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Antihyperlipidemic Effectivity of Sweet Orange Peel Extract (*Citrus sinensis*) on Triglyceride Levels in Male Mice (*Mus musculus*) Induced by High Cholesterol

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ABSTRACT

Hyperlipidemia is a significant risk factor for atherosclerosis in arteries and cardiovascular disease, especially coronary heart disease (CHD), the leading cause of global mortality. Treatment of hyperlipidemia can be done through chemical treatment. However, statins have potentially dangerous side effects such as myopathy and rhabdomyolysis, so a therapeutic solution with minimal toxicity is needed. One of the potential treatments is *Citrus sinensis* orange peel, which contains antihyperlipidemic bioactive compounds, namely hesperidin, and naringenin. The aim of this research is to determine the effectivity of sweet orange peel extract (*Citrus sinensis*) on high cholesterol-induced male mice (*Mus musculus*) triglyceride levels. *Citrus sinensis* orange peel was extracted by maceration method using 50% ethanol as solvent. Lipid profile measurements were performed three times using the GPO-PAP method. The concentration of orange peel extract in the treatment group (P1, P2, P3) was 10%, 5%, and 1%. The positive control received 40 mg atorvastatin treatment, the negative control received 1% CMC Na, and the control received abnormal treatment. Antihyperlipidemic therapy with *Citrus sinensis* sweet orange peel extract has a strong relationship/influence on triglyceride levels in mice. Sweet orange peel extract (*Citrus sinensis*) has antihyperlipidemic effectiveness in reducing blood triglyceride levels in mice (*Mus musculus*) induced by high cholesterol. The effective dose is 1% dose.

INTRODUCTION

Hyperlipidemia is a significant risk factor for atherosclerosis in arteries and cardiovascular disease, especially coronary heart disease (CHD) (Iqbal *et al.,* 2013). The World Health Organization (WHO) states that CHD is one of the leading causes of mortality globally (Setyaji *et al.,* 2018). CHD ranks the second most common cause of death after stroke, and an estimated 15 out of 1,000 Indonesians suffer from this disease (Dwizella *et al.,* 2018).

Currently, the primary therapy for hyperlipidemia is through chemical treatments such as statins, niacin, and fibric acid derivatives (fibrate). The drugs most often prescribed are statins. However, statins have potentially dangerous side effects, including myopathy and rhabdomyolysis at 3-6 months of use, and increase when given together with certain drugs such as fibrates and nicotinic acid. The reported incidence is about 0.00–3.34 in 100,000 person-year. Statins also are reported to increase the risk of New-Onset Diabetes (NOD) in patients who are at risk for type 2 diabetes mellitus and should not be used in lactating mothers (Gunawan and Nafriadi, 2013). Based on exposure to these side

effects, a therapeutic solution is needed using materials that have minimal toxicity, one of which is natural ingredients, namely orange peels.

Orange peel is a natural ingredient that contains antihyperlipidemic bioactive compounds, namely hesperidin and naringenin. The hesperidin compound is most abundant in *Citrus sinensis* compared to other oranges. The results of a previous study in 2019 showed that sweet orange peel (*Citrus sinensis*) has bioactive ingredients which can significantly reduce triglyceride levels in the blood of type 2 diabetic rats through the mechanism of inhibition of HMG-CoA reductase enzyme activity, which is the exact mechanism of action as statin class drugs. The dose used in the study was 100 mg/kg body weight (BW) hesperidin isolated from *Citrus sinensis* (L.) peels (Rekha *et al.,* 2019).

Based on the explanation described above, the researchers aimed to conduct a study to determine the antihyperlipidemic effectiveness of sweet orange peels (*Citrus sinensis*) in mice. Based on previous studies, there has not been a detailed and specific study on the effectiveness of sweet orange peel extract (*Citrus sinensis*) on reducing blood triglyceride levels in mice when induced by high cholesterol feed (B2 feed with additional slurry), so the researchers are interested in examining the anti-effectiveness hyperlipidemia of sweet orange peel extract (*Citrus sinensis*) on triglyceride levels of male mice (*Mus musculus*) induced with high cholesterol.

METHODS

The materials used were *Citrus sinensis* sweet orange peel, 50% ethanol, avicel MCC NF, triglyceride lipid profile reagent purchased from Glory Diagnostic No lot 16702, 10% EDTA anticoagulant, and distilled water.

The tools used are a 70 mesh test sieve size 212μm, digital balance Analytical Balances brand BEL, macerator, evaporator Heating Bath brand B-100 BUCHI Heidolph, microwave, magnetic stirrer brand 18-one, centrifugal brand Eppendorf mini spin Aosheng, photometer Thermo scientific genesis 10S -UV vis, one med syringe 3 ml, micropipette 10*μm*, micropipette 1000*μm,* test tube rack, and test tubes.

Preparation of Experimental Animals

The animal experiment was approved by the Ethics Committee for the Care of Animals and Animal Experiments of Tanjungpura University Pontianak No. 4691/UN22.9/PG/2021. Animals in this experiment used were *Mus musculus* (male mice) with BW ± 25 grams and aged 2–3 months. The mice were randomly divided into six experimental groups, which were 3 control groups (normal, positive, negative) and 3 treatment groups (P1, P2, P3). The animals were housed in the animal facility at Tanjungpura University at a temperature of 22 ± 1°C and 50% to 60% relative humidity, with a 12 h light–dark cycle (light on at 7:00 a.m.)

Preparations for the maintenance of experimental animals were done before the study began, such as preparing cages, feeding places, drinking places, and feeding. For the maintenance of test animals, a feeding place for mice, drinking bottles for mice, boxes for mice, and standard feed B2 (551) were needed. Mice were acclimatized for seven days. After that, the cholesterol induction treatment was done with feed B2 and slurry (Dulcich, 2013).

Preparation of *Citrus sinensis* Peel Test Material

The steps for making orange peel simplicia were washing, peeling, dehydrating, grinding, and sifting the orange peel powder. The simplicia was then macerated using 50% ethanol with a ratio of 1:3 for three days. The macerate was filtered and then macerated 3 times with the same ratio using cloth twice and with filter paper once. The macerate obtained was then concentrated using a rotary evaporator, followed by a water bath until a thick extract was obtained.

Dosage Calculation of Sweet Orange Peel Extract (*Citrus sinensis*)

Sweet orange peel extract was dissolved in as much as 0.625 g in 25 ml of distilled water to obtain a concentration (w/v) of 2.5%. The dose of orange peel extract used is every 25 mg/25 grBB for 1 ml. Variations in the concentration of orange peel extract in the treatment group (P1, P2, P3) were 10%, 5%, and 1%, respectively. Administration was done orally, using a mouse sonde 2x every day.

Induction and Treatment Test Group

Induction of hyperlipidemia in mice with a slurry made from 20% quail egg yolk, 10% beef fat, and 20% used cooking oil. The slurry was given orally through a sonde, while the standard feed given to mice was feed B2 (551). Furthermore, different treatments were conducted in each group.

Measurements of Mice's Obesity and Triglyceride Index

Obesity index measurements were made every Saturday from the first week to the sixth week. The obesity index was measured using the Rōhrer index formula, namely BW/(naso-canal length) 3 x 10^3.

Triglyceride measurements were done three times, that is on the 8th day (second week, before induction of high-fat diet), the 30th day (the fifth week after induction of high-fat diet), and on the 42nd day (end of the sixth week, after hyperlipidemia therapy). Lipid profile measurements were made three times using the enzymatic calorimetry Glycerol-3-Phosphatase-Oxidase-Paminophenazone (GPO-PAP) method.

RESULTS AND DISCUSSION

Extract Manufacturing Process

Results of determination of plant samples were made in the Herbarium Medanese laboratory, Faculty of Science and Mathematics, North Sumatera University, which identified that the sample used in this study was genuine *Citrus sinensis* (L.) Osbeck.

The average weight of each sweet orange is 215 g of orange. From 200 g of oranges, the weight of the skin is 30 g, so for 40 kg of oranges, ± 5 kg of orange peel is obtained. Next, the oranges were dehydrated for 14 days by air drying method. Air drying was done in Tarutung, North Sumatra, with an average temperature in Tarutung city of 19-23^{0}C and humidity of 65-80% for 8-12 hours. The results of air drying are presented in Table 1.

Sweet orange peel of as much as 1.7 kg was processed into simplicia with various stages. The first step was collecting raw materials; then sorting was done to separate raw materials damaged or unfit for use in making extracts and then slicing. Chopping was done to simplify the drying process. The simplicia was dried in the sun, but indirectly, with the top covered with a black cloth to avoid direct exposure to ultraviolet light because ultraviolet light is a catalyst to speed up the reaction so that the bioactive content of the orange peel is not damaged (Kartikasari *et al.,* 2019).

After the simplicia became dry, it was refined again by chopping it smaller or in a blender to reduce the particles and speed up the extraction process. The optimal particle size for *Citrus sinensis* peel is 0.2 mm (M'hiri *et al.,* 2014). Sweet orange peel extraction was done using the maceration method. This method was chosen because the process is easy and is one of the cold extraction methods. This method is conducted by immersing simplicia in a solvent at room temperature to minimize the damage or degradation of metabolites (Kartikasari *et al.,* 2019). The solvent used in the research maceration method is 50% ethanol. 50% ethanol solvent was chosen because the extraction was reported to be able to dissolve almost all organic compounds present in the sample, has volatile properties and is quickly released from the extract. Besides that, 50% ethanol extraction also produced the highest hesperidin content. The 50% ethanol concentration showed the highest IC50% value, 0.382 mg/ml (M'hiri *et al.,* 2014). Simplicia was macerated using 50% ethanol with a ratio of 1:3 for 3 days.

Table 1. Results of Dehydration to Weight of Orange Peel

Days to-	Total Orange Peel Weight
1	4.5 kg
2	4 kg
3	3.7 kg
4	3.4 kg
5	3 kg
6	2.4 kg
7	2.1kg
8	2 kg
9	1.9 kg
10	1.9 kg
11	1.8 kg
12	1.8 kg
13	1.7 kg
14	1.7 kg

When maceration occurs, storage was done in a place protected from direct light exposure to prevent light-catalyzed reactions or discoloration. The results of the filtrate obtained were then concentrated using a rotary vacuum evaporator and a water bath. Thickening using a rotary vacuum evaporator causes a decrease in the vapor pressure of the solvent so that the solvent will evaporate below its typical boiling point so that the phytochemical components contained in the extract are not damaged due to excessive heating. A water bath aims to make the remaining viscous liquid evaporate until a thick extract is obtained (Widuri, 2018).

After concentration with Waterbath, a viscous extract weighing 262.2 g of orange peel extract and 386.9 g of alcohol was obtained with a yield of 16%. Determination of the yield of this extract aims to compare the results of the simplicia extract with the simplicia used. The amount of extract indicates the active compound contained in the extract (Kartikasari *et al.,* 2019). A total of 1.7 kg of dried sweet orange peel was mashed using a blender and sifted using mesh no. 70 US Standard at 212 µm. The result was 1.64 kg of orange peel simplicia. Furthermore, the orange peel simplicia was macerated using 50% ethanol to produce 620 g of macerate. The macerate was evaporated, and 226.2 g of orange peel extract and 386.9 g of alcohol were obtained.

Effect of High Cholesterol Feed on Body Weight and Obesity Index of Mice

The high-fat diet used in this study consisted of 10% beef tallow, 20% cooking oil, and 20% quail egg yolk mixed in 120 ml and administered orally. Quail eggs are included in the category of very high cholesterol levels because the egg yolk cholesterol level is 844 mg. Quail egg yolk also functions as a slurry emulsifier, a unifying agent for oil and water, so that the slurry was made from beef tallow and used cooking oil which can blend with distilled water. The cooking oil used contains carcinogenic free radicals. The heating process can change the oil's physicochemical properties, accelerate triglyceride hydrolysis, and increase free fatty acids. Another ingredient used to improve lipid profile levels was beef tallow. Beef fat contains many saturated fatty acids such as lauric, stearate, palmitate, and myristate. The content of saturated fatty acids is 50.3% (Gunawan *et al.,* 2018; Megantara *et al.,* 2017).

Weight gain in mice can occur when the calories consumed by food are more than the calories expended as energy by the activities of the mice, including the physiological processes of the body so that the excess energy will be stored in adipose tissue, which will add to the mass of the body (National Heart Lung and Blood Institute, 2020). Being overweight (obesity) was measured using the parameter body mass index (BMI). The BMI of the mice in this study was determined using the Rōhrer index (Ardiansyah *et al.,* 2018).

Body weight is one indicator that describes the development of mice related to extrinsic (feed and drink) and intrinsic (genetic, metabolic, physiological) factors. Mice's BW was observed every week for six weeks. Data from the calculation of the average BW can be seen in Figure 1.

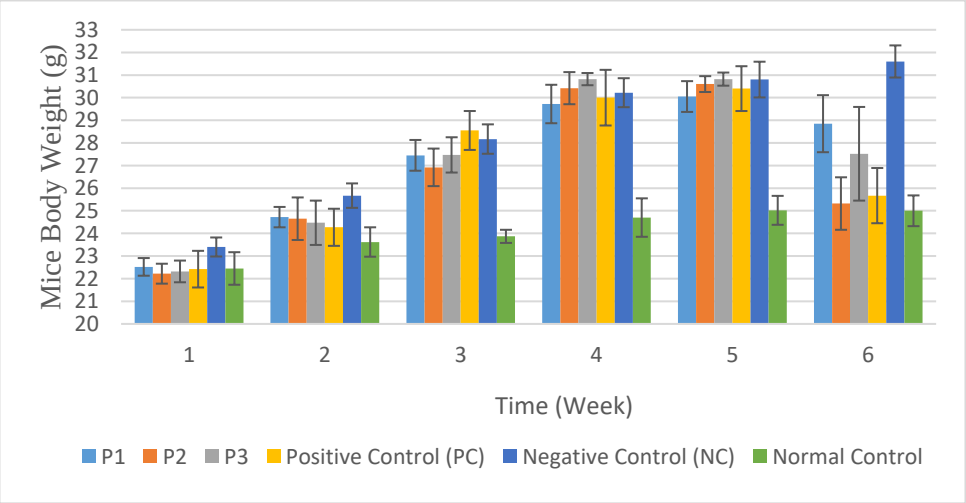


Figure 1. Graph of Mice Average Body Weight.

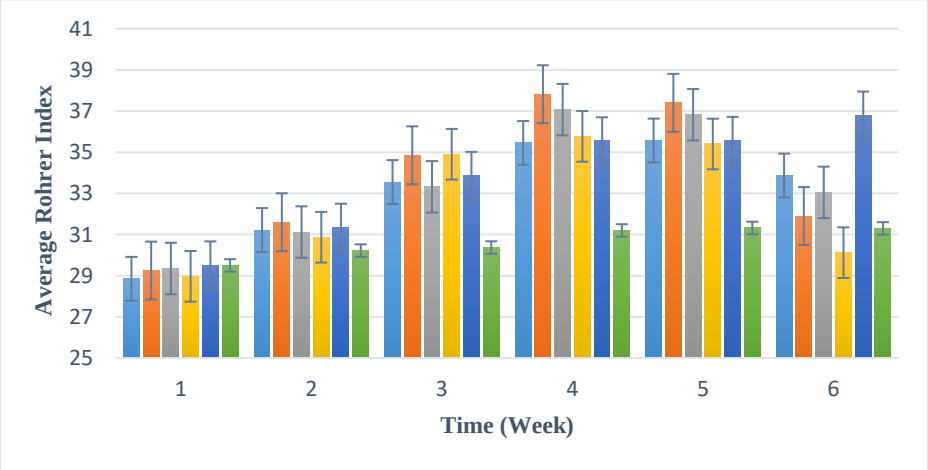


Figure 2. Graph of Average Mice Rōhrer Index.

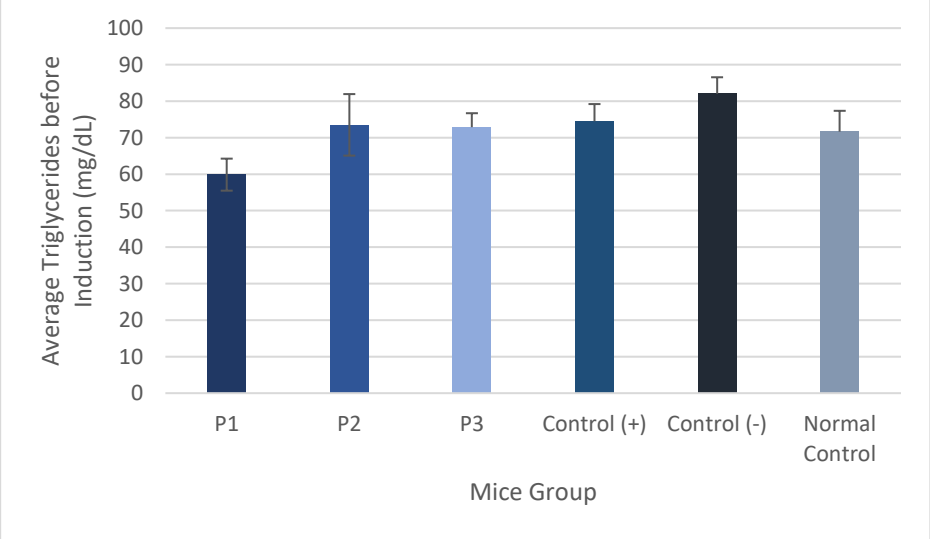


Figure 3. Graph of Mice Triglyceride Levels before Induction of Hyperlipidemia.

The mice obesity index (Rōhrer index) is one of the screenings to measure whether mice are obese. Data from the calculation of the average body weight are presented graphically in Figure 2.

Body weight (BW) and naso-canal length measurements were performed every week to determine the mice obesity index (Rōhrer index). The first week of measurement results during the acclimatization period showed that all groups of mice had an average obesity index (Rōhrer index <30). After the acclimatization period, the five groups of mice (P1, P2, P3, positive control, and negative control) were given a high-cholesterol slurry containing quail egg yolk, beef fat, and cooking oil.

The regular control group that was only fed B2 (551) also experienced an increase in body weight and an increase in the obesity index, reaching the obesity category. Feed B2 (551) contains 4% fat. The average value of the increase in BW in the normal control group was 0.75 kg/week.

Initial mice triglyceride profile (first week)

Measurement of the initial triglyceride profile of the mice was conducted before the seven-day acclimatization period, in which the mice were only given standard feed B2 (551) and water. The profile of triglyceride measurements in the first week before acclimatization and induction of high cholesterol feeds are presented graphically in Figure 3.

Acclimatization as a period of adaptation and conditioning was done before the experiments started to fulfill the mice's comfort

aspects during the study period to minimize research bias on experimental animals. Mice were placed in cages measuring 24 cm x 18 cm x 20 cm, and the cages were cleaned before entering the acclimatization period. During acclimatization, the mice were given B2(551) pig feed and metal-free water.

Mice triglyceride profile after cholesterol induction

Induction of high cholesterol slurry in experimental animals was done for 28 days. Examination of the lipid profile after the hypercholesterolemia induction period was then carried out again. The induction of high-cholesterol feed causes all mice to become obese. The results of triglyceride measurements after induction of high cholesterol feed are presented graphically in Figure 4.

The results of lipid profile measurements after induction of hypercholesterolemia showed an increase in triglyceride values in groups P1, P2, K+, and K. These changes were inversely proportional to group P3 where there was a decrease in triglyceride values.

Mice triglyceride profile after antihyperlipidemic therapy.

The final triglyceride profile measurement was conducted after antihyperlipidemic therapy. The results of triglyceride measurements in the sixth week after being treated with sweet orange peel extract (*Citrus sinensis*), atorvastatin, 1% CMC Na, and distilled water are presented graphically in Figure 5.

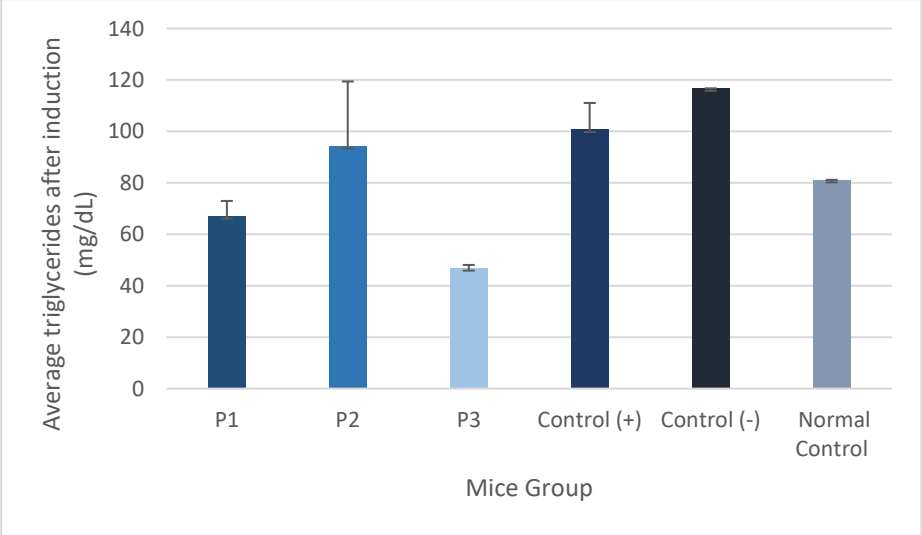


Figure 4. Graph of Triglyceride Levels after Cholesterol Induction.

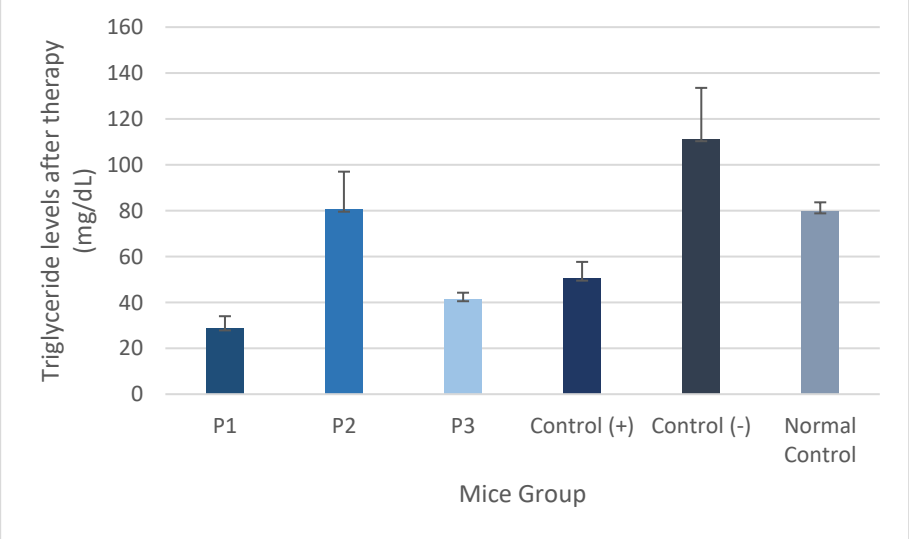


Figure 5. Triglyceride Levels after Antihyperlipidemic Therapy.

A high-cholesterol diet-induced mice. Then mice entered antihyperlipidemic therapy for two weeks with group P1 (10% *Citrus sinensis* skin extract), P2 (5% *Citrus sinensis* skin extract), P3 (10% *Citrus sinensis* skin extract), and K+ (atorvastatin 40 mg). Based on the measurement results, the triglyceride levels in the treatment group decreased. Meanwhile, mice in the K- group (CMC Na 1% and distilled water) continued to experience an increase in triglyceride values, as did mice in the normal control group which did not receive treatment.

Based on the results of obesity index measurements, in the first week before acclimatization, all mice were included in the normal category. In the second and third weeks, the mice were induced with high cholesterol feed, which caused them to gain weight, and almost all of them became obese (Rōhrer index > 30); only two mice in the normal control group were not obese. Increased differences in the response of mice can also occur in the same group and treatment due to genetic variation in mice, especially in the Quantitative Trait Loci (QTL), which is thought to have 63 variations of 190 total possible chromosomes that affect the weight of mice (Leamy *et al.,* 2019).

This high cholesterol induction treatment has been shown to increase blood cholesterol levels in mice, which has been proven in previous studies. Giving high-cholesterol feed in the form of quail egg yolk and broiler chicken feed in white mice (*Mus musculus*) causes mice to become hypercholesterolemic. Similar studies also found that mice fed high-cholesterol diets show increased body weight and serum levels of total cholesterol and triglycerides (Ahn *et al.,* 2019). It was also shown in rats that induction of high-fat diets using quail egg yolks, cooking oil, and goat fat caused mice to become hypercholesterolemic (Ardiani, 2017).

Antihyperlipidemic Effectiveness of Sweet Orange Peel Extract (*Citrus sinensis*) on Mice Triglyceride Levels

The results of measuring the lipid profile after induction of hypercholesterolemia showed an increase in triglyceride values, as seen in groups P1, P2, K+, and K-. This value is because the induction of high cholesterol slurry increases the lipid profile in the blood of experimental animals. This study's results align with research and theory, which indicate that high-fat food intake (including saturated fat, for example, used in cooking oil) will increase triglyceride levels because triglycerides are the main lipid component in food intake. Triglycerides can be stored in adipose tissue, skeletal muscle, liver, and intestines. In obese conditions, triglycerides can be stored in large quantities for months, so obesity in mice is a risk factor for increased triglyceride levels (Rial *et al.,* 2019).

The research data on blood serum triglyceride levels of mice after administration of *Citrus sinensis* orange peel extract therapy were analyzed using SPSS 25.0 (IBM Corp., Chicago, IL.) and tested for normality using the Shapiro-Wilk test because the number of samples was 24 (<50). The normality test resulted in a sig. p>0.05, which shows that the triglyceride data obtained is typically distributed, then its homogeneity is tested on the Levene test with a yield of sig. p>0.05, which indicates homogeneous data.

After going through the normality and homogeneity test stages, the paired T-test was conducted.

The results of the paired T-test show that the sig. (2-tailed) <0.05, which can be interpreted that there is a significant difference between triglyceride levels in the pre-test and post-test treatment of hyperlipidemia using sweet orange peel extract (*Citrus sinensis*).

Table 2. Paired Sample T-Test Results

Paired Samples Test									
		Paired Differences					t	df	Sig. (2-tailed)
		Means	Std. Deviation	Std. Error Means	95% Confidence Interval of the Difference				
					Lower	Upper			
Pair 1	PRETEST - POSTTEST	14.95375	15.65441	3.19544	8.34347	21.56403	4.680	3	.000

Table 3. Pearson Correlation Test Results

Correlations			
		PRE-TEST	**POST-TEST**
PRE-TEST	Pearson Correlation	1	.859**
	Sig. (2-tailed)		.000
	N	24	24
POST-TEST	Pearson Correlation	.859**	1
	Sig. (2-tailed)	.000	
	N	24	24
**. Correlation is significant at the 0.01 level (2-tailed).			

The increase in triglycerides in the P1, P2, K+, and K- groups was inversely proportional to the P3 group, where there was a decrease in triglyceride values. These triglyceride values happened because, during the hypercholesterolemia induction period, sweet orange peel extract (*Citrus sinensis*) was given to determine whether *Citrus sinensis* orange peel could provide a protective effect against hyperlipidemia. The P3 group is likened to a person who eats high-fat foods while consuming *Citrus sinensis* orange peel extract. The decrease in triglyceride levels in the P3 group indicated that *Citrus sinensis* peels had a protective effect. This effect proves previous research and publications that *Citrus sinensis* peel has a role in preventing atherosclerosis, cardioprotective, and cytoprotective effects on oxidative Stress. This protective effect can occur due to the primary mechanism of hesperidin which is involved, especially inhibition of lipid oxidation and regulation of gene expression that provide a protective effect including antithrombotic, anti-ischemic, antioxidant, and vasorelaxant with three main actions including increasing coronary vasodilation (vasorelaxant), reducing the ability of platelets to in the blood to clot (antithrombotic), and prevent LDL oxidation (antioxidant). The antioxidant and anti-inflammatory properties of the citrus flavonoids in *Citrus sinensis* play a key role in their activity against atherosclerosis (Etebu and Nwauzoma, 2014).

After the induction period of high cholesterol feed, mice were given therapy with *Citrus sinensis* orange peel extract in the treatment group and atorvastatin in the positive control group. In the treatment groups P1, P2, and P3, there was a decrease in triglyceride levels. This experiment proves that administering *Citrus sinensis* orange peel extract can reduce triglyceride levels. *Citrus sinensis* peel contains hesperidin and naringenin, which have antihyperlipidemic activity. Hesperidin and naringenin help lower blood cholesterol and triglyceride levels by inhibiting HMG-CoA reductase enzyme activity, suppressing APOB secretion in the liver, and suppressing plasma carnitine palmitoyl-O-transferase (CPT), which is involved in the transport of free fatty acids (Ji *et al.,* 2019).

Effective Dosage of Sweet Orange Peel Extract (*Citrus sinensis*)

The mean triglyceride levels of mice before and after administration of *Citrus sinensis* orange peel extract successively in the P1, P2, and P3 treatment groups were 67.1 mg/dL to 28.8 mg/dL, 94.32 mg/dL to 80.56 mg/dL, 46.87 mg/dL to 41.52 mg/dL, the percentage of decreasing triglyceride levels was 57%, 15%, 11%, respectively. In the control group C(+), C(-), and C(N) was 100.75 mg/dL to 50.5 mg/dL, 116.75 to 111.29 mg/dL, 81.17 mg/dl to 79.82 mg/dL, the percentage of decreasing triglyceride levels were 49%, 4.67%, 1.67%, respectively.

Based on Duncan's further test of analysis, the effective dose in this study was dose P1 (dose of 10% sweet orange peel extract), followed by dose P3 (1% dose, but at P3, when inducing high cholesterol feed, sweet orange peel extract was given). The results of Duncan's analysis were used in the Least Significance Different (LSD) post-hoc follow-up test.

The decrease in triglyceride levels in this study may be because sweet orange peel extract (*Citrus sinensis*) contains a flavone group that is known to have antihyperlipidemic activity, especially hesperidin, and naringenin which work with several mechanisms such as inhibiting the HMG-CoA reductase enzyme so that it can reduce triglyceride levels in the blood, increase the activity of Lipoprotein Lipase (LPL), an enzyme attached to capillary endothelial cells which hydrolyze triglycerides into fatty acids and glycerol. Hydrolysis of triglycerides will lower blood triglyceride levels. In addition, a decrease in triglyceride levels can also be caused by the

presence of saponins in sweet orange peel extract (*Citrus sinensis*), which bind to fat present in the intestinal lumen and form insoluble complex compounds that the intestinal mucosa and saponins cannot absorb can also increase production and secretion. Bile also expedites fat metabolism to lower blood triglyceride levels (Chen *et al.,* 2013; Mallick and Khan, 2016; Putri *et al.,* 2017).

In the LSD post-hoc follow-up test, the positive control group (atorvastatin therapy) had no significant difference with the P2 dose group (5%), which was induced by high cholesterol. This research is in line with previous studies, which found that the statin group had a moderate effect on reducing triglycerides, whereas its main effect is on low-density lipoprotein (LDL) reduction (National Heart Lung and Blood Institute, 2020).

CONCLUSIONS

Sweet orange peel extract (*Citrus sinensis*) has antihyperlipidemic effectiveness in lowering blood triglyceride levels in mice (*Mus musculus*) induced high cholesterol with an effective dose of 1% because 1% dose was able to provide an 11% decrease during antihyperlipidemic therapy and was able to make the P3 group the only group that experienced a decrease in triglycerides at the time of induction of high cholesterol.

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CONFLICT OF INTEREST

There is no conflict of interest.

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