract from each concentration and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

Standard solution of simvastatin was taken 2 μl from each concentration 0 ppm, 0.0010 ppm, 0.0014 ppm, 0.0018 ppm, 0.0022 and 0.0026 ppm. Each 2 μl solution was mixed with 364 μl 1x assay buffer, 24 μl HMG-CoA substrate, 8 μl NADPH and 2 μl enzyme. The mixture was then measured at 37 °C with a spectrophotometer UV (Multiscan Go, Thermo Scientific, USA) at 340 nm wavelength and the absorbance was read every 15 seconds for 5 minutes.

The data from spectrophotometric was analyzed to determine the enzyme activity (Sigma-Aldrich, 2013), using this equation:

$$\text{Specific activity} = \frac{(\Delta A(\text{sample}/\text{min}) \times \text{TV})}{12.44 \times \text{V enzyme} \times 0.6 \times \text{LP}}$$

https://doi.org/10.1016/j.heliyon.2019.e01485

where ΔA: Change of absorbance, TV: Total volume of the reaction in ml, 12.44: coefficient of NADPH, V enzyme: volume of enzyme used in the assay, 0.6: Enzyme concentration in mg-protein, LP: Lightpath in cm.

2.4. Statistical analysis

All test scores were presented as mean values of inhibition ±standard deviation from two replications. The percent of inhibition was obtained from the activity without inhibitor minus activity with inhibitor divided by activity without inhibitor. For statistical data analysis, each group was compared using independent sample T-test with 95% level of confidence.

2.5. Antioxidant assays

Antioxidant activities of the extracts were assayed by three different methods, which were the DPPH method, the FRAP method, and beta-carotene method. The DPPH method states the antioxidant activity as the oxidation inhibition by referring to Chandra and Dave [15] and Shafazila et al. [16]. The antioxidant potency was measured using % Scavenging effect. The antioxidant assay using FRAP reagent refers to Benzie and Strain [17] where the antioxidant capacity stated as μmoles Trolox/g dry powder. The beta-carotene assay was done according to Utami et al. [18]. The antioxidant potency of the sample was expressed as the concentration with exhibit 50% of the antioxidant activity (EC_{50}).

2.6. Total phenolic content

Extract solution of bay leaves was prepared in different concentration: 0 ppm, 10 ppm, 25 ppm, 50 ppm, 150 ppm, 300 ppm, 600 ppm, and 1200 ppm. Each solution of bay leaves extract was pipetted 100 μl and was mixed with 300 μl of 2% sodium carbonate, 1.58 ml of deionized water, and 100 μl of 10% Folin-Ciocalteu reagent. The absorbance of the reaction mixture was observed at 750 nm (Multiscan Go, Thermo Scientific, USA) after 30 min incubation at room temperature. Gallic acid was used as a standard [19]. The data were expressed as ppm gallic acid equivalents.

3. Results & discussion

Choosing the right extraction method is one of the supporting factors in the success of a therapy, including lowering cholesterol level in the blood. This can be caused by the solubility of secondary metabolites in plants depending on the type of solvent and temperature used during extraction. From the phytochemical screening results, both bay leaves ethanol extract (percolation method and soxhletation method) contain alkaloid, flavonoid, saponin, tannin, steroid.

https://doi.org/10.1016/j.heliyon.2019.e01485

The results of inhibition potency and IC_{50} of bay leaves ethanolic extract obtained by percolation and soxhlet extraction method towards HMG-CoA Reductase enzyme activity can be seen in Tables 1 and 2. Simvastatin, the first generation of statins, was used as a reference compound in this research. The inhibition potency of simvastatin toward HMG-CoA Reductase enzyme is shown in Fig. 1. The IC_{50} value of simvastatin measured in this study was 0.00238 ± 0.00004 μg/mL, which is smaller than the values found in the former researches which were about 0.00376−0.00778 μg/mL [7, 20, 21]. These values (49.50 ± 0.700 μg/mL for extract obtained by percolation, and 15.50 ± 0.707 μg/mL for extract obtained by Soxhlet extraction) were significantly different ($p > 0.05$) if compared to the IC_{50} of simvastatin. The potency of ethanolic extract of bay leaves in inhibiting HMG-CoA Reductase is smaller when compared with simvastatin, where the ability of simvastatin in inhibiting HMG-CoA Reductase about six thousand to twenty thousand times greater than the ethanolic extract of bay leaves.

Several other reports have also reported the potency of plant extracts in HMG-CoA Reductase inhibition. *Opuntia ficus-indica* (L) Miller extract was reported by Ressaissi et al. [22] to have IC_{50} 20.3 μg/ml and said as to have moderate potency. Ademosun et al. [23] reported that grapefruit peels had an IC_{50} on HMG-CoA Reductase activity 0.11 μg/ml. *Vernonia condensata* extract showed the IC_{50} value of 271.7 μg/ml [24] and *Gnetum gnemon* extract had an IC_{50} value on HMG-CoA Reductase of 400 μg/ml [25]. There are also studies that have assayed the potency of several isolated chemical contents of the plants in HMG-CoA Reductase inhibition, and it was reported that the compounds inhibit the enzyme activity with the IC_{50} value 8.34−149.6 μg/ml [22, 26]. Based on these several studies it can be stated that certain plant extract is said to have HMG-CoA Reductase inhibition potency in the range value of IC_{50} between 0.1 to 400 μg/ml [22, 23, 25, 27]. Thus, the ethanol extracts of bay leaves are also a potent HMG-CoA Reductase inhibitor.

Table 1. The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by percolation method.

Concentration (μg/ml)	% of Inhibition		Mean	SD	IC50 (μg/ml)
	n1	n2			
0	0	0	0	0	n1 = 50.00
10	28.49	21.03	24.760	5.275	n2 = 49.00
25	47.10	42.59	44.845	3.189	
50	57.10	57.03	57.065	0.049	
150	64.40	67.02	65.710	1.853	
300	66.24	74.66	70.450	5.954	
600	82.24	83.28	82.760	0.735	
					Mean ± SD = 49.50 ± 0.700

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Table 2. The inhibition of HMG-CoA Reductase of ethanol extract of bay leaves obtained by Soxhlet method.

Concentration (μg/ml)	% of Inhibition		Mean	SD	IC50 (μg/ml)
	n1	n2			
0	0	0	0	0.000	n1 = 15.00
10	47.17	48.55	47.860	0.976	n2 = 16.00
25	54.72	56.16	55.440	1.018	
50	69.81	66.67	68.240	2.220	
150	79.25	76.09	77.670	2.234	
300	88.68	84.42	86.550	3.012	
600	101.9	97.10	99.500	3.394	
					Mean ± SD = 15.50 ± 0.707

The potency of ethanol extract of bay leaves obtained by Soxhlet extraction is three times higher than the potency of ethanol extract of bay leaves obtained by percolation. This showed that the Soxhlet process was able to extract more active constituent that responsible for the inhibition of HMG-CoA Reductase and that the active constituents are stable under heating. It is suspected that these active constituents are polyphenolic compounds such as gallic acid, eugenol, kaempferol and quercetin [28]. Some studies have shown that polyphenolic compounds (luteolin, quercetin, and isorhamnetin) contained in many plant extracts play a role in inhibiting HMG-CoA Reductase activity [22, 27]. The phenolic compound of grapefruit peels (genistein and daidzein) showed inhibition of HMG-CoA Reductase activity competitively against HMG-CoA as substrate [23]. Flavonoids, in specific, are stated by Lee et al. [12] to have the ability to inhibit the activity of the HMG-CoA Reductase. The research conducted by Anggraeni [29] which states that at the same concentration

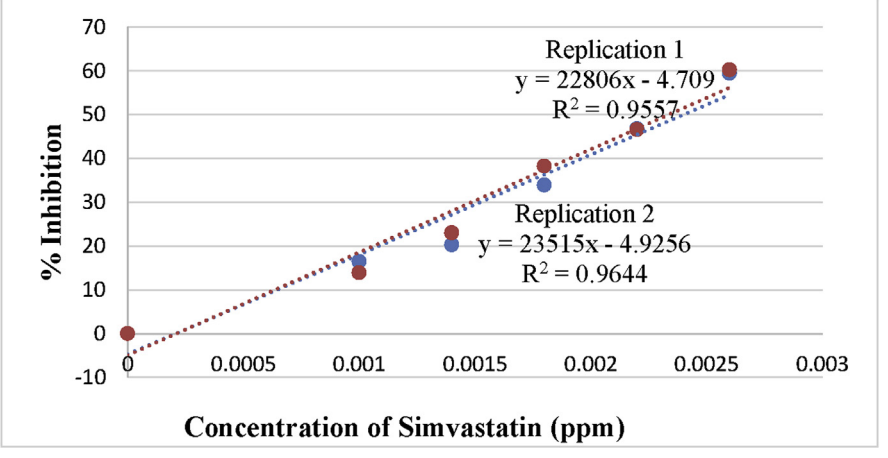

Fig. 1. Graphic of HMG-CoA Reductase inhibition by simvastatin.

https://doi.org/10.1016/j.heliyon.2019.e01485

(10 μg/ml) quercetin and rutin are able to inhibit the activity of HMG-CoA Reductase respectively 41.10% and 60.17 % also support this hypothesis. However, other studies have not mentioned the inhibition kinetics of other flavonoid groups.

The hypothesis that the inhibition of HMG-CoA Reductase in ethanol extract of bay leaves was due to the polyphenolic content was proved by searching the correlation between the inhibition activity and the total phenolic content in the extract. Besides that, we also measured the antioxidant activity of each extract to study the correlation of it to inhibition activity and types of extract. The total phenolic content and antioxidant activity of each concentration involved in the measurement of HMG-CoA Reductase inhibition activity were reported in Tables 3 and 4. The total phenol in the soxhlet extract is greater than the total phenol in the percolation extract, which in accordance with the inhibition of HMG-CoA Reductase activity pattern. The antioxidant activity of each extract, measured by DPPH, FRAP and beta-carotene method, was compared to gallic acid and quercetin (Table 5). The DPPH and beta-carotene method gave the same pattern results, which showed that the antioxidant activity of Soxhlet extract was higher when compared to the percolation extract. These results also in line with the inhibition of HMG-CoA Reductase activity pattern. The FRAP method in the other way gave a different result, which showed that the antioxidant activity of the percolation method is higher than that of the Soxhlet method. This could be caused by the difference in the mechanism of the assay. FRAP method assay was based on the reduction of ferric ion to ferrous ion. Not all of the Fe^{3+} reductants are antioxidant, and some antioxidants are not able to reduce Fe^{3+} [30].

Table 3. Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by percolation method.

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	1.9960A	0A	0.0000A
10	53.6B	3.5532B	0A	10.2513B
25	56.0B	3.8627B	0A	8.0186C
50	61.4C	5.1647C	0.9625A	13.1217D
150	99.0D	8.5790D	8.3633B	7.9707E
300	150.4E	24.9729E	21.3933C	12.5075F
600	193.7F	43.3887F	22.2472C	21.2928G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.
Values with the same letter are not significantly different ($P < 0.05$).
[a] IC50 was the concentration of substance that provides 50% inhibition.
[b] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.
[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

https://doi.org/10.1016/j.heliyon.2019.e01485

Table 4. Total phenolic content and antioxidant activity of ethanol extract of bay leaves obtained by Soxhlet method.

Concentration (μg/ml)	Total phenol content (ppm)	Antioxidant activity		
		DPPH method[a]	FRAP method[b]	Beta-Carotene method[c]
0	0.0A	2.2224A	0A	0A
10	35.4B	4.1808B	0A	14.9736B
25	90.8C	5.1574C	0A	15.2237C
50	92.4C	10.0685D	0A	18.4625D
150	139.0D	20.1246E	0A	20.6429E
300	187.9E	46.5714F	4.2877B	27.6990F
600	201.8F	66.9863G	19.1348C	29.0379G

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.
Values with the same letter are not significantly different ($P < 0.05$).
[a] IC_{50} was the concentration of substance that provides 50% inhibition.
[b] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.
[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

The correlation analysis between each factor in this research was done by 3D linear analysis using SigmaPlot 12.5. The results of the analysis were shown in Tables 6, 7, and 8. Table 6 showed the correlation between extraction method (expressed in concentration, x-axis) and total phenolic content (y-axis) towards antioxidant activity. The level of correlation was shown by the R^2 value. The results showed that there is a high correlation between the extraction method and total phenolic content towards antioxidant activity. The higher to total phenolic content in both extracts will cause the increase in the antioxidant activity.

Table 5. Antioxidant activity value of ethanol extract of bay leaves obtained by Soxhlet Method.

Samples	Antioxidant activity		
	DPPH method (IC_{50} − ppm)[a]	FRAP method (FRAP value − ppm)[b]	Beta-Carotene method (EC_{50} − ppm)[c]
Gallic Acid	23.87 ± 0.00A	10.60 ± 0.01A	24.87 ± 0.24A
Quercetin	48.87 ± 0.00B	21.94 ± 0.00B	98.44 ± 0.39B
Bay leaves ethanolic extract - percolation	888.08 ± 0.05C	295.00 ± 0.02C	2965.62 ± 0.65C
Bay leaves ethanolic extract - soxhlet	437.89 ± 0.03D	684.00 ± 0.03D	2230.35 ± 1.20D

Data were obtained from three independent experiments, each performed in triplicates (n = 9) and represented as mean ± SD.
Values with the same letter are not significantly different ($P < 0.05$).
[a] IC_{50} was the concentration of substance that provides 50% inhibition.
[b] FRAP value was calculated as Ferrous Equivalents, the concentration of trolox/quercetin or extracts which produced an absorbance value equal to that of 1 mM Fe_2SO_4.
[c] EC_{50} represents the effective concentration at 50% of total antioxidant activity.

https://doi.org/10.1016/j.heliyon.2019.e01485

Table 6. Correlation between extraction method and total phenolic content towards antioxidant activity.

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 0.8310 + 0.445x + 0.0742y	0.9890
	FRAP	f = 0.7649 + 0.0419x+0.0196y	0.9663
	Beta − Carotene Bleaching	f = 3.7012 + 0.0068x + 0.0652y	0.8511
Soxhlet	DPPH	f = 5.2176 + 0.0288x + 0.20083y	0.9137
	FRAP	f = 1.2690 + 0.0465x−0.0501y	0.9949
	Beta − Carotene Bleaching	f = 5.1409 + 0.0032x + 0.1196y	0.9156

Table 7. Correlation between extraction method and total phenolic content towards percent of HMG-CoA Reductase inhibition.

Extraction method	Function	R^2
Percolation	f = 3.9241−0.0955x + 0.6945y	0.8688
Soxhlet	f = 15.4733−0.0299x + 0.4829y	0.8871

Table 8. Correlation between extraction method and antioxidant activity towards percent of HMG-CoA Reductase inhibition.

Extraction method	Antioxidant method	Function	R^2
Percolation	DPPH	f = 38.8052−0.3180x−3.1319y	0.6154
	FRAP	f = 32.6035 + 0.0486x + 1.1740y	0.6006
	Beta − Carotene Bleaching	f = 15.5054 + 0.0362x + 2.6778y	0.7075
Soxhlet	DPPH	f = 43.3496 + 0.2689x−2.2742y	0.5670
	FRAP	f = 43.5523 + 0.0533x + 1.3197y	0.5750
	Beta − Carotene Bleaching	f = 1.4981−0.0057x + 3.4218y	0.9759

Table 7 showed the correlation between extraction method (concentration, x-axis) and total phenolic content (y-axis) towards percent of HMG-CoA Reductase inhibition. There was also a strong correlation between each factor towards the inhibition of HMG-CoA Reductase activity, but the concentration of extract gave a different effect against the inhibition of HMG-CoA Reductase activity when compared to the total phenolic content. It can be explained that the increase of the concentration of extract will cause the increase also in the total phenolic content, but not all of the phenolic compounds in the extract act as an inhibitor of HMG-CoA Reductase. Thus, some of the phenolic compounds in the extract may act as an activator of the HMG-CoA Reductase.

Correlation between extraction method (concentration, x-axis) and antioxidant activity (y-axis) towards percent of HMG-CoA Reductase inhibition was shown in Table 8. The results of the 3D linear analysis showed a poor correlation between the concentration of extract and antioxidant activity towards the inhibition of HMG-CoA

https://doi.org/10.1016/j.heliyon.2019.e01485

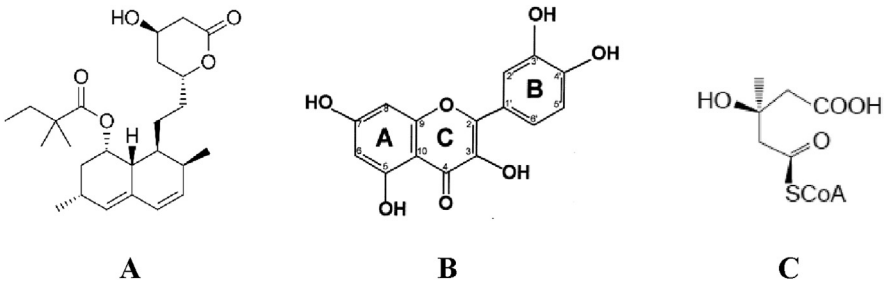

Fig. 2. Structure of simvastatin (A), flavonoid (B), and HMG-CoA (C) [31, 32, 33].

Reductase activity. Thus, though the HMG-CoA Reductase catalyze the reduction-oxidation activity, its inhibition mechanism was not related to the antioxidant mechanism. We conclude that antioxidant compounds might be contributes to inhibit HMG-CoA Reductase but does not go through in the reduction-oxidation mechanisms.

Based on these results, it can be concluded that the inhibition of HMG-CoA Reductase activity by the percolation and soxhlet extracts are caused by the phenolic compounds in the extracts, and it was suspected due to the flavonoids compounds. Further research needs to be done to confirm this report. The relationship between the flavonoid structure (Fig. 2B) with its activity as an enzyme inhibitor of HMG-CoA Reductase is due to the presence of -OH groups in C3 ′, C4′, and C5. It is also caused by the C=O group at C4. These groups play a role in forming hydrogen bonds with amino acids from HMG-CoA Reductase through hydrophobic interaction [26]. It is suspected that these groups play a role in their activity inhibiting the HMG-CoA Reductase enzyme because they have similarities in the pharmacophores group of the simvastatin. In the simvastatin structure (Fig. 2A) there is an -OH group and a C=O group (a pharmacophore group) that will form a bond with the enzyme, so that the enzyme work becomes inhibited. The C=O group in lactone ring of simvastatin will be hydrolyzed to become an active form (acid). The hydrolyzed simvastatin will then bind to the HMG-CoA Reductase by hydrogen bonding with the amino acids located on the active site of the enzyme. The structure of the hydrolyzed simvastatin in the lactone ring corresponds to the structure of the HMG-CoA substrate (Fig. 2C) so that the enzyme is able to bind with simvastatin and form the complex of enzymes.

Declarations

Author contribution statement

Lanny Hartanti, Sumi Wijaya: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data; Wrote the paper.

https://doi.org/10.1016/j.heliyon.2019.e01485

Stefania Maureen Kasih Yonas, Josianne Jacqlyn Mustamu: Performed the experiments; Wrote the paper.

Henry Kurnia Setiawan: Conceived and designed the experiments; Analyzed and interpreted the data; Contributed reagents, materials, analysis tools or data.

Lisa Soegianto: Contributed reagents, materials, analysis tools or data.

Funding statement

This work was supported by the Ministry of Research, Technology and Higher Education of the Republic of Indonesia.

Competing interest statement

The authors declare no conflict of interest.

Additional information

No additional information is available for this paper.

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