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Original Article

The role of Indonesianin-5 in HMG CoA reductase and sterol regulatory element binding protein-2 expressions of hypercholesterolemic rat

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Abstract

Increased cholesterol level increases the risk of heart disease and stroke. Sterol Regulatory Element Binding Protein-2 (SREBP-2) is a transcription factor for the synthesis of cholesterol genes. SREBP-2 can induce the expression of HMG CoA Reductase (HMGR). HMGR is a key cholesterol synthesis enzyme. Indonesianin-5, derived from Mahogany seeds (*Swietenia macrophylla* K), is a flavonoid. Indonesianin-5 is known to have a hypocholesterolemic effect. This study aimed to analyze the mechanism of Indonesianin-5 to decrease LDL and cholesterol levels and the expression of hepatic SREBP-2 and HMGR. *Rattus norvegicus* were grouped into 5 groups: normal group (N), Hypercholesterolemia group (HC), Hypercholesterolemic groups given simvastatin (HC+Sim), 10 mg of Indonesianin-5 (HC+ 10 Ind-5) and 90 mg of Indonesianin-5 (HC+ 90 Ind-5). *In silico* analysis was used to determine the effects of Indonesianin-5. The expressions of SREBP-2 and HMGR were analyzed by the RT-PCR method. Indonesianin-5 inhibited the activity of the HMGR enzyme. Expression of SREBP-2 was high after induction of Indonesianin-5 ($p<0.05$). The expression of HMGR was high after induction of Indonesianin-5 ($p<0.05$). Indonesianis-5 inhibits HMG CoA reductase and increases gene expression of SREBP-2 and HMG CoA reductase.

Keywords: Indonesianin-5, SREBP-2, HMG CoA reductase, RT-PCR, anti-hypercholesterolemia.

Introduction

Hypercholesterolemia is a condition of increasing total fasting cholesterol levels without increasing triglyceride levels in the blood [1]. Based on Balitbang, the Ministry of Health of the Republic of Indonesia in the 2013 RISKESDAS showed that 35.9% of Indonesia's population over 15 years old had abnormal cholesterol levels [2]. High cholesterol level is a risk for heart disease and stroke [3].

Most of the cholesterol level in the body is a product of auto-synthesis in the liver [4]. Auto synthesis mechanism is regulated by cholesterogenic genes, particularly HMGR enzyme and SREBP-2. SREBP-2 is a transcription factor for genes involved in the synthesis and excretion of cholesterol. Nuclear SREBP-2 is an active protein that can transcribe Sterol Regulatory Element (SRE) genes to the RNA of some specific proteins for cholesterol regulation. One of the proteins is HMGR [5]. HMG CoA reductase is a rate-limiting step of the synthesis of cholesterol. This enzyme has the function to reduce 3-hydroxy-3-methylglutaryl (HMG) CoA to become a mevalonate compound [6, 7].

Statin compounds like simvastatin or atorvastatin have been known as anti-hypercholesterolemia agents.

These compounds will inhibit HMGR enzymes' activity to decrease cholesterol's endogenous auto-synthesis. This mechanism will decrease hepatic cholesterol [8, 9].

Some Flavonoids have activity as anti-hypercholesterolemia agents. Indonesianin-5 (7-hydroxy-2-(4-hydroxy-3-methoxy-phenyl) -chroman-4-on) is one of the flavonoid compounds [10]. Based on research conducted by Prasetyastuti *et al.* (2019) and Ayunda *et al.* (2019), Indonesianin-5 derived from mahogany seeds (*Swietenia macrophylla* K) can improve lipid profiles and antioxidant activity in hyperlipidemic rats [11, 12]. However, it has yet to be discovered how the mechanism and the molecular influence of mahogany flavonoids actually improve lipid profiles. Accordingly, in this study, we analyzed the mechanism of Indonesianin-5 supplementation by docking analysis and the role Indonesianin-5 on the expressions of HMGR and SREBP-2, which are important factors in cholesterol metabolism.

Material and methods

Animal experiment

Hypercholesterolemia model and flavonoid supplementation

Study subjects were Rattus norvegicus, Wistar strain, weighing 180–210 grams, aged eight weeks. The animals were acclimatized for a week in a cage and given normal feed, namely AIN-93. The composition is shown in Table 1. After the acclimatization, animals were divided into the following groups: Normal group (N) given 0.5 mL/Kg saline, Hypercholesterolemic group (HC) is untreated hypercholesterolemic rats, Hypercholesterolemic groups given simvastatin 80 mg/70 Kg BW (HC+Sim), Hypercholesterolemic groups given 10 mg/200 g BW of Indonesianin-5 (HC+ 10 Ind-5) and Hypercholesterolemic groups given 90 mg/200 g BW of Indonesianin-5

(HC+ 90 Ind-5). Each group had treatment orally for four weeks. The hypercholesterolemia diet contained about 1% pure cholesterol (Table 1) and was given for seven days [13]. Lipid levels in rat blood were measured by enzymatic CHOD-PAP methods. This method used the Cholesterol FS Diagnostic System Kit (DiaSys, Germany) to confirm that rats had hypercholesterolemia. The livers were removed for SREBP-2 and HMGR gene expression analysis.

In silico analysis

Molecular docking and ADME analysis

In silico molecular docking of the inhibitory peptides and HMG CoA reductase was the preliminary step involved in the preparation of HMG CoA reductase as the protein molecule (PDB ID: 1hwk1) which was obtained from the Research Collaboratory for Structural Bioinformatics Protein Data Bank (RCSB PDB, http://www.rcsb.org/pdb/home/home.do). Simvastatin (PubChem CID:54454) was used as an inhibitor ligand to compare the affinity energy of Indonesiasin-5. Ligands underwent geometry optimization by Avogadro. Protein optimization by removing the polar hydrogen group and water used AutoDock version 4.0. Autogrid was done in the native ligand position on the binding

Table 1: Composition diet.

	AIN 93 M (standar) (g)	Pakan induksi hiperlipidemia (g)
Tepung jagung	620.692	620.692
Casein	140	140
Fat (corn oil)	40	40
sucrose	100	100
Serat (CMC)	50	50
Mineral mix (AIN 93-MX)	35	35
Vitamin mix (AIN 93-VX)	10	10
L-Cystenin	1.8	1.8
Choline bitartrat	2.5	2.5
Pure Cholesterol	0	10
Cholic acid	0	2

site by arranging the grid coordinates 40x40x40. Ligand tethering of the protein was done by regulating genetic algorithm parameters with 100 runs of Genetic Algorithm criteria. The analysis was done by studying Gibs Energy and inhibition constant. Parameters were classified based on the lowest energy or cluster. The ligand interaction and active site of protein complexed were visualized using Discovery Studio visualizer version. Indonesianin-5 was analyzed by the SwissADME server (http://www.swissadme.ch) from the Swiss Institute of Bioinformatics supported by ChemAxon) for physiochemistry, lipophilicity, solubility, and pharmacokinetics analysis.

RNA extraction, cDNA synthesis and RT-PCR

The following method was used to analyze the expression of SREBP-2 and HMG CoA reductase with beta-actin as an internal control. Previously, RNA was isolated from the liver using the Tri-RNA Favorgen (Favorgen, Taiwan), chloroform, isopropanol and 75% alcohol. The RNA results were converted to cDNA using Thermo Scientific RevertAid First Strand cDNA Synthesis Kit #K1622 (Waltham, MA USA). RT-PCR was a method for gene expression analysis used GoTag Green Master Mix, Promega #M7122 (Madison, USA), primers (Macrogen, Seoul) and NFW. Amplification of SREBP-2 and HMGR genes used primers listed in Table 2.

cDNA was amplified to the following conditions: 95˚C for 3 seconds (initial denaturation), 95˚C for 30 seconds (denaturation), 58˚C (SREBP-2), 62˚C (HMGR) and 62˚C (*β actin*) for 60 seconds (annealing), 72˚C for 1 minute (extension), and 72˚C for 10 minutes (last extension) for 40 cycles. The PCR products were analyzed in 2% agarose gel Genedirex #mb755-0100 (German) along with a 100 bp DNA Hyperladder Bioline #33029 (London). Expressions of the gene were quantified with densitometry analysis using ImageJ software. β actin was used as a housekeeping gene.

Statistical analysis

Gene expressions were measured by calculating band density in agarose by ImageJ of the target gene and internal control. Data were normality tested by Shapiro-Wilk tests. Normally distributed data were analyzed by ANOVA test and post hoc Tukey test to measure the difference between the groups.

Results

Molecular docking of Indonesianin-5

Molecular docking was used to analyze the activity of indonesianin-5. HMGR is a rate-limiting step of cholesterol synthesis, and some antihypercholesterolemia drugs inhibit its activity. Docking result of Indonesianin-5 on HMGR enzyme compared to simvastatin Docking result which has well-known acts as an antihypercholesterolemic and can inhibit HMGR. Data are presented in Table 3.

Based on the above data, Indonesianin-5 has Gibs energy similar to simvastatin with three amino acids in active sites as binding sites of Indonesianin-5 just simvastatin. Indonesianin-5 has low energy to binding on active sites, which means that the affinity of Indonesianin-5 is high. However, the inhibitory constant (Ki) of Indonesianin-5 is higher than simvastatin. Figure 1 shows the interaction of the amino acids in active sites with simvastatin (1b) and Indonesianin-5 (1a).

Expression SREBP-2 and HMGR increased by Indonesianin-5

SREBP-2 expression was lower in the hypercholesterolemia group than the normal group, but it was not statistically significant ($p>0.05$). Data are presented in

Table 2: Gene primers for amplification.

Gen		Primer
SREBP-2	Forward	AGCATACCGCAAGGTGTTC
	Reverse	CAGGTGTCTACTTCTCCGTGT
HMGR	Forward	CTTGACGCTCTGGTGGAATG
	Reverse	AGTTGGAAGCACGGACATA
Beta-actin	Forward	TGTGGATTGGTGGCTCTATC
	Reverse	AGAAAGGGTGTAAAACGCAG

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Table 3: Comparation *in silico* analysis of Indonesianin-5 and simvastatin.

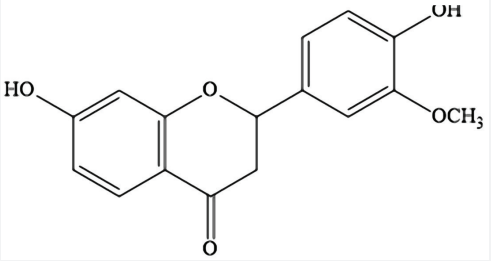
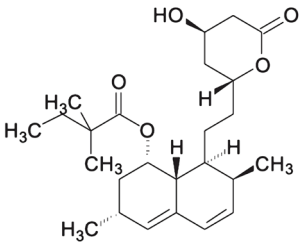
Parameter		
	Indonesianin-5	**Simvastatin**
Gibs energy (KJ/mol)	-4.00	-4.50
Amino acid binding site	Ala682, Lys692, **Ser684**, Asp690, **Arg590**, **Val683**	Lys691, Cys688, **Ser684**, **val683**, **Arg590**, Ser661, Asn658
Type of binding	Van der Waals: Ser684; Hydrogen Bond: Lys692, Val683, **Arg590**, Asp690; Pi Bond: Arg590; Pi donor hydrogen bond: Ser684, Lys692; Pi-sigma bond: Ala682	Van der Waals : Ser684; Hydrogen Bond : **Arg590** Ser661, Asn658; Carbon hydrogen Bond: Val683, Cys688; Alkyl bond: Lys691
IC50 (Ki)	1.18 mM	505.58 µM

Figure 2. Simvastatin and Indonesianin-5 increased the expression of the SREBP-2 gene more than the hypercholesterolemic and normal groups. Enhancement of SREBP-2 expression was lower in the hypercholesterolemia group given Indonesianin-5 than the hypercholesterolemia group given simvastatin, but the difference was not statistically significant ($p>0.05$). This result is the same as the HMGR expression that is presented in Figure 3.

Figure 3 shows that HMGR expression in the hypercholesterolemia group was lower than in the normal group, but the expression between the two was not significantly different. HMGR gene expression was significantly different Indonesianin-5 supplementation and simvastatin compared to the hypercholesterolemia group ($p<0.05$). HMGR gene expression in the hypercholesterolemic groups given 10 mg/200 BW, 90 mg/200 g BW of Indonesianin-5, and simvastatin were not statistically different ($p>0.05$).

Based on data (Table 4), Indonesianin-5 has a higher solubility than the simvastatin drug. At the same time, the lipophilicity of simvastatin is higher than indonesianin-5, which is 3.74. Both active compounds, indonesianin-5 and simvastatin, have high permeability to the gastrointestinal tract.

Discussion

The research results showed that The Gibs energy of Indonesianin-5 was similar to simvastatin to inhibit the activity of HMG CoA reductase enzyme. It appeared to be the main mechanism of Indonesianin-5 to decrease LDL lipid and total cholesterol levels in a study conducted by Ayunda *et al.*, 2019. The expression of SREBP-2 and HMG CoA reductase gene high after Indonesianin-5 administration.

Based on antihypercholesterol activity that had been published before, we did *in silico* analysis. *In silico* analysis showed that Indonesianin-5 can inhibit the activity of HMG CoA reductase enzyme. Indonesianin-5 can bind to active sites of HMGR, but the Gibs energy for binding was lower than simvastatin. When the Gibs energy has a negative value, then the ligand-protein binding occurs spontaneously. According to recent research, ΔG determines the stability of protein-ligand complex [14]. This mechanism may increase expression of SREBP-2 dan HMGR genes. This appeared to be the main mechanism of Indonesianin-5 to decrease LDL lipid and total cholesterol levels.

In silico analysis showed that there are five types of binding of Indonesianin-5 and HMG CoA reductase which are hydrogen bonds, Van der Waal bonds, pi bonds, pi donor hydrogen bonds, and pi sigma bonds. Whereas there are four types of binding of simvastatin and HMG CoA reductase, which are hydrogen bonds, Van der Waal bonds, alkyl bonds and carbon hydrogen bonds. There is some similarity between Indonesianin-5 and simvastatin. Both molecules have Van der Waals bonds to one amino acid in a specific position, which is serine 684. Active sites for binding of HMG to HMG reductase were previously found to be lys735,

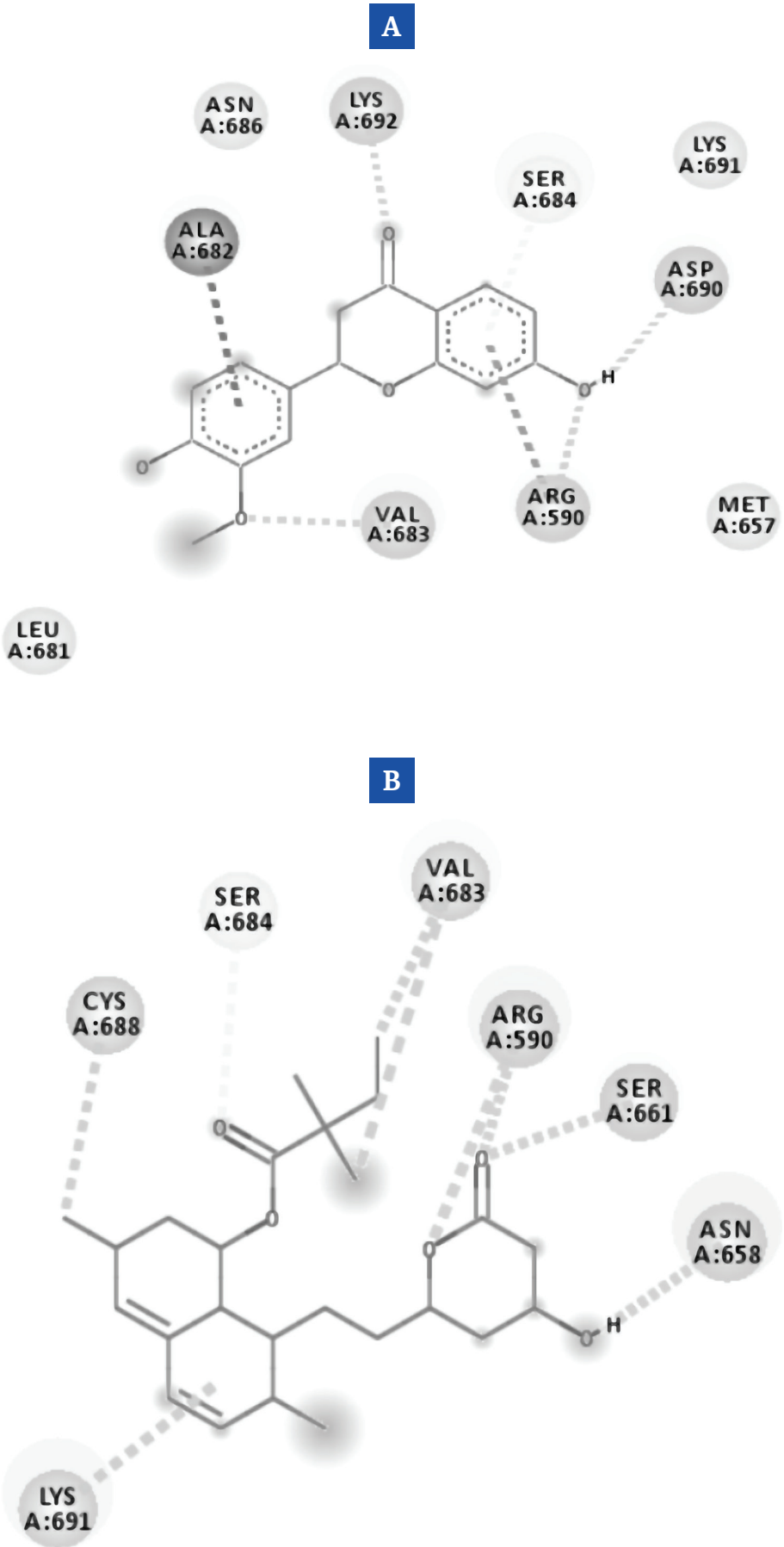


Figure 1: Interaction of Indonesianin-5 (A) and simvastatin (B) with amino acids in active sites of HMG CoA reductase. 3D picture of interaction Indonesianin-5 and HMG CoA reductase (C) and bond distance indonesianin-5 and HMG-CoA reductase (D).

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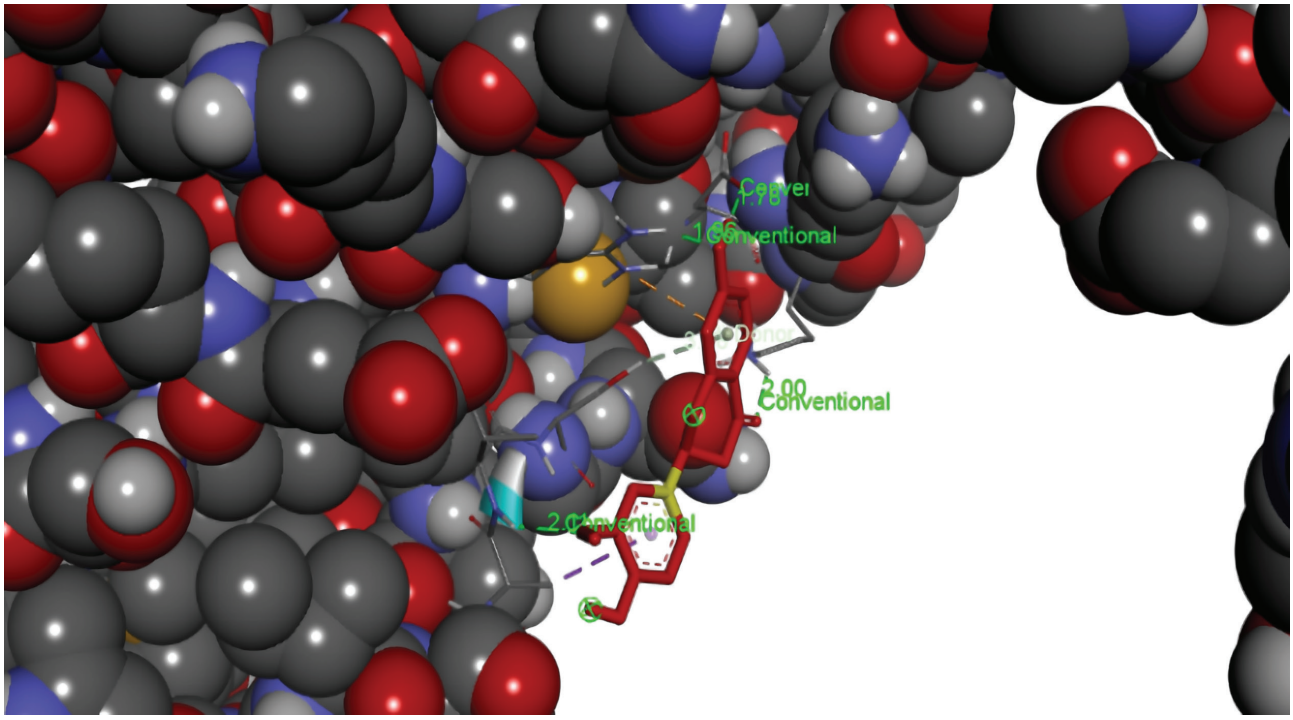
C



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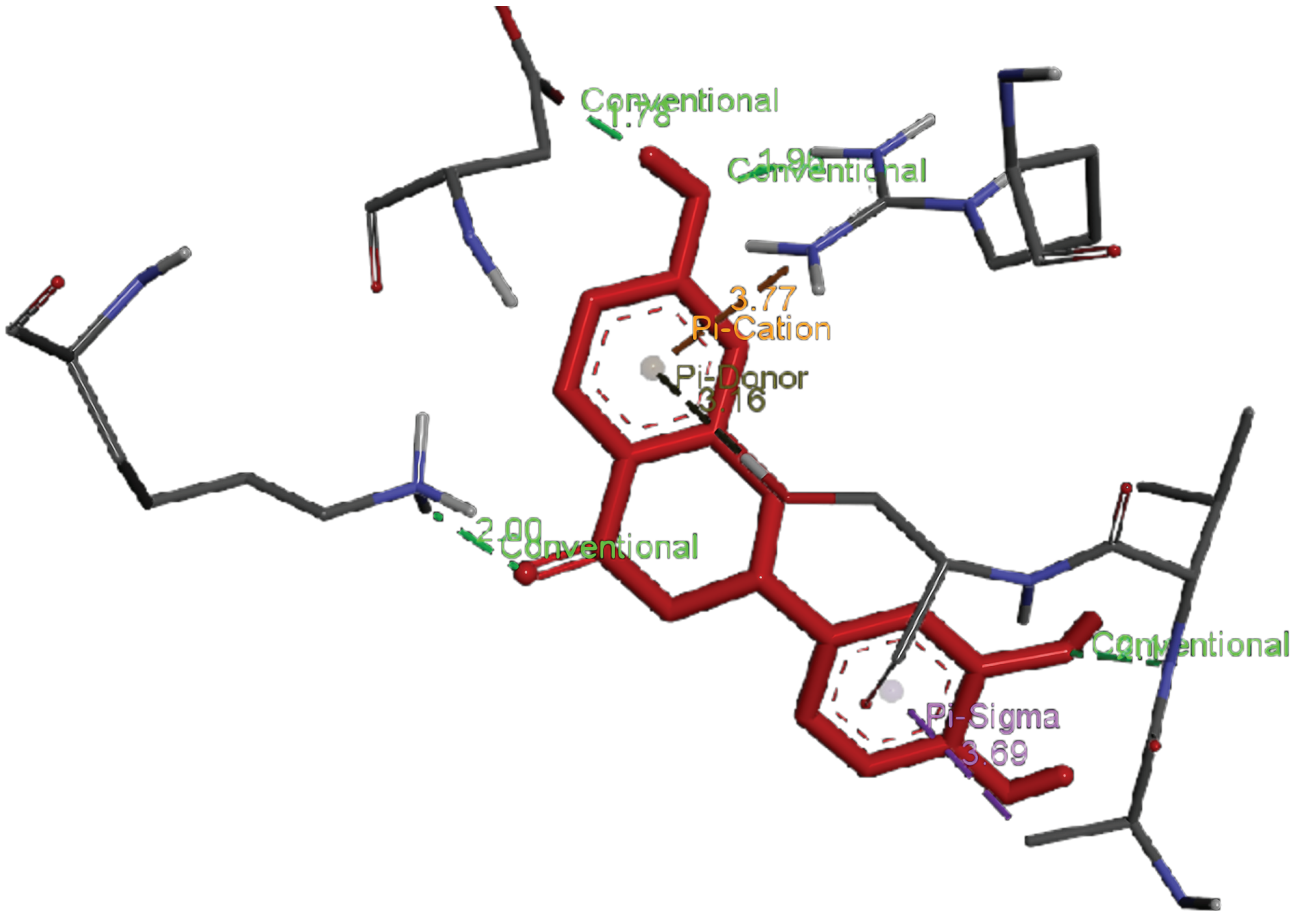

Figure 1: Continued.

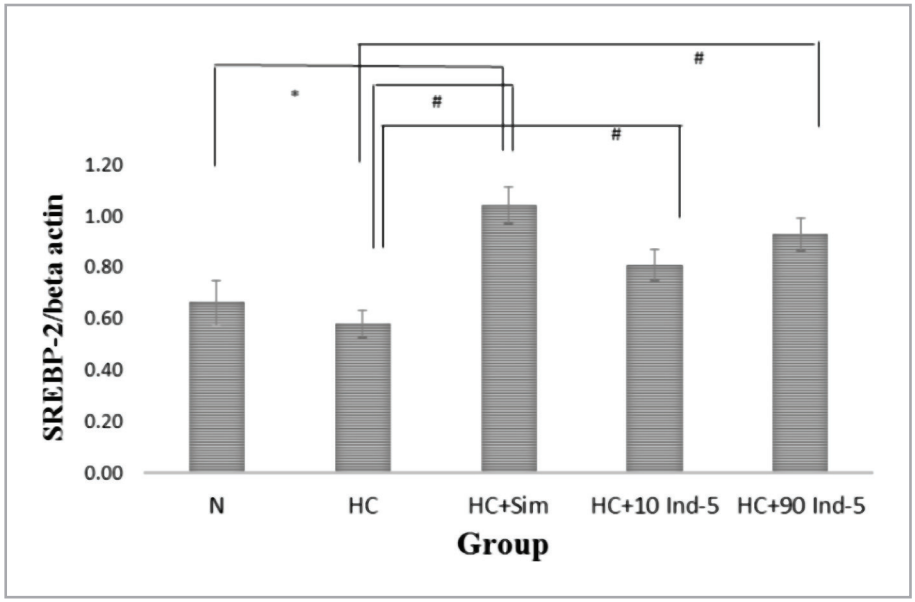

Figure 2: Graph of ratio expression of SREBP-2. Data distribution was tested using Saphiro Wilk (p>0.05), data were presented in the mean (n=6) and ±SEM. Comparison of expression of SREBP in each group by One way of ANOVA p<0.05 with Post hoc analysis using Tukey test. *p<0.05 *vs*. N, #p<0.05 *vs*. HC. N: Normal group, HC: Hypercholesterolemic, HC+Sim: Hypercholesterolemia+Sim. HC+10 Ind-5: Hypercholesterolemia diet given with 10 mg of Indonesianin-5, HC+ 90 Ind-5: Hypercholesterolemia diet given with 90 mg of Indonesianin-5.

Ser684, Lys692, Leu853, Asp690, Arg590, Lys691, Glu559, and Asn755 [15]. Binding sites of simvastatin to active sites of HMGR were on R590 and S684. Binding sites of Indonesiani-5 to active sites of HMGR were on Lys 692, Ser684, Asp690, and Arg590. Based on this result, Indonesianin-5 acted as a competitive inhibitor to HMGR, like simvastatin, because it binds to substrate-binding sites. This process is illustrated in Figure 1. Carbonyl and hydroxyl group of Indonesianin-5 bond to amino acids of HMGR enzyme with hydrogen bond with a bond length of approximately 2.1 A. Indonesianin-5 also has covalent bonding, pi cation bond, pi donor bond and

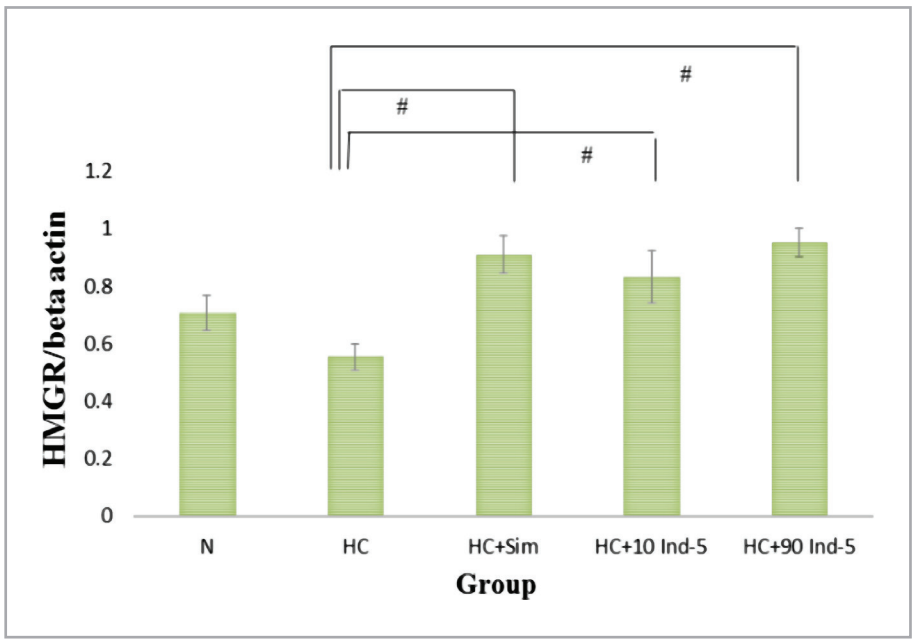

Figure 3: Graph of ratio expression of HMGR. Data distribution was tested using Saphiro Wilk (p>0.05), and data were presented as the mean (n=6) and ±SEM. Comparison of expression of SREBP in each group by One way ANOVA P<0.05 with Post hoc analysis using Tukey test. #p<0.05 *vs*. HC. N: Normal group, HC: Hypercholesterolemic, HC+Sim: Hypercholesterolemia+Sim. HC+10 Ind-5: Hypercholesterolemia diet given with 10 mg of Indonesianin-5, HC+90 Ind-5: Hypercholesterolemia diet given with 90 mg of Indonesianin-5.

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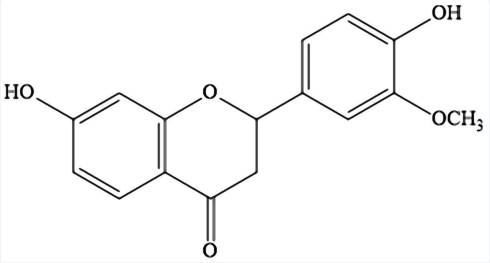
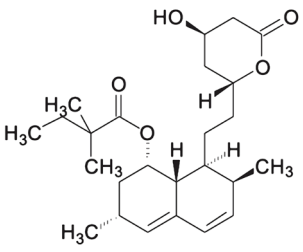
Table 4: Comparison of bioavailability Indonesianin-5 and Simvastatin (*in silico* analysis).

Parameter		
	Indonesianin-5	**Simvastatin**
Lipophilicity (log Po/w)	2.14	3.74
GI absorption	High	High
Solubility	Soluble	Moderately soluble

pi-sigma bond with the benzene group of Arg590 and Ala 682. The bond length is 3.77 A, 3.16 A, and 3,69 A.

Based on the *in silico* analysis result (Table 2), Indonesianin-5 has Ki that is about twice larger than simvastatin. Ki values are used to compare some inhibitors of the same enzyme. Ki values that are lower indicate this inhibitor was more effective in inhibiting enzyme activity and needs less dose or concentration for inhibition [16]. Indonesianin-5 had a higher Ki than simvastatin. This value indicates that the antihypercholesterol activity of indonesianin-5 requires a high dose to have the same activity as simvastatin activity in inhibiting the HMGR enzyme.

The result also shows that indonesianin-5 can absorb through the gastrointestinal tract but has lipophilicity lower than simvastatin. High lipophilicity exhibits a high ability of the molecule to penetrate the membrane cell [17]. HMGR is an intracellular enzyme that inhibits it from entering cells. It showed that simvastatin has a higher ability to enter cells than indonesianin-5. It might cause the activity of indonesianin-5 to be lower than simvastatin.

Based on antihypercholesterol activity that had been published before and *in silico* analysis. After that, we analyzed the molecular effect of indonesianin-5. After administration of Indonesianin-5, SREBP-2 and HMGR expressions were higher than the normal and hypercholesterolemia groups. Research conducted by Bok *et al.* in 1999 showed that the administration of naringenin and hesperidin, which are flavanones, is known to inhibit HMG CoA reductase enzyme activity similar to statins [18].

Naringenin compounds are also known to increase the expression and maturation of SREBP- 2. High expressions of SREBP-2 increase LDLR expression, an important protein for cholesterol uptake. SREBP-2- 2 is a transcription factor for genes involved in cholesterol synthesis, so increased expression and activity of SREBP-2 can induce expression of HMG CoA reductase which is one of the enzymes involved in cholesterol synthesis [19, 20]. Kartawijaya *et al.* (2016) found that genistein, one of the flavonoids, upregulated mRNA and protein of LDLR. Increasing LDLR genes and proteins are because of the increase of the nuclear SREBP-2 and binding SREBP-2 by JNK signaling [21]. Indonesianin-5 causes high SREBP-2 and HMGR expression. This compound, by *in silico* analysis, can inhibit the activity of HMG CoA reductase. This mechanism, such as simvastatin, is thought to be the mechanism that causes Indonesianin-5 to increase the expression of SREBP-2 and HMGR.

After administration of simvastatin, SREBP-2 and HMGR expressions were higher than the normal and hypercholesterolemia groups. Statin treatment in this study showed increased HMGR and SREBP-2 gene expression. This finding is in line with a study conducted by Roglans *et al.* (2002) that found statins increase HMGR and SREBP-2 genes [9]. Statin also increases hepatic cholesterol by increasing reuptake cholesterol from the blood by increasing LDLR and ACAT genes by inhibiting the activity of HMGR. The research by Schonewille *et al.* in 2016 also showed that statin treatment increased HMGR and SREBP-2. Increasing SREBP-2, increasing LDLR for cholesterol uptake, Abcg 5, Abcg8, and Cyp7a1. Abcg 5, Abcg8, and Cyp7a1 are genes for bile synthesis. This study also revealed that statin treatment increases endogenous cholesterol by biosynthesis of cholesterol but not by increasing plasma cholesterol. This occurred because of the high activity of bile synthesis and bile excretion to the intestines [8].

Results of this study also showed that the homeostatic mechanism was still occurring by the expression

of HMGR and SREBP-2 in the hyperlipidemia group, which tended lower than the normal group. This result is because cholesterol and its metabolites become suppressors for SREBP-2 activation. These results indicate that one week of induction did not disturb molecular regulation; instead, there were only metabolic changes.

While many variables are related to cholesterol metabolism, lipoprotein and toxicity were not analyzed in this study such as LDLR and Cyp7a1 to more show the mechanism of Indonesianin-5 as an anti-hypercholesterolemia agent. Accordingly, more research is needed to explain this complex interaction. Interventions for a high-cholesterol diet must last longer than a week so it can better explain molecularly the effects and mechanisms involved in a high-cholesterol diet and anti-hypercholesterol substances.

Conclusion

Based on our research results, Indonesianin-5 can inhibit HMG CoA reductase enzymes to generate an increased expression of SREBP-2 and HMG CoA reductase. The most effective dose was 90 mg/200 BW, which had a high percentage of activity toward LDL and total cholesterol levels with a competitive inhibitory mechanism.

Acknowledgments

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Conflict of interest

The authors declare no conflict of interest.

Ethics approval

The Medical and Health Research Ethics Committee approved the experimental protocol of the Faculty of Medicine, Public Health and Nursing, Universitas Gadjah Mada (FKKMK UGM) number KE/FK/0729/EC on July 19th, 2018.

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