Pakistan Veterinary Journal

ISSN: 0253-8318 (PRINT), 2074-7764 (ONLINE)
DOI: 10.29261/pakvetj/2022.023

RESEARCH ARTICLE

Nanoparticles of *Kirinyuh* (*Chromolaena odorata* (L.) R.M.King & H.Rob.) Leaves Extract as a Candidate for Natural Remedies Lowering Hypercholesterol: In Silico and In vivo Study

Shafira Salwa Salsabil[1], Vania Putri Ardana[1], Raden Roro Prakasita Budi Larastiyasa[1], Inarotul Wardah Pratiwi[2], Rizky Aprillia Widianti[2] and Anggi Muhtar Pratama[3*]

[1]Faculty of Veterinary Medicine, Universitas Gadjah Mada, Sleman, Yogyakarta, 55281, Indonesia; [2]Faculty of Mathematics and Natural Science, Universitas Gadjah Mada, Sleman, Yogyakarta, 55281, Indonesia; [3]Department of Pharmacology, Faculty of Veterinary Medicine, Universitas Gadjah Mada, Sleman, Yogyakarta, 55281, Indonesia
*Corresponding author: anggi.muhtar.p@mail.ugm.ac.id

ARTICLE HISTORY (21-427)

Received: October 26, 2021
Revised: March 17, 2022
Accepted: April 02, 2022
Published online: April 22, 2022

Key words:
Anti hypercholesterolemia
Kirinyuh leaves
Nanoparticles
Molecular docking
In silico study.

A B S T R A C T

Hypercholesterolemia in recent years has become a serious problem because of its cause of almost half of cases of ischemic heart disease. Alternative medicine most people usually use also has a weakness, namely weak pharmacological effects. This study aims to optimize the pharmacological effect of *kirinyuh* leaves as an antihypercholesterolemic agent by making nanoparticles preparations. This study used in silico and in vivo studies, the in silico study was done to predict the potential of flavonoid, one of *kirinyuh* leaves' compounds which can inhibit HMG-CoA reductase (HMGCR) enzyme, that have a role of cholesterol making. In vivo study was done to hypercholesterolemia mouse model (n=24) who were given of *kirinyuh* leaves extract each with 100 mg (T1) and 200 mg/200 g body weight (BW) (T2) dose, and *kirinyuh* leaves extract in nanoparticle formulas each 1 mL (T3) and 2 mL/200 g BW (T4) every day for 2 weeks. Blood was snipped at the end of treatment for lipid profile analysis. Molecular docking's result showed that flavonoid compounds can reduce hypercholesterolemia by interacting with HMGCR enzymes thus the formation of cholesterol in the liver can be inhibited, therefore T1-T4 are capable to improve blood lipid profile significantly compared to control ($P<0.05$). The best result is shown in T4 that gave 2 mL/200 g BW dose of nanoparticle formulation with the most reduction of total cholesterol and lipid profile between all the treatment groups. It can be concluded that nanoparticle formulation has better pharmacologist effect.

To Cite This Article: Salsabil SS, Ardana VP, Larastiyasa RRPB, Pratiwi IW, Widianti RA, Pratama AM 2022. Nanoparticles of *kirinyuh* (*Chromolaena odorata* (L.) R.M.King & H.Rob.) leaves extract as a candidate for natural remedies lowering hypercholesterol: in silico and in vivo study. Pak Vet J, 42(3): 397-403. http://dx.doi.org/10.29261/pakvetj/2022.023

INTRODUCTION

Hypercholesterolemia is a condition when cholesterol levels in blood plasma are high which is also characterized by an increase in Low Density Lipoprotein (LDL), triglycerides, and a decrease in High Density Lipoprotein (HDL) in the blood (Rawat *et al.,* 2020). According to the World Health Organization (WHO), hypercholesterolemic conditions in the long term can cause ischemic heart disease and coronary heart disease which are closely related to the deaths of 17.9 million people in the world (Threapleton *et al.,* 2015). Treatments that are still often used are statin, but as we already know, chemical drugs have various side effects for the body.

Several treatment innovations for hypercholesterolemia have been carried out, one of them by utilizing herbal plants. A research conducted by Nouri and Abad (2013) has succeeded in proving that tomatoes and tomato paste can improve the condition of hypercholesterolemia within one month, but giving these tomatoes takes a long time for this hypercholesterolemic condition to return to normal. Baskaran *et al*. (2015) also proved that gendola leaves extract can reduce cholesterol in the blood within a period of four weeks, but the use of this herbal plant is still not optimal because gendola leaves is a seasonal plant whose growth is strongly influenced by weather conditions.

Ikewuchi and Ikewuchi (2011) stated that *kirinyuh* leaves has a diuretic effect that causes a decrease in blood pressure, body weight, prevent diseases such as diabetes

mellitus, dyslipidemia, hypertension, and obesity. This leaves is familiarly consumed with steeping method. According to Michel *et al*. (2020) the use of aqueous extract of *kirinyuh* leaves can be used intra-gastrically to experimental animals and results in reductions in total cholesterol (TC), LDL, non-HDL, VLDL, and triglycerides. Idoko *et al.* (2018) also stated that *kirinyuh* fresh leaves aqueous extract possesses hypolipidemic and hypoglycemic ability.

This study was conducted by having the extract being made as an ethanolic extract. In accordance with Ugwoke *et al*. (2017), the ethanol extract of *kirinyuh* leaves contain higher values/percentage of the phytochemicals present in the extract. The use of 70% ethanol solvent allows to produce high-level yields of the extraction owing to its similar polarity with most of the components in the plant (Rahardhian *et al.,* 2019). The 70% ethanol solvent can remarkably dilute phytochemical compounds since the solvent contained optimum water content (30%) that could help in the extraction process. For this reason, we chose to make the extract with 70% ethanol as the solvent.

Kirinyuh leaves are considered to be an alternative to herbal plants that can be used as natural therapeutic ingredients to cure hypercholesterolemia conditions because they contain several compounds including alkaloids, flavonoids, tannins, and steroids (Vijayaraghavan *et al.,* 2017). These compounds will act as antioxidants which can lower cholesterol levels in the blood. *Kirinyuh* leaves' flavonoid compounds have hypolipidemic properties that can inhibit the activity of the HMGCR enzyme which causes excess cholesterol formation. Therefore, flavonoid compounds in *kirinyuh* leaves can inhibit cholesterol synthesis in the body (Bao *et al.,* 2016).

In silico study was conducted to confirm the interaction between flavonoids and HMGCR enzymes to form stable complexes. Great result in in silico study indicates that flavonoids can actually inhibit cholesterol synthesis. This study is important as a preliminary before proceeding to in vivo.

Unfortunately, the use of *kirinyuh* leaves in the form of steeping is still not able to optimize the active compounds contained in these plants, therefore it is necessary to innovate preparations that are able to provide optimal natural treatment for hypercholesterolemic patients. This research was conducted to maximize the pharmacological effect of *kirinyuh* leaves ethanol extract as an antihypercholesterolemic agent and make it more effective by making *kirinyuh* leaves ethanol extract in polymer nanoparticle preparations. Nanoparticle serves as a drug delivery system which aims to control drug delivery, therefore the drug can reach the intended receptor. Reducing the particle size of the extract will expand the absorption surface so that the rate of solution increases and accelerates drug absorption (Garg *et al.,* 2019). This process gives advantages to nanoparticles of *kirinyuh* leaves ethanol extract to have good pharmacological effects even in smaller doses, therefore it is expected to reduce side effects from the medicine.

This study aims to determine the potential of flavonoid compounds in *kirinyuh* leaves as antihypercholesterolemic agents through in silico test and compare the effect of giving *kirinyuh* leaves extract with *kirinyuh* leaves extract nanoparticles in improving blood lipid profile through in vivo tests.

MATERIALS AND METHODS

Materials: The tools used are dry blender, 70 mesh filter, cloth, beaker glass, erlenmeyer, water bath, magnetic stirrer, volumetric flask, measuring cup, mouse cage, rat drinking place, Particle Size Analyzer, and a set of laptops equipped with Chimera applications, AutodockTools, Discovery Studio, GaussianView, and SPSS. The materials used were *kirinyuh* leaves, chitosan, sodium tripolyphosphate, comfeed-AD II, 99% sigma cholesterol, cholic acid, 70% ethanol, 99% v/v acetic acid, polysorbate 80, and distilled water.

Molecular docking: The receptor used was HMGCR (ID 2Q1L) downloaded from the Protein Data Bank. This method consists of separation of receptors from other unnecessary molecules and ligands, optimization, re-docking, matching the optimized ligand using flavonoid compounds in *kirinyuh* leaves based on Vijayaraghavan *et al*. (2017) and statin as commercial medicine for hypercholesterolemia (Table 1) with the best conformation of the native ligand, tethering of the ligand compounds to the to the active site of the HMGCR receptor, analyzing the docking results and analyzing the parameters amino acid residues and hydrogen bonds.

Plant determination: *Kirinyuh* leaves was obtained from Bantul Regency, Special Region of Yogyakarta. The plant was identified at Faculty of Biology, Universitas Gadjah Mada, Yogyakarta, Indonesia.

Extraction and nanoparticles preparation: The simplicia of *kirinyuh* leaves was extracted by maceration method using 5 litre of 70% ethanol. Extract of *kirinyuh* leaves was kept in the refrigerator. Nanoparticle of *kirinyuh* leaves extract was made by ionic gelation method. The particle size of nanoparticles was examined by using particle size analyzer.

Cholesterol induced rats: A total of 24 Sprague-Dawley rats aged 2 - 2,5 months with a body weight of 150 - 200 grams were divided into 6 groups with 4 rats in each group. The rats were divided into 6 groups: control 1 (C1): no treatment, control 2 (C2): high fat diet, treatment 1 (T1): extract 100 mg/200 g BW, treatment 2 (T2): extract 200 mg/200 g BW, treatment 3 (T3): nanoparticles 1 mL/200 g BW, treatment 4 (T4): nanoparticles 2 mL/200 g BW. All the rats except C1 were given by 0,3 gr of cholesterol sigma grade ≥99% and 0,03 cholic acid for 6 weeks orally. After the experimental animals was proven to experience hypercholesterolemia, based on the results of the examination of total cholesterol levels and lipid profiles in blood that exceed normal limits in the 6th week, the extract and nanoparticles of *kirinyuh* leaves extract would be given to rats by oral route for 14 days. During that time, all the rats were still given standard feed and water on an ad libitum basis.

Total cholesterol level and lipid profile examination: The total cholesterol test used the Cholesterol Oxidase-

Peroxsidase Aminoantypirin (CHOD-PAP) method, while the lipid profile test consisted of testing the levels of triglycerides, LDL, and HDL. Triglycerides were tested by the Glyserol Peroxidase Phosphat Acid (GPO-PAP) method, while HDL and LDL were tested by the precipitation method.

Histopathological examination: To measure the microscopic changes of liver cells, a scoring system was used refers to the semi-quantitative scoring system of Meyerholz and Beck (2018), with the scoring value of necrosis and fatty degeneration: 1 = < 25%, the value of 2 = 25-<50%, the value of 3 = 50-<75%, and value 4 = 75-100%. The assessment was carried out by observing five different fields of view.

Statistical analysis: Qualitative analysis was carried out descriptively on plant identification, nanoparticle characterization and in silico test. Quantitative analysis on lipid profile examination was carried out using the Shapiro-Wilk method followed by One-way ANOVA. Comparative non-parametric test was carried out for histopathological examination with Chi square for histopathological examination.

RESULTS

Molecular docking result: The visualization of HMGCR Receptor (PDB ID 2QIL) and native ligand are presented in Figure 1. Interaction between flavonoid compounds from *kirinyuh* leaves and HMGCR enzyme receptor (ID 2Q1L) (Fig. 2) with a value of negative Gibbs free energy (Table 2), showed binding site amino acid residue. The enzymes of HMGCR contains catalytic amino residues that play a role in the catalytic activity of HMGCR enzymes, namely histidine (His-866), lysine (Lys-735, and Lys-691), aspartate (Asp-690, and Asp-767), and glutamate (Glu-559), as well as other contributing amino acids include asparagine (Asn-755), tyrosine (Tyr-479), and serine (Ser-864). The strength of the hydrogen bond interaction can stabilize the bond between ligand and the receptor showed in Table 3. Other interactions that can support bond stability between the ligand and the receptor are electrostatic interactions and van der walls interactions (Table 3).

Characterization of nanoparticle of *kirinyuh* leaves extract: A total of 1 kg of simplicia produced 105.11 grams of extract, so the yield of the extract was 10.51%. To improve the pharmacological effect, *kirinyuh* leaves was formulated into nanoparticle using ionic gelation method. Nanoparticle of *kirinyuh* leaves was characterized with Particle Size Analyzer (PSA), based on the result, the nanoparticle size of *kirinyuh* leaves extract was 20.3 nm and the Polydispersity Index (PI) value was 0.150.

Total cholesterol and lipid profile findings: Total cholesterol and lipid profile were examined from the hypercholesterolemic group rats (C2-T4) to ensure that the rats had hypercholesterolemia. Based on the results of the examination, rats in the hypercholesterol group had higher total cholesterol (TC), triglycerides, and LDL as much as 200.24 mg/dL, 142.68 mg/dL, 76.19 mg/dL, respectively, while HDL was found in the lower level as much as 24.75 mg/dL. After the rats were confirmed to experience hypercholesterolemia, rats from hypercholesterolemic group except from the control group (C1 and C2)

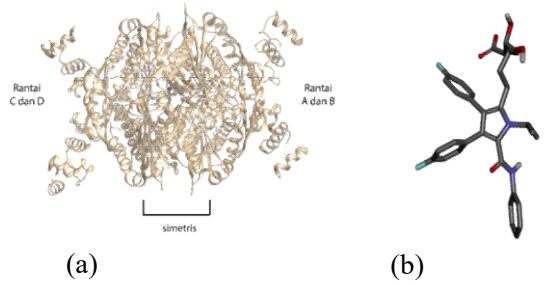

Fig. 1: HMGCR Receptor (PDB ID 2Q1L) (a) and native ligand (b).

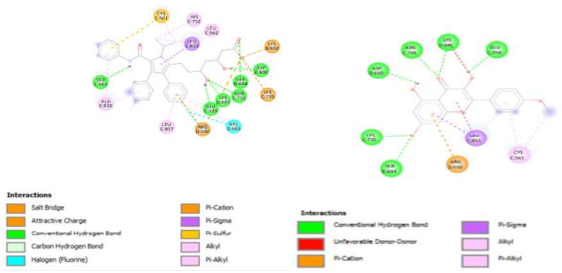

Fig. 2: Interaction between *native ligand* (a) and compound 4 (b) with HMGCR receptor.

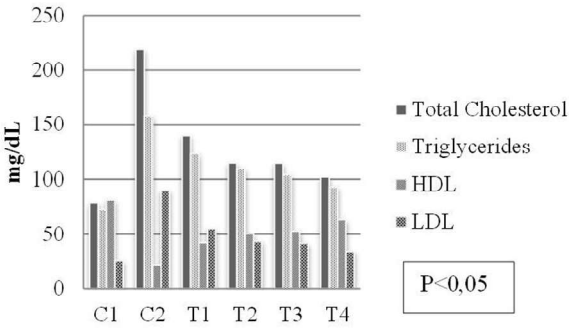

Fig. 3: The average results of measurements of lipid profiles and total cholesterol in mice after given *kirinyuh* leaves extract and nanoparticles (Description: Control 1 (C1): no treatment; Control 2 (C2): high fat diet; Treatment 1 (T1): high fat diet + *kirinyuh* leaves extract 100 mg/200 g body weight of rats; Treatment 2 (T2): high fat diet + *kirinyuh* leaves extract 200 mg/200 g body weight of rats; Treatment 3 (T3): high fat diet + nanoparticles of *kirinyuh* leaves extract 1 mL/200 g body weight of rats; Treatment 4 (T4): high fat diet + *kirinyuh* leaves extract nanoparticles 2 mL/200 g of body weight of rats.

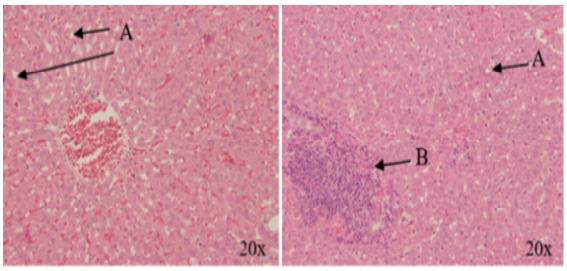

Fig. 4: HE-stained liver tissue: Description: **A**: Fat vacuole; **B**: Necrosis

Table 1: Flavonoid compounds (FC) in *Kirinyuh* Leaves (Vijayaraghavan et al., 2017)

Structure number	Flavonoid compound
1	(FC) *Odoratenin*
2	(FC) *Aromadendrin 4 methyl ether*
3	(FC) *Eriodictyol 7,4 dimethyl ether*
4	(FC) *Kaempherol 4 methyl ether*
5	(FC) *Naringenin 4 methyl ether*
6	(FC) *Scultellarein tetramethyl ether*
7	(FC) *Quercetin*
8	(FC) *Sinensetin*
9	Statin

Table 2: Values of Bond Free Energy

Compound	Gibbs Free Energy (kkal/mol)
Native ligand	-8.89
1	-5.08
2	-3.96
3	-4.28
4	-4.71
5	-4.85
6	-2.75
7	-3.59
8	-4.55
9	-5.03

Table 3: Amino acid residues to the HMGCR receptor (PDB ID 2Q1L)

Ligand	Bond types	Residue
Native ligand	*Hydrogen Bond*	*Asp-690, Lys-691, Lys-735, Asn-755, Ser-684, Glu-559,Ser-565, Lys-692*
	Electrostatic	*Arg-590 (2 bond), Lys-692, Lys-735*
	Hydrophobic	*Leu-853 (2 bond), His-752, Ala-856 (2 bond), Leu-857, Val-683*
Compound 1	*Hydrogen Bond*	*Lys-691, Lys-735, Asn-755, Ser-684, Cys-561*
	Electrostatic	*Arg-590,*
	Hydrophobic	*Leu-853 (2 bond), Leu-857, Cys-561*
Compound 2	*Hydrogen Bond*	*Asp-690, Lys-691, Lys-735, Asn-75, Glu-559, Lys-692 (2 bond)*
	Electrostatic	*Arg-590 (2 bond)*
	Hydrophobic	*Leu-853 (3 bond)*
Compound 3	*Hydrogen Bond*	*Lys-691, Asn-75, Asp-690, Arg-590, Asn-658, Ala-751*
	Electrostatic	*Arg-590, Glu-559*
	Hydrophobic	*Met-657 (2 bond), Cys-561*
Compound 4	*Hydrogen Bond*	*Asp-690, Lys-691, Lys-735, Asn-755 (2 bond), Ser-684, Glu-559, Ser-565*
	Electrostatic	*Glu-559, Arg-590*
	Hydrophobic	*Leu-853 (3 bond), Cys-561*
Compound 5	*Hydrogen Bond*	*Lys-735, Asn-755, Lys-691, Asp-690, Ser-684*
	Electrostatic	*Arg-590*
	Hydrophobic	*Leu-853 (3 bond), Cys-561*
Compound 6	*Hydrogen Bond*	*Lys-691, Asn-755, Ser-565, Asp-690*
	Electrostatic	*Glu-559*
	Hydrophobic	*Ala-856 (4 bond), Lys-691, Leu-857, Val-683, Leu-853 (2 bond)*
Compound 7	*Hydrogen Bond*	*Lys-691, Asn-755, Glu-559, Asp-690, Cys-561*
	Electrostatic	*Arg-590*
	Hydrophobic	*Leu-853 (2 bond), Cys-561*
Compound 8	*Hydrogen Bond*	*Lys-735, Asn-755, Ser-684, Asp-690, Asn-658, Gly-650*
	Electrostatic	*Arg-590, Glu-559 (2 bond)*
	Hydrophobic	*Leu-853 (2 bond), Cys-688, Lys-691, Lys-692, Met-657, Cys-561 (2 bond)*
Compound 9	*Hydrogen Bond*	*Lys-735, Arg-590, Ser-684*
	Hydrophobic	*Val-683, Met-657, Ala-856, His-752, Leu-853*

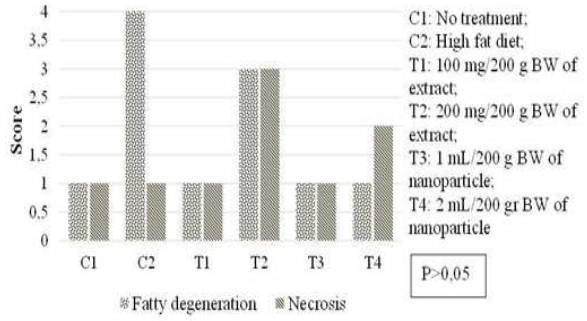

Fig. 5: Graph of histopathological analysis in rat liver.

Table 4: Histopathological Examination Results of Rat Liver per Group

Treatment	Score	Description
Control 1	1 = degeneration	Fatty degeneration <25%
	1 = necrosis	Necrosis <25%
Control 2	4 = degeneration	Fatty degeneration 75-100%
	1 = necrosis	Necrosis <25%
Treatment 1	1 = degeneration	Fatty degeneration <25%
	1 = necrosis	Necrosis <25%
Treatment 2	2-3 = degeneration	Fatty degeneration 25% - <75%
	3 = necrosis	Necrosis 50% - <75%
Treatment 3	1 = degeneration	Fatty degeneration <25%
	1 = necrosis	Necrosis <25%
Treatment 4	1 = degeneration	Fatty degeneration <25%
	2 = necrosis	Necrosis 25% - <50%

would be getting treatment with *kirinyuh* leaves (T1 and T2) and nanoparticle of *kirinyuh* leaves (T3 and T4) for 14 days. Total cholesterol and lipid profile were examined again from all groups after the 14 days treatment showed in Figure 3. The statistical analysis was carried out by using One-way ANOVA method for total cholesterol and profile lipid examination, it revealed a significant decrease ($P<0.05$) in all of the groups compared to control groups (C1 and C2).

Histopathological findings: Based on the results of histopathological examination, the liver tissue of the rats showed necrosis and fat degeneration. The result is presented in Figure 4.

DISCUSSION

Fat derived from food will be absorbed by the intestinal lumen in the form of chylomicrons which will circulate in the blood vessels and hydrolyzed by endothelial lipoprotein lipase into nonesterified fatty acids and glycerides, then chylomicrons are absorbed in the liver (Shattat, 2014). Based on Göbel *et al.* (2020), the first two reversible reactions in the liver are catalyzed by

thiolase and HMG-CoA synthase. This leads to the condensation of two molecules of acetate to form acetoatyl-CoA which is condensed with a third molecule of acetate to form 3-hydroxy-3methylglutaryl-CoA coenzyme A (HMG-CoA). The following reaction is a key regulatory point in cholesterol synthesis and is catalyzed by the enzyme HMGCR which reduces HMG-CoA to mevalonate (Göbel *et al.,* 2020). Once the product from the mevalonate pathway has been obtained, the process is continued by condensation of six molecules of isopentenyl pyrophosphate to form squalene which is then cyclized and converted into cholesterol through several steps (Göbel *et al.,* 2020).

Most of lipid metabolism happened in the liver, the histopathological changed in the liver can be seen because of the administration of cholesterol orally. Histopathological examination was carried out by observing liver cells through microscopic images of rat's liver with a magnification of 50 micrometers and based on Figure 4, the types of changes observed in the liver of the rats were necrosis and fat degeneration. Fat degeneration is characterized by the presence of fat vacuoles and the cell nucleus being pushed to the edge, while necrosis is characterized by the absence of a cell nucleus and cell membrane that is not clearly visible (Iswari *et al.,* 2020).

In liver tissue, the highest fatty degeneration was found in the C2 group (high cholesterol diet group without treatment) around 75-100% in five fields of view (Figure 5). This group was likened to rats that had hypercholesterolemia before being given treatment. Rats after given treatment experienced a decrease in the number of fatty degeneration cells by 25-50% in all groups (T1, T2, T3, and T4), these groups were given *kirinyuh* leaves extract and nanoparticle of *kirinyuh* leaves extract. The T2 group experienced the smallest decrease in the number of fatty degenerated cells, while T1, T3, and T4 had the largest decrease in the number of fatty degenerated cells. According to Casey *et al*. (2021), the accumulation of lipids in the liver is the cause of fat degeneration that can progress to necrosis. This occurred due to the administration of a high-cholesterol diet.

Necrosis is a form of cell death (Iswari *et al.,* 2020). Based on the observation of liver tissue, the most necrotic cells were found in the T2 group with necrosis range 50-<75%. Followed by T4 which experienced less necrosis, which was between 25-<50% in five fields of view. Several etiologies of hepatic necrosis include toxins, drug-induced injury, viral infections, ischemia, and metabolic diseases (Butler et al., 2018). The appearance of necrosis in the T4 liver does not necessarily indicate toxicity. According to Asomugha *et al*. (2015), the estimated toxicity of *kirinyuh* leaves extract is >5000 mg/kg BW, while the content of the extract in nanoparticles at a dose of 2 mL/200 g BW is only 2 mg, so the occurrence of necrosis in the treatment 4 group with 2 mL/200 g BW of nanoparticles was predicted to happen due to a high cholesterol diet. According to Iswari *et al*. (2020), massive lipid droplets in hypercholesterolemic patients have the potential to increase the oxidation of unsaturated fatty acids which can damage hepatocytes and trigger necrosis.

However, it is important to fix the hypercholesterolemic condition with some treatments. The rats were given *kirinyuh* leaves extract and nanoparticle of *kirinyuh* leaves orally for 14 days. *Kirinyuh* leaves contain flavonoid compounds (Table 1) that have the potential to inhibit the limiting enzyme of the mevalonate pathway (HMGCR enzyme) by interacting with its receptor (ID 2Q1L), therefore the activity of cholesterol synthesis through the mevalonate pathway is disrupted.

Flavonoids in *kirinyuh* leaves can inhibit the HMGCR enzyme in the D chain by forming hydrogen bond interactions with amino acid residues such as lysine, aspartate and glutamate, according to the results of the in silico study. These residues are the main amino acid residues that play a role in inhibiting the catalytic activity of the HMGCR enzyme (Lateef *et al.,* 2019). Therefore, this inhibition will slow down cholesterol synthesis and result in a decrease in TC, LDL, triglycerides, and an increase in HDL according to the results in the treatment group 1 up to 4.

Gibbs energy is the energy required for ligands to bond with protein receptors with the value of bond free energy from molecular docking presented in Table 2. Based on the results, all of the compounds have a value of negative Gibbs free energy. The negative gibbs free energy is more valuable which means that the bond between the ligand and the receptor has a good stability (Du *et al.,* 2016). It also showed that the flavonoid compounds' free energy is not much different from statin as a commercial antihypercholesterolemic medicine. According to the Gibbs free energy, there are 3 flavonoids that have the lowest energy, namely odoratenin, kaempherol, and naringenin. Odoratenin has the lowest energy which means it is the closest compound to the native ligand which is even lower than statins, commonly used medicine for anti-hypercholesterolemia. This shows the stability of the odoratenin interaction with the receptor is better than statins.

The strongest interaction between ligands and receptors is hydrogen bond. The strength of the hydrogen bond interaction can stabilize the bond between ligand and the receptor. Other interactions that can support bond stability between the ligand and the receptor are electrostatic interactions and van der walls interactions (Lateef *et al.,* 2019). Visualization of ligand docking results with receptors from hydrogen bonding, electrostatic, and hydrophobic interaction on Table 3 can be seen that in general the ligand compounds had an interaction major catalytic with amino acid residues of the lysine, aspartate, and glutamate classes. The key amino acid residues that play a role are Asp-690, Lys-691, Lys-735, Asn-755, Ser-684, Glu-559. These amino acid key residues act as HMGCR inhibitor. Based on the interaction of the ligand with amino acid, it can be seen that the flavonoid compounds of *kirinyuh* leaves had the potential as hypercholesterol reducing agent.

Meanwhile, when considered from the number of the hydrogen bond interaction between ligand and receptor, Kaempherol 4 methyl ether is the flavonoid that has the most role as an HMGCR inhibitor because it binds to 7

amino acid residues. The interaction of Kaempherol 4 methyl ether is greater than Statin which is known as a commercial antihypercholesterol medicine that only binds to 3 amino acids residues (Table 2). When compared with the native ligand, Kaempherol 4 methyl ether has 5 key amino acids residues which are the same as the native ligand, while statins only have 1 key amino acids residues are the same as the native ligand. Odoratenin has hydrogen bonds with 5 amino acids and has the same 3 key amino acids as the native ligand. While Naringenin 4 methyl ether has hydrogen bonds with 5 amino acids and has the same 4 key amino acids as the native ligand. Based on the interaction of ligands with key amino acids residues, it can be seen that the flavonoid compounds of *kirinyuh* leaves have the potential as hypercholesterol reducing agents with kaemppherol 4 methyl ether as the most likely flavonoid.

Gibbs energy value of kaempherol 4 methyl ether is the third most negative and has the most hydrogen bond interactions with amino acids residues which means that the bond between the ligand and the receptor has good stability. Based on previous research by Kong *et al*. (2013), they reported that kaempherol is a potential anti-atherogenic agent that prevents vascular inflammation. Their in vivo study of rabbits fed with ten weeks of a high-cholesterol diet aimed to determine the anti-inflammatory effect of kaempherol as an atherosclerosis medicine. The results showed that the cholesterol level and arteriolar lesions of rabbits were significantly reduced. A study by Zeka *et al*. (2017) reported that flavonoids such as kaempherol, naringenin, myricetin, and epigallocatechin gallate (EGCG) can inhibit HMG-CoA reductase activity.

It also supported with the result of in vivo study which showed improvement of the blood cholesterol levels with the decrease of TC, LDL, triglyceride, and an increase in HDL can be seen in Fig. 3. The lowest TC examination results were found in group T4 that was given the nanoparticles of *kirinyuh* leaves extract at a dose of 2 mL/200 g BW rats with an average TC level of 102.21 mg/dL (Fig. 3). This value was followed by treatment group 3, 2, and 1 with the value of 114.63 mg/dL; 114.8 mg/dL; and 139.63 mg/dL, respectively. This happened in all treatment groups because *kirinyuh* leaves contain flavonoid compounds that have the potential to inhibit the limiting enzyme of the mevalonate pathway by interacting with the HMGCR receptor (ID 2Q1L) (Göbel *et al.,* 2020) therefore the cholesterol synthesis activity through the mevalonate pathway can be disrupted, according to the result of in silico study.

Low Density Lipoprotein is often referred as 'bad cholesterol' because it has a function in carrying 60-70% cholesterol and have a functions in transporting cholesterol from the liver to peripheral tissues (Elshourbagy *et al.,* 2014), an increase in LDL levels in the blood is an indication of hypercholesterolemia (Rawat *et al.,* 2020). This significant decrease in LDL can be seen in all of the treatment groups (T1-T4), respectively, amounting to 55.4 mg/dL; 43.54 mg/dL; 41.78 mg/dL; and 34.44 mg/dL ($p<0.05$). The T4 group had the greatest decrease in the mean LDL level compared to the other groups. This value was much lower when compared to the group 2 who were given cholesterol diet without any treatments with the average LDL levels of 90.56 mg/dL. The decrease in all treatment groups occurred because *kirinyuh* leaves flavonoid compounds that inhibit the HMGCR enzyme can reduce LDL in the blood by increasing hepatic LDL receptor activity which causes an increase in LDL particle removal (Zeka *et al.,* 2017).

The best increase in the average blood HDL level was shown in the T4 group with the value of 63.31 mg/dL. According to Nurhidajah *et al.* (2019), normal HDL levels in the blood are in the range of ≥35 mg/dL. Based on the results, the treatment group has normal HDL levels. The HDL level has an important role in reverse cholesterol transport (RCT) where excess cholesterol is removed from peripheral vessels and transported back to the liver for elimination, HDL also has functions as antioxidant, endothelial anti-inflammatory or vasodilation, antithrombotic, and cytoprotective by providing strong protection. from oxidative damage due to oxidized LDL (Elshourbagy *et al.,* 2014), therefore the increase in HDL levels in all treatment groups can help reduce cholesterol and prevent diseases caused by hypercholesterolemia. The increase in HDL was then followed by treatment group 3, 2, and 1 which were 53.04 mg/dL; 50.57 mg/dL; 42.4 mg/dL, respectively.

In the examination of the average triglyceride levels, all treatment groups (T1-T4), both the extract and nanoparticle extract treatments from *kirinyuh* leaves, experienced a significant decrease based on their average triglycerides levels and the treatment group 4 had the best results of 93.32 mg/dL (Figure 3). The decrease in average triglycerides was followed by treatment group 3, 2, and 1 which were 123.29 mg/dL; 109.21 mg/dL; 104.15 mg/dL, respectively. According to Nurhidajah *et al*. (2019), the normal range of rat's triglycerides level is between 26-145 mg/dL, therefore all the treatment groups have returned to normal. Triglycerides have a function in the transport and storage of fatty acids in the body, a decrease in blood cholesterol levels occurs due to increased activity of the lipase enzyme which affects the increase in lipolysis of triglycerides in adipose tissue (Alves-Bezerra and Cohen, 2017). Flavonoid compounds contribute to increase the activity of the lipase enzyme and affecting in the increase of triglycerides lipolysis in adipose tissue (Bao *et al.,* 2016).

The best reduction results can be seen in the group given nanoparticles of *kirinyuh* leaves extract 2 mL/200 g BW which has a nanoparticle size of 20.3 nm. The absorption of nanoparticles is also faster because the nanoparticles function as a drug delivery system (Garg *et al.,* 2019). Nanoparticles formulation can help to increase the absorption ability and reduce the dose of treatment. Nanoparticle of *kirinyuh* leaves' size is 20.3 nm. It is indicated that the nanoparticle preparation has been successful because it has fulfilled the nanoparticle size requirements, which are in the range of 10 to 1000 nm and also fulfilled the requirements for a good nanoparticle size in a drug delivery system, which is less than 300 nm (Ismik *et al.,* 2020). The result of the PI value obtained is 0.150 which is less than 0.5, it shows that the

nanoparticles have a narrow particle distribution, and the particle size can be said to be homogeneous (Tzeyung *et al.,* 2018).

From the results above, *kirinyuh* leaves extract nanoparticles showed the best results in improving cholesterol levels in the blood. It is supported with the results of in silico study with its negative gibbs free energy and interactions with the amino acid residues; reduction in TC, LDL, and triglycerides; increase in HDL; and no significant change in histopathological findings from liver. It is in accordance with Idoko *et al*. (2018) from their previous research, that *kirinyuh* leaves extract have the potential to reduce cholesterol levels in blood. Moreover, the nanoparticle preparation from this research can optimize the effect *kirinyuh* leaves extract to reduce cholesterol levels in blood as a drug delivery system (Garg *et al.,* 2019). Thus, this research can be used as initial research to support another research on *kirinyuh* leaves extract nanoparticles as natural remedies in the future.

Conclusion: Based on this findings, *kirinyuh* leaves product in the form of nanoparticle demonstrated anti hypercholesterolemic activity at 2 mL/200 grams BB dose has the most decrease in TC, LDL, and triglycerides as well as the largest increase in HDL compared to the other groups ($P<0.05$), the interaction with key amino acid residues (sp-690, Lys-691, Lys-735, Asn-755, Ser-684, Glu-559) showed that flavonoid compounds can reduce hypercholesterolemia by inhibiting HMGCR enzymes, so that the formation of cholesterol in the liver can be inhibited and its negative gibbs free energy indicates that the bond between the ligand and the receptor has a good stability.

Acknowledgement: The author would like to thank the Directorate of Learning and Student Affairs, Director General of Higher Education, Ministry of Education, Culture, Research and Technology Republic Indonesia and Universitas Gadjah Mada.

Authors contribution: All authors contributed in the study design, interpreted the data, and wrote the manuscript. IWP and RAP performed molecular docking and the making of nanoparticle. SSS and VPA performed the histopathological and lipid profile analysis parts of the study. RRPBL analyzed the statistical result. AMP reviewed and edited the manuscript.

REFERENCES

Alves-Bezerra M and Cohen DE, 2017. Triglyceride metabolism in the liver. Compr Physiol 8:1-8.

Asomugha RN, Ezejiofor AN, Okafor PN, *et al.,* 2015. Acute and cytotoxicity studies of aqueous and ethanolic leaf extracts of Chromolaena odorata. Pak J Biol Sci 18:46-9.

Bao L, Hu L, Zhang Y, *et al.,* 2016. Hypolipidemic effects of flavonoids extracted from *Lomatogonium rotatum*. Exp Ther Med 11:1417-24.

Baskaran G, Salvamani S, Ahmad SA, *et al.,* 2015. HMG-CoA reductase inhibitory activity and phytocomponent investigation of *Basella alba* leaf extract as a treatment for hypercholesterolemia. Drug Des Dev Ther 9:501-17.

Butler DC, Lewin DN, and Batalis NI, 2018. Differential diagnosis of hepatic necrosis encountered at autopsy. Acad Forensic Pathol 8:256-295.

Casey CA, Donohue Jr TM, Kubik JL, *et al.,* 2021. Lipid droplet membrane proteome remodeling parallels ethanol-induced hepatic steatosis and its resolution. J Lipid Res 62:1-19.

Du X, Li Y, Xia YL, *et al.,* 2016. Insights into protein–ligand interactions: mechanisms, models, and methods. Int J Mol Sci 17:144.

Elshourbagy NA, Meyers HV and Abel-Meguid SS, 2014. Cholesterol: The Good, the Bad, and the Ugly – Therapeutic Targets for the Treatment of Dyslipidemia. Med Princ Pract 23:99-111.

Garg U, Chauhan S, Nagaich U, *et al.,* 2019. Current Advances in Chitosan Nanoparticles Based Drug Delivery and Targetin. Adv Pharm Bull 9:195-204.

Göbel A, Raunera M, Hofbauera LC, *et al.,* 2020. Cholesterol and beyond - The role of the mevalonate pathway in cancer biology. Biochim Biophys Acta Rev Cancer 1873:1-19.

Idoko A, Ikpe VPO, Rita ON, *et al.,* 2018. Leaf extract of *Chromolaena odorata* (linn) in albino wistar rats fed different concentrations of cholesterol enriched diet. Univers J Pharm Res 3:37-42.

Ikewuchi JC and Ikewuchi CC, 2011. Anti-cholesterolemic effect of aqueous extract of the leaves of *Chromolaena odorata* (l) king and robinson (asteraceae): potential for the reduction of cardiovascular risk. Pas J Sci Tech 12:385-9.

Ismik D, Mansuroglu DS, Bulus E, *et al.,* 2020. The Use of chitosan nanoparticles obtained by ionic gelation method as a drug delivery system. J Mater Electron 20:6-11.

Iswari RS, Dafip M and Rifa'I M, 2020. Biochemical and histopathology analysis of liver damage in hypercholesterolemic rats induced by tomato extract. Biosaintifika 12:438-45.

Kong L, Luo C, Li X, *et al.,* 2013. The Anti-Inflammatory effect of kaempferol on early atherosclerosis in high cholesterol fed rabbits. Lipids Health Dis 12:1-12.

Lateef T, Naeem S, and Qureshi SA, 2019. In-silico studies of HMG-Co A reductase inhibitors present in fruits of *Withania coagulans* Dunal (Solanaceae). Trop J Pharm Res 19:305-12.

Meyerholz DK and Beck AP, 2018. Fundamental concepts for semiquantitative tissue scoring in translational research. ILAR J 59:13-7.

Michel J, Rani NZA, and Husain K, 2020. A Review on the potential use of medical plants from Asteraceae and Lamiaceae plant family in cardiovascular diseases. Front Pharmacol 6:1-26.

Nouri MHK and Abad ANA, 2013. Comparative study of tomato and tomato paste supplementation on the level of serum lipids and lipoproteins levels in rats fed with high cholesterol. Iran Red Crescent Med J 15:287-91.

Nurhidajah R, Astuti and Nurrahman, 2019. Black rice potential in HDL and LDL profile in sprague dawley rat with high cholesterol diet. IOP Conference Series: Earth and Environmental Science. Semarang, Indonesia; 28-29 November pp:1-7.

Rahardhian MRR, Murti BT, Wigati D, *et al.,* 2019. Solvent concentration effect on total flavonoid and total phenolic contents of *Averrhoa bilimbi* leaf extract. Pharmaciana 9:137-44.

Rawat S, Kumar S, Arora M, *et al.,* 2020. Assessment of atherogenic index of plasma, non-HDL cholesterol and other cardiac indices as risk factors for CVD in type II diabetes. Int J Health Clin Res 3:137-41.

Shattat GF, 2014. A review article on hyperlipidemia: types, treatments and new drug targets. Biomed Pharmacol J 7:399-409.

Threapleton, Burley VJ, Greenwood DC, *et al.,* 2015. Dietary fibre intake and risk of ischaemic and haemorrhagic stroke in the UK Women's Cohort Study. Eur J Clin Nutr 69:467-74.

Tzeyung AS, Shadab M, Bhattamisra SK, *et al.,* 2019. Fabrication, optimization, and evaluation of rotigotine-loaded chitosan nanoparticles for nose-to-brain delivery. Pharmaceutics 11:1-17.

Ugwoke CEC, Orji J, Anze SPG, *et al.,* 2017. Quantitative phytochemical analysis and antimicrobial potential of the ethanol and aqueous extracts of the leaf, stem and root of *Chromolaena odorata* (Asteraceae). Int J Pharmacogn Phytochem Res 9:207-14.

Vijayaraghavan K, Rajkumar J and Seyed MA, 2017. Efficacy of *Chromolaena odorata* leaf extracts for the healing of rat excision wounds. Vet Med 62:565-78.

Zeka K, Ruparelia K, Arroo RRJ, *et al.,* 2017. Flavonoids and Their Metabolites: Prevention in Cardiovascular Diseases and Diabetes. Diseases 5:6-8.