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Antioxidants and Antihyperlipidemia Test of Ethanol Extract of Indonesian Plant *Sambang Getih* Leaves (*Hemigraphis Bicolor* Boerl.) In Hyperlipidemia Mice

Rika Sari Dewi*, Ni Made Dwi Sandhiutami*, Dea Anisa Sarsono, Doreensia Putri Cuinita

Fakultas Farmasi Universitas Pancasila, Srengseng Sawah Jagakarsa – South Jakarta, Indonesia 12640

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***Correspondence:** Ni Made Dwi Sandhiutami, Faculty of Pharmacy, Universitas Pancasila, South Jakarta, Indonesia. E-mail: dwisandhiutami@univpancasila.ac.id
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Abstract

AIM: *Sambih getih leaves* (SGL) contain flavonoids acting as an antioxidant and antihyperlipidemia. The purpose of this study is to determine the antioxidant activity and antihyperlipidemia effect of Ethanol Extract of Sambang Getih Leaves (EESGL).

METHODS: EESGL was tested for antioxidant activity in vitro and in vivo. Antihyperlipidemia test of SGL is done by looking at blood lipid parameters, that are total cholesterol, triglyceride, low-density lipoprotein (LDL), and high-density lipoprotein (HDL). 2,2-diphenyl-1-picrylhydrazyl (DPPH) of SGL and atorvastatin showed that Inhibition Concentration (IC50) values are 24.15 and 86.75 ppm. In vivo test was divided into six groups: Normal, negative, positive (atorvastatin), low dose 487.5, medium dose 975, and high dose 1950 mg/kg dose groups.

RESULTS: Measurements of Malondialdehyde (MDA) plasma levels of mice on that groups on 21st day were 1.91 ± 0.51; 5.86 ± 0.57; 2.18 ± 0.55; 4.33 ± 0.16; 3.08 ± 0.06; and 1.82 ± 0.19 nmol/mL. Measurements of Superoxide Dismutase (SOD) activity for each group were 193.29 ± 3.36; 89.41 ± 2.84; 192.46 ± 4.25; 175.53 ± 1.71; 191.53 ± 3.34; and 192.83 ± 2.88 U/mL. Results of total cholesterol level of positive (atorvastatin), low, medium, and high dose group were 62.16%, 40.50%, 48.55%, and 59.52%. The decrease in triglyceride levels was 48.94%, 27.78%, 34.27%, and 54.02%. The decrease in LDL levels was 82.77%, 57.43%, 65.18%, and 77.67%. Increased HDL levels were 20.24%, 17.94%, 17.08%, and 17.84%.

CONCLUSION: Based on the results obtained, it can be concluded that the EESGL have antioxidant activity and antihyperlipidemia but is not as good as atorvastatin.

Introduction

The vast majority 87.8% of deaths in HICs (High-income countries) in 2019 were due to NCDs (Non-communicable diseases), with heart disease, dementia, and stroke the leading causes. The main clinical entities of the disease are coronary heart disease (CHD), ischemic stroke, and peripheral arterial disease. The cause of the disease is multifactorial, some of which can be modified. One of the modifiable risk factors is dyslipidemia. There is a strong association between dyslipidemia and cardiovascular disease which is relatively equal between Asian and non-Asian populations in the Asia Pacific region [1]. Data in Indonesia based on the 2017 Biomedical Riskesdas Report show that the prevalence of dyslipidemia based on total cholesterol concentration >200 mg/dL is 39.8% [2]. Hyperlipidemia is a condition of increased concentrations of various lipids in the blood. Lipids or fats are energy-rich substances, which serve as a major source of metabolic processes. Fat-breaking process in addition to generating energy also produces by products and free radicals. By increasing the breakdown of fat as an energy source, it will accelerate the process of fat or cholesterol synthesis in the body, increasing oxygen and Nicotinamide Adenine Dinucleotide Phosphate (NADPH) consumption, then increasing the superoxide radical (O_2*) generation [3]. The hyperlipidemic condition can also increase the amount of reactive oxygen species (ROS). This excessive amount of ROS results in lipid peroxidation resulting in MDA and may decrease the capacity of intracellular antioxidant enzymes, SOD, and catalase [4].

Free radicals are compounds that do not have electron pairs so tend to pair with other compounds. Free radicals are also considered to play a role in the process of the occurrence of various degenerative diseases. Free radicals can attack multiple unsaturated fatty acids (Polyunsaturated Fatty Acids, PUFAs) containing at least three double bonds, and this reaction is called lipid peroxidation. This is because lipids are considered one of the most sensitive molecules to free radicals.

Antioxidants are electrons or reductants that can inhibit oxidation reactions by binding to

free radicals and highly reactive molecules so that cell damage can be inhibited [5]. Under normal circumstances, the body has a systematic strategy to counteract the formation of free radicals, such as SOD enzymes and catalase. The famous exogenous antioxidant is an antioxidant of polyphenolic compounds, flavonoids [6].

Sambang getih is a native plant of Indonesia and is generally found to grow wild or planted in the yard and garden as an ornamental plant. SGL contain several chemical compounds such as flavonoids, polyphenols, and tannins based on studies capable of inhibiting cholesterol synthesis, cellular esterification of cholesterol, triglycerides, and phospholipid synthesis, as well as inhibiting the activity of 3-hydroxy-3-methylglutaryl coenzyme A (HMG-CoA) reductase enzyme [7]. Phenolic compounds are also known to play a significant role in antioxidant activity.

The purpose of this study is to determine the antioxidant activity and antihyperlipidemia effect of EESGL. Antioxidant activity and antihyperlipidemia effects of ethanol extract of *sambang getih* leaves (*Hemigraphis bicolor* Boerl.) tested by looking at the parameters of antioxidants *in vitro* using DPPH and *in vivo* using the levels of MDA plasma and the activity of the enzyme SOD and lipid parameters in the blood that are total cholesterol, triglycerides, LDL, and HDL in hyperlipidemic mice.

Materials and Methods

Materials

Ethanol extract of *sambang getih* leaves, Atorvastatin tablets of Truvaz brand PT. Kalbe Farma, EDTA, phosphate buffer pH 7, methanol pro analysis, DPPH (Sigma-Aldrich, 257621), sodium carbonate buffer pH 10.2, epinephrine solution (Sigma, C-109) 0.01 M, distilled water, trichloroacetic acid (TCA, T0699) 20%, Tiobarbiturat Acid (TBA) (Merck, 361941) 0.67%, 1,1,3,3 tetraethoxypropane (TEP) (Sigma, 19445), magnesium powder, 1% hydrochloric acid, concentrated hydrochloric acid, amyl alcohol P, iron (III) chloride 1 solution %, Sodium acetate powder, 80% yolk, 65% fat 5% sucrose, cholesterol kit reagent (Biolabo), triglyceride kit reagent (Biolabo), and HDL reagent kit (Labtest). Micropipet, analytical scale (AND GR-200), capillary pipe, sonde gastric, syringe, cotton, glassware, effendorf tube, microlab 300, centrifugation tools, UV-VIS spectrophotometry (Genesys 10 UV), refrigerators, water bath, chocolate bottle, dropper pipette, aluminum foil, syringe, mortar, and animal scales.

Methods

Submission of the ethics committee

This study has been studied and passed the ethical review by the Medical Research Ethics Committee of the Faculty of Medicine University of Indonesia.

Plant collection and determination

The collection of plants obtained from the Research Center for Medicines and Aromatics (BALITRO) was then determined by plants.

Preparation of sambang getih leaves ethanol extract

Sambang getih leaves were washed and dried. Simplicia of *sambang getih* leaves is sifted using sieve number 20/40. A total of 500 g Simplicia powder of *sambang getih leaves* are macerated 7 times in 5 L of 70% ethanol solvent for 5–6 days. The result of the maceration is filtered with gauze, then the filtrate is put into the rotatory evaporator and evaporated in the water bath until a viscous extract is formed.

Phytochemical screening

The extract was tested for qualitative content of flavonoids, saponins, and tannins.

In vitro test of sambang getih leaves ethanol extract

The principle of this test is based on the hydrogen-capture reaction of antioxidants by free radical DPPH. Previously prepared DPPH solution, blank solution, parent atorvastatin solution, and series of test solutions. The antioxidant activity test was performed by: The atorvastatin solution and the test solution were incubated at 37°C for 30 min. The uptake was measured at a 515 nm wavelength using a UV-VIS spectrophotometer [8].

In vivo test of sambang getih leaves ethanol extract

In vivo test is done to see antioxidant activity (MDA and SOD) and the effects of antihyperlipidemia (total cholesterol, triglycerides, LDL, and HDL) in ethanol extract of leaves *sambang getih*.

a. Maintenance and preparation of experimental animals: mice were adapted in the laboratory for 1 week.
b. Induction of hyperlipidemia in mice: the feed in the form of emulsions and made freshly each day during inducing. Given orally to mice at a

Table 1: Distribution of animal groups

Group	Treatment
I (normal control)	Given standard feed and aquadest
II (positive control)	Given feeding inducer 0.35 g/20 g for 14 days, then given atorvastatin 0.052 mg/20 g/day for 7 days
III (negative control)	Given feed inducer 0.35 g/20 g BW for 14 days
IV (low dose group)	Given feeding inducer 0.35 g/20 g BW for 14 days, then given EESGL low dose (487.5 mg/kg BW) for 7 days
V (medium dose group)	Given feeding inducer 0.35/20 g BW for 14 days, then given EESGL meidum dose (975 mg/kg BW) for 7 days
VI (high dose group)	Given feeding inducer 0.35/20 g BW for 14 days, then given EESGL high dose (1950 mg/kg BW) for 7 days

EESGL: Ethanol Extract of *Sambang Getih* Leaves.

dose of 17.5 g/kg twice a day (morning and evening) for 14 days.

c. Division of groups: 30 mice were divided into six groups, each consisting of 5 mice. The distribution of animal groups are shown in Table 1.

d. Blood sampling

Blood sampling from each group was performed on days 0, 14, and 21 for measurement of antioxidant activity (MDA) and antihypercipidemia (total cholesterol, triglyceride, LDL, and HDL). Blood was taken through the eye's orbital sinus using a capillary pipe of approximately 0.5 mL by piercing the tip of the eye of the mice with capillaries until blood flows through the pipe. The blood was then deposited into eppendorf tubes containing EDTA anticoagulants, then the blood was centrifuged for 10 min at a rate of 3000 rpm and separates the plasma obtained.

Table 2: The results of phytochemical screening of ethanol extract of *Sambang Getih* leaves

Serial number	Group of compounds	EESGL
1	Flavonoids	+
2	Saponin	+
3	Tanin	+

Description: +: Give positive reaction, −: Give a negative reaction.

e. Measurement of MDA levels

A 200 μL plasma (a clear solution) added 1.0 mL of TCA 20% and 2 mL of TBA 0.67%. The solution was homogeneously mixed and heated over a water bath with a temperature of 90–100°C for 10 min. After that centrifuge at 3000 rpm for 10 min. The pink filtrate was measured at 532 nm wavelength using a UV-VIS spectrophotometer. MDA levels were calculated using standard MDA curves with concentrations of 0; 0.025; 0.05; 0.1; 0.2; 0.4; 0.8; and 1.6 nmol/mL [9].

f. Measurement of SOD levels

On the 21st day, the mice were dislocated, then dissected and taken away. Mice hearts were crushed using a mortar, then extracted with phosphate buffer pH 7 at a ratio of 1:10 in a bowl of ice cubes (in the cold). The extract results in a cold state centrifuged at a rate of 3000 rpm for 10 min. Take as many as 100 mL filtrate into another test tube, add 2800 mL of sodium carbonate buffer pH 10.2 and 100 mL of epinephrine solution into a test tube. The same way is also done for aquadest (blank). Then, the absorption was read at a wavelength of 480 nm in the min 1, 2, 3, and 4 [10].

g. Measurement of total cholesterol

Prepared tube blanks, standard tubes, and sample tubes consisting of distilled water and cholesterol reagents of 1000 μL each. Then, 10 μL of aqua distillate was inserted into the blank tube, 10 μL cholesterol standard was inserted into the standard tube and 10 μL of mice blood plasma was included in the sample tube then mixed homogeneously, and incubated for 10 min at room temperature. Absorption is read at 505 nm wavelength.

h. Measurement of triglyceride

Prepared tube blanks, standard tubes, and sample tubes consisting of distilled water and cholesterol reagents of 1000 μL each. Then, 10 μL aqua distillate was inserted into the blank tube, 10 μL standard triglyceride was inserted into the standard tube and 10 μL of mice blood plasma was included in the sample tube then mixed homogeneously, and incubated for 10 min at room temperature. Absorption is read at 505 nm wavelength.

i. Measurement of HDL

Prepared tube blanks, standard tubes, and sample tubes consisting of distilled water and cholesterol reagents of 1000 μL each. Then, as much as 100 μL of aqua distillate was inserted into the blank tube, 100 μL cholesterol standard was inserted into the standard tube and 100 μL of mice blood plasma was inserted in the sample tube then mixed homogeneously, and incubated for 10 min at room temperature. Absorption is read at 505 nm wavelength.

j. Measurement of LDL

Measurement of LDL levels was done indirectly by the formula

$$\text{LDL} = \text{Total Cholesterol} - \left[\frac{\text{Tryglicerida}}{5} + \text{HDL}\right]$$

Results and Discussion

Plant determination

Plant determination is done at the Center for Biological Research Herbarium Bogoriense LIPI-Cibinong Botanical Field, Bogor, Indonesia to know the truth of the type of plant used in the study. The result of the determination shows that the plants used in the study are *Sambang getih* leaves (*Hemigraphis bicolor* Boerl.) Family of Acanthaceae.

Table 3: Level of antioxidant by 2,2- diphenyl-1-picrylhydrazyl [8]

Intensity	IC_{50} (ppm)
Very strong	<50
Strong	50–100
Medium	101–150
Weak	>150

Screening phytochemicals

Phytochemical screening is performed to determine the content of secondary metabolites contained in the extract as a compound that has biological activity. The result of phytochemical screening on EESGL showed the presence of flavonoids, saponins, and tannins. The results of phytochemical screening of ethanol extract of Sambang Getih leaves are shown in Table 2. According by research was done Made Ria Defani, SGL contains flavonoids and polyphenols in the leaves and roots that are useful in some medication. Tannins are also found in the leaves, while saponins and tannins are also found in the stems of plants [11].

In vitro test of EESGL

The testing activity of EESGL *in vitro* using the method of DPPH. DPPH is an oxidizing agent that can be used as a free radical in antioxidant activity testing. Testing was done by calculating the IC_{50} value.

The test results showed IC_{50} values for EESGL at 24.15 ppm and 86.75 ppm for atorvastatin. This shows that the EESGL can reduce DPPH radical and damping strength classified as a very strong antioxidant, and atorvastatin is considered a strong antioxidant. Level of antioxidant by 2,2-diphenyl-1-picrylhydrazyl are shown in Table 3.

DPPH radical damping ability in EESGL is associated with compounds contained in *sambang getih* leaves such as flavonoids, polyphenols, and tannins. Flavonoid compounds, polyphenols, and tannins can donate hydrogen, the antioxidant activity of these compounds is achieved by the discontinuation of the chain radical reaction that occurs [12].

In some studies, antioxidant activity generally uses Vitamin C as a positive control. In this study, antioxidant activity using atorvastatin as a positive control. Atorvastatin is a newer synthetic statin with an active metabolite which can inhibit HMG-CoA reductase equivalent to a parent compound. As a result, atorvastatin has half resistance to HMG-CoA reductase (20–30 h) and reduces cholesterol synthesis greater than simvastatin [13]. Compounds that have antioxidant activity will react with DPPH by electrons from antioxidant compounds to DPPH, this reaction causes a decrease in the color intensity of the purple DPPH solution. The greater concentration of the sample the less intensity of the purple color of the DPPH solution which can be measured uptake using a UV-VIS spectrophotometer at 515 nm wavelength which is the maximum wavelength of DPPH.

In vivo test of EESGL

Measurement of MDA levels

The principle of measuring MDA levels is by reacting two molecules of TBA with one MDA molecule under acidic conditions to produce a pink, pink MDA-TBA complex can be measured by UV-VIS spectrophotometers at a wavelength of 532 nm.

Figure 1 shows that plasma MDA levels of each group showed an increase in plasma MDA levels in the negative control group compared with positive control group MDA levels, treatment group low dose of 487.5 mg/kgBW, treatment group medium dose 975 mg/kgBW and the treatment group high dose 1950 mg/kgBW on day-to-21. In the negative control group, irradiated induced hyperlipidemia mice led to an increase in free radicals in the body. One of the targets attacked by free radicals is unsaturated fatty acids that form the product of lipid peroxidation which is MDA whose existence is very toxic in the cell. High MDA products are evidence of low antioxidant status in the body so they cannot prevent the reactivity of free radical compounds.

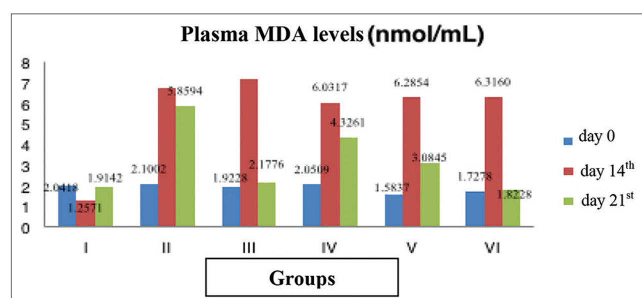

Figure 1: Plasma MDA levels in the animal group. Description - I: Normal control group. II: The negative control group induced hyperlipidemia. III: Positive control group given atorvastatin 2.6 mg/kgBW. IV: Treatment group low dose 487.5 mg/kgBW. V: Treatment group medium dose 975 mg/kgBW. VI: Treatment group high dose 1950 mg/kgBW

The results of this study indicate that the negative control increased levels of MDA, this is due to feeding induction of hyperlipidemia until day 14 and then given drinking water and feed only so MDA levels in the negative control group did not decrease significantly. According to research conducted by Sunaryo *et al*. hyperlipidemia will increase oxidative stress [13]. This is due to the increased number of ROS. The group given antioxidant EESGL and atorvastatin showed the prevention of unsaturated fat oxidation. Atorvastatin causes a significant reduction in oxidative stress determined by MDA concentrations. Flavonoids and polyphenols are exogenous antioxidant compounds that can counteract free radicals and inhibit lipid peroxidation. The tested group had decreased MDA levels similar to other research, which said that *sambang getih* leaves can decrease the MDA level of mice with oxidative stress [14].

Prevention of unsaturated fat oxidation in the group given EESGL is more effective and safer than the group given atorvastatin, but atorvastatin can prevent oxidation of unsaturated fats. This suggests that the EESGL has an effective ability as an antioxidant.

Measurement of SOD levels

SOD is one of the enzymatic antioxidants. SOD catalytic reaction of superoxide anions to hydrogen peroxide (H_2O_2) and oxygen (O_2). SOD enzyme activity has an important role in the body's defense system, especially in the activity of reactive oxygen compounds [4].

Figure 2 shows that the measurement results of the SOD activity of mice liver in each group showed a decrease in the mean activity of the SOD liver of mice in the negative control group compared with the positive control group and treatment group. The low activity of SOD in the negative control group is due to the increase of free radicals from hyperlipidemic feed-induced mice, where hyperlipidemic conditions can lead to free radical production produced beyond the body's capacity to counteract [13].

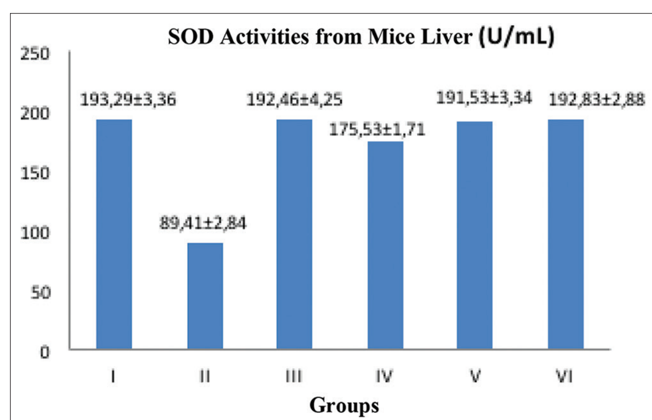

Figure 2: SOD activities from mice liver against the animal group

When SOD in the body is normal, then free radical levels will also be normal. However, when free radicals in the body increase levels of SOD in the body decrease as many of the SOD enzymes are in use. While the average SOD activity in the three treatment groups was higher than the negative control group, it increased SOD due to the presence of antioxidants in flavonoid compounds. Components of flavonoid compounds may work synergistically together with SOD enzymes in neutralizing free radicals so that SOD levels do not decrease as in negative controls [4]. Flavonoids may inhibit the enzyme responsible for the production of radicals anion superoxide (O_2*) and end-radical reactions and quenches superoxide anion (O_2*) which is the work of SOD, so the use of SOD is not too high and may radicals free of excess.

Measurement of total cholesterol levels

Figure 3 shows changes in the total cholesterol levels of mice during the study. On the day 0 where before being treated, all groups are still at normal levels of total cholesterol. The average results of total cholesterol on days 0 ranged from 66.04 ± 1.85 mg/dL–71.33 ± 6.89 mg/dL. Then, there was an increase in total cholesterol on day 14 for negative, positive, low-dose (487.5 mg/kg BW), medium

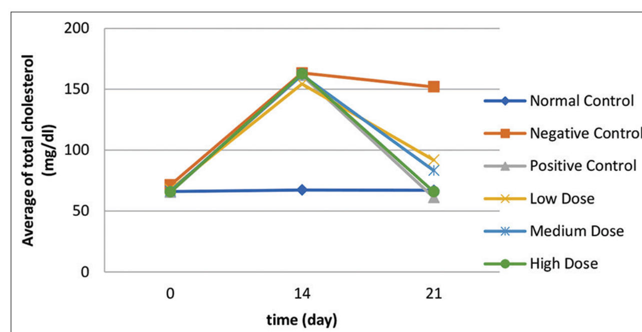

Figure 3: Average of cholesterol levels in the animal group on days 0, 14 and 21

dose (975 mg/kg BW), and high dose (1950 mg/kg BW). Increased total cholesterol occur due to feeding-induced dyslipidemia. Blood cholesterol levels of mice on day 14 increased significantly when compared with total cholesterol on day 0, except in the normal control group, because the normal control group was not given any treatment, given only standard feed and drink. Furthermore, each group of mice was given a treatment of dosage for 7 days except normal control and negative control. On the 21st day, the percentage decrease in total cholesterol level in positive control, low dose (487.5 mg/kg BW), medium dose (975 mg/kg BW), and high dose (1950 mg/kg BW); there were 62.16%, 40.50%, 48.55%, and 59.52%, respectively. This is due to the cessation of additional feeding induction and the provision of EESGL and atorvastatin as a positive control.

Measurement of triglyceride levels

Figure 4 is a graph of the change of triglyceride levels of mice during the research on the day 0 before being treated, on the 14th day when given the treatment of inducing dyslipidemia, and on the 21st day that was given the treatment of dosage EESGL. On day 0, all groups were still in normal condition, after feeding of dyslipidemia induced for 14 days

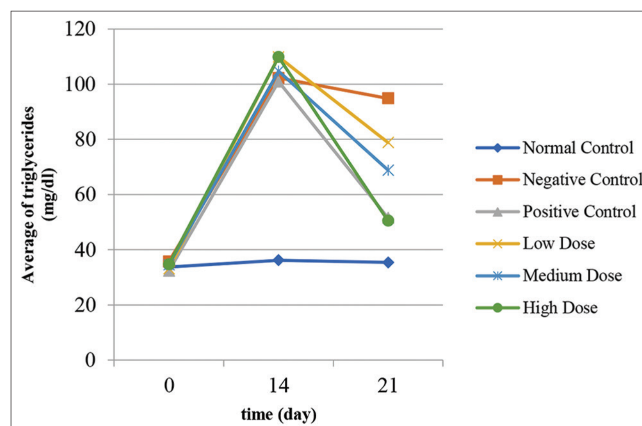

Figure 4: Average of triglycerides levels in the animal group on days 0, 14, and 21. Description - I: Normal control group. II: The negative control group induced hyperlipidemia. III: Positive control group given atorvastatin 2.6 mg/kgBW. IV : Treatment group low dose 487.5 mg/kgBW. V: Treatment group medium dose 975 mg/kgBW. VI: Treatment group high dose 1950 mg/kgBW

increased triglyceride levels in all groups except the normal control group with mean levels ranging from 36.17 ± 2.96 mg/DL–102.25 ± 7.84 mg/dL. Increased levels are due to the provision of sucrose solution that may turn into triglycerides. The triglyceride levels at day 14 increased significantly when compared with the normal control group. Then, on day 21, all groups were treated for 7 days except the normal control group was given standard feeding and beverage treatment only. In the positive group treated with atorvastatin suspension with a dose of 0.052 mg/20 g BW mice decreased triglyceride levels with an average range of 51.56 ± 6.52 mg/dL. In all groups (except normal controls), decreased triglyceride levels were caused by the cessation of induced supplementary feeding with antidislipidemia assaying atorvastatin as positive control and three dose variations from EESGL.

Measurement of HDL levels

Figure 5 is a graph of changes in HDL levels of mice during the research on the 0th day before being treated, on the 14th day when given the treatment of dyslipidemia-induced feed, and on the 21st day that was given the treatment of EESGL. On day 0, all groups were still in normal condition, after 14 days of induced dyslipidemia, decreased HDL levels were noticed in all groups except the normal control group with mean levels ranging from 22.95 ± 2.32 mg/dL–24.39 ± 4.34 mg/dL. Decrease in levels due to the mixture of feed (pellets) with animal fat 5%. HDL levels at day 14 decreased significantly when compared with the normal control group. Then, on day 21, all groups were treated for 7 days unless the normal control group was given standard feeding and beverage treatment only. In the positive group treated with atorvastatin suspension at 0.052 mg/20 g, the mice experienced an increase in HDL levels with an average range of 24.39 ± 4.34 mg/dL. In all groups (except normal controls), HDL levels increased due to cessation of induced feeding supplementation with antidyslipidemia assaying atorvastatin as positive control and three dose variations from EESGL.

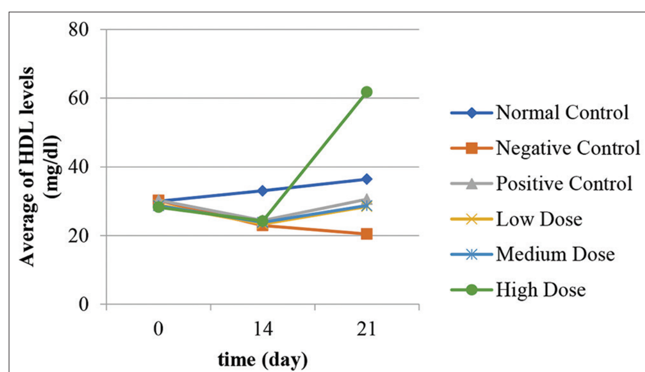

Figure 5: Average of HDL levels in the animal group on days 0, 14, and 21

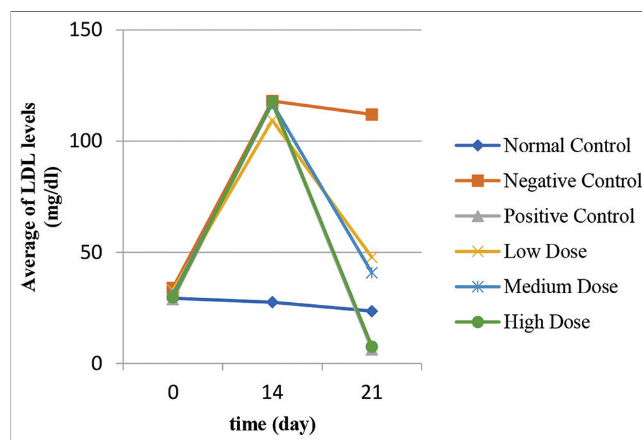

Figure 6: Average of LDL in the animal group on days 0, 14 and 21

Measurement of LDL levels

Figure 6 shows the change in LDL levels of mice during the study. On the 0th day where before being treated, all groups are still at normal levels of LDL. The average LDL yield of mice on day 0 ranged from 26.38 ± 7.02 mg/dL–33.91 ± 5.25 mg/dL. Then, an increase in LDL levels of mice on day 14 for negative, positive, low-dose (487.5 mg/kg BW), moderate dose (975 mg/kg BW), and high dose (1950 mg/kg BW). Increased LDL occur due to feeding-induced dyslipidemia. LDL blood levels of mice on day 14 increased significantly when compared with LDL levels of mice on day 0, except in the normal control group, because the normal control group was not given any treatment given only standard feed and drink. Furthermore, each group of mice was given a treatment of dosage for 7 days except normal control and negative control. On day 21, the percentage decrease in LDL levels in positive control, low dose (487.5 mg/kg BW), moderate dose (975 mg/kg BW), and high dose (1950 mg/kg BW) was 82.77%, 57.43%, 65.18%, and 77.67%, respectively. This is due to the cessation of additional feeding induction and the provision of EESGL and atorvastatin as a positive control.

Conclusion

The ethanol extract of *sambang getih* leaves that were tested *in vitro* had a very strong antioxidant activity and give its effect by decreasing MDA levels at a medium dose of 975 mg/kg and high dose 1950 mg/kg. The ethanol extract of *sambang getih* tested for SOD activity in the liver of mice at same doses, it was able to increase the activity of the SOD liver of mice approaching normal concentration. The ethanol extract of sambang getih leaves when used in different doses has protective hypolipidemic effect by lowering total cholesterol, triglycerides, and LDL levels while increasing HDL levels.

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