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THE EFFECT OF ROSELLA DRIED CALYX INFUSION (*HIBISCUS SABDARIFFA*) IN LOWERING LDL CHOLESTEROL SERUM LEVEL IN MALE *SPRAGUE DAWLEY* RATS EXPOSED TO CIGARETTE SMOKE

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ABSTRACT

Background: Cigarettes increase cardiovascular risks by modifying lipid profiles through oxidation, alternating lipoprotein composition, and fat metabolizing enzyme activity. Vascular dysfunction can be primarily identified by LDL cholesterol composition shift, one of the atherosclerosis predictor. Rosella is a multifunctional herb widely used as a pleasant tea. Aside from its rich components, anthocyanin, pectin, PCA and hibiscus acid are playing the role keys in lowering LDL cholesterol through lipid metabolism enzyme activity modification. Rosella is easy to find, relatively cheap, tasteful and also categorized as food-grade FDA approved. **Aim:** To investigate the effect of rosella infusion in decreasing LDL cholesterol serum on cigarettes exposed male *Sprague dawley* rat. **Methods:** This study was a true experimental study design with post-test only controlled group design. After 7 days of adaptation, a total of 30 male Sprague dawley rats were randomly divided into 5 groups (n=6 per group); KN received no special treatment; KP exposed to 4 rods of cigarette smoke/day; experimental groups (P1, P2, P3) exposed to 4 rods of cigarette smoke/day and rosella dried calyx infusion with 250, 500 and 1000 mg/kg respectively for 30 consecutive days. LDL cholesterol levels were measured using the CHOD-PAP method. Collected data were analyzed statistically using One Way ANOVA Test. **Results:** There is a significant difference of LDL levels between all experimental groups (p=0,00) in the post-hoc test. **Conclusion:** These lines of evidence suggest that rosella infusion significantly decreases LDL Cholesterol serum level in cigarettes exposed *Sprague dawley* rats in a dose-dependent manner.

Keywords: *Cigarettes smoke, LDL cholesterol, Rosella calyx infusion*

INTRODUCTION

For years, cigarettes have been famous for causing major health, social, economy and environmental burden. On the health sector, smoking leads to lifestyle preventable diseases, such as COPD (Chronic Obstructive Pulmonary Disease), perinatal abnormalities, CHD (Coronary Heart Disease), ACS (Acute Coronary Syndrome), certain neoplasms, stroke and even sudden death.[1–4] It was all because cigarettes contain toxic substances, particularly nicotine that modify lipid in many ways.

After inhaled, nicotine activates nAchRs, a transmembrane ligand-gated ion channel protein, causes LDL accumulation in arterial walls through 3 factors, which are the increasement of LDL influx to arterial walls (by accelerating TGRLs' synthesis and secretion), the increasement of LDL retention (by altering lipid metabolic enzymes activity) and decelerating LDL efflux from arterial walls (by lowering HDL level).[5] Through these mechanisms, nicotine promotes a hyperlipidemic effect, primarily by worsening lipolysis and free fatty acid release. Free fatty acid will be uptaken by liver for further synthesis of VLDL (Very Low Density Lipoproteins) and LDL (Low Density Lipoproteins).[6–8] Altered lipid profile leads to worsened atherogenicity, an early sign of cardiovascular dysfunction, which can be measured by LDL cholesterol level.[9]

It's unfortunate that although most smokers had already known the harmful effects of smoking, the number of smokers are keep increasing each year in Indonesia.[1,10] Rosella is multifunctional Indian herbs that widely used as a pleasant tea. Aside from its rich components, anthocyanin, pectin, PCA (Procatheuic Acid) and hibiscus acid plays the key role in lowering LDL Cholesterol, one of atherosclerosis predictor, through several mechanisms.[11] Rosella is easy to find, relatively cheap, tasteful and also "food grade" FDA approved with LD50 over 5000 mg/kg (oral).[12] There hasn't been any research presenting the effect of Rosella infusion to decrease LDL Cholesterol serum level in cigarette smoke-exposed rats.

METHODS

Study Design

This study used true experimental with post-test only controlled group design. Experimental

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animals' procurement and treatment were conducted in Nutrition and Dietary PAU Gadjah Mada University Yogyakarta Laboratorium for 30 days.

Preparation of Rosella infusion

Fresh rosella calyxes were sorted to find the good one, then washed with running water. After thoroughly washed, rosella calyxes are dried with indirect sunlight. Rosella calyx was obtained from Pasar Beringharjo, Yogyakarta in a dried rosella calyx form. Crushed calyxes are taken as much as 2.5, 5 and 10 gram into 3 beaker glass. 100 mL aquadest added into each beaker glass. Based on a study presenting anthocyanin thermal kinetic degradation in rosella calyx [13] and a study of optimizing rosella beverage formulation[14], beaker glass I, II, III then placed into a waterbath, wait until the temperature reaches 85^{o}C, continue for 10 minutes while stirring. Using the filter paper, the infusion is filtered while it's hot. The infusion is cooled at room temperature then stored at 4^{o}C in a dark bottle until used.

Animal Models

The study established on 30 male *Sprague dawley* rats with the following criteria: healthy, 2 months old, weighed 150-200 g. After 7 days of adaptation, samples divided into 5 randomized groups: 2 control groups (KN, KP) and 3 experimental groups (P1, P2, P3) with a total of 6 rats/group. All groups are given unlimited access to COMFEED standardized meal and water, placed on a stainless steel cage with 12 h light-dark cycle. KN receives no special treatment; KP exposed to 4 rods of cigarette smoke/day; P1, P2, P3 exposed to 4 rods of cigarette smoke/day and given rosella infusion dose 250, 500, 1000 mg/kg respectively (1mL/100g). None of the rats had diarrhea, fatigue, or died during the study. The study used nonfilter kretek cigarette containing 2,5 mg nicotine and 39 mg tar.

Laboratory Analysis

LDL Cholesterol level is measured in Nutrition and Dietary PAU Gadjah Mada University Yogyakarta Laboratorium using CHOD-PAP methods by DiaSys. Samples obtained from male *Sprague dawley*'s plexus retroorbital under anesthesia. 100 µL blood from plexus retroorbital mixed with 1000 µL LDL precipitate reagent kit then incubated for 15 minutes at room temperature (20 - 25^{o}C). Centrifuge at 2500 g for 20 minutes so supernatant will be formed. Cholesterol standard diluted 1:10 with NaCl (9 g/L). After diluted, standard is treated like supernatant. The former 100 µL supernatant formed were mixed with 1000 µL cholesterol reagent kit, then incubated for 10 minutes at room temperature or 5 minutes at 37^{o}C. Diluted cholesterol standard is taken as much as 100 µL then mixed with 1000 µL cholesterol reagent kit, followed by incubation for 10 minutes at room temperature or 5 minutes at 37^{o}C. Blanko was made by mixing 10 µL aquadest with 1000 µL reagent. Shortly, LDL cholesterol serum measurement material are listed in table 1.

Table 1. LDL cholesterol serum measurement material

Material	Blanko	Standard	Sample
Supernatant		-	100 µL
Standard		100 µL	-
Cholesterol reagent	1000 µL	1000 µL	1000 µL
Aquadest	10µL	-	-

Read the value of OD (*Optical Density*) in 500 nm or 546 nm Hg wave. LDL cholesterol level obtained by calculating the following formula:

$$\text{Supernatant cholesterol (mg/dL)} = \frac{\Delta \text{ OD Sample}}{\Delta \text{ OD Standard}} \times \text{Standard consentration (200 mg/dL)}$$

LDL cholesterol(mg/dL) = total cholesterol(mg/dL) – supernatant cholesterol (mg/dL)
LDL cholesterol (mmol/L) = LDL cholesterol (mg/dL) x 0.02586

Statistical Analysis

Collected data were statistically processed using the SPSS software. Data normality analyzed using Sapphiro-Wilk test. Intergroup data were analyzed using One-way ANOVA proceed with Levene's Post Hoc Equal Variances Assumed Bonferroni. The value degree of significance is $p<0.05$.

Ethics Approval

The study protocol was approved by The Faculty of Medicine Diponegoro University Ethics Committee with license No. 107/EC/H/KEPK/FK-UNDIP/VII/2019.

RESULTS

The average weight baseline characteristic of all samples (KN; KP; P1; P2; P3) were 168.67; 174.83; 175.33; 175.17; 176.50 gram respectively. Data were normally distributed (p=0.184) and homogeneous (p=0.444). On the other hand, the weight characteristic of all samples (KN; KP; P1; P2;

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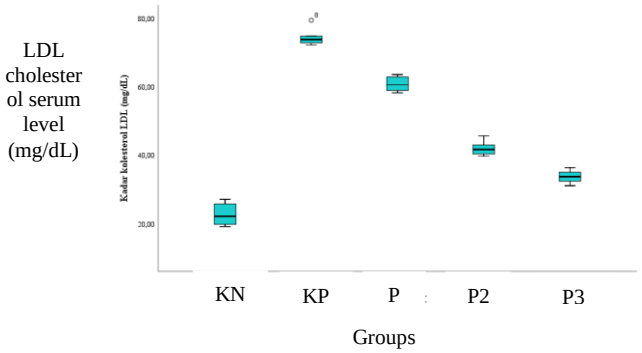
P3) at the end of the study were 173.17; 179.67; 180.17; 180.17; 180.83; 184.87 gram respectively.

The result of LDL cholesterol level serum were shown in Graphic 1 and Table 1.



Graphic 1. Boxplot LDL cholesterol level serum

Table 2. LDL cholesterol level serum (mg/dL)

Groups	n=30	Mean±Deviation standard	p
KN (Negative Control)	6	22.74 ± 3,23	
KP (Positive Control)	6	74.50 ± 2,60	
P1 (Trial group 1)	6	60.82 ± 2,19	0.00
P2 (Trial group 2)	6	42.05 ± 2,17	
P3 (Trial group 3)	6	33.77 ± 1,92	

One Way ANOVA test , significance value p<0.05

Table 2 shows that cigarette smoke elevates LDL cholesterol level. This study supports the antihyperlipidemic effect of rosella infusion. P1 (rosella infusion dose 250 mg/kg) lowers LDL cholesterol serum level as much as 18.36 %. P2 (rosella infusion dose 500 mg/kg) lowers LDL cholesterol serum level as much as 43.56 %. P3 (Rosella infusion dose 1000 mg/kg) lowers LDL cholesterol serum level as much as 54.67 %. It shows up that rosella infusion lowers LDL cholesterol serum in a dose-dependent manner despite the decline of LDL cholesterol level by 1000 mg/kg rosella infusion doesn't do as much as the lower doses of rosella infusion.

For hypothesis analysis, One Way ANOVA test was applied. Intergroup data has a significant difference (p=0.00). The result of Post Hoc Bonferroni Test were shown in Table 3.

Table 3. Post Hoc Bonferroni Test

Kelompok	KN	KP	P1	P2	P3
KN	-	p= 0.00*	p= 0.00*	p= 0.00*	p= 0.00*
KP		-	p= 0.00*	p= 0.00*	p= 0.00*
P1			-	p= 0.00*	p= 0.00*
P2				-	p= 0.00*
P3					-

p= significant value, *significance regarding p<0.05.

Table 3 shows that cigarette smoke significantly increases LDL cholesterol serum level. Rosella infusion possessed an antihyperlipidemic effect by lowering LDL cholesterol level in a dose-dependent manner although the biggest dose used in this study (1000 mg/kg) hasn't made the decline reached the normal LDL cholesterol level.

DISCUSSION

Nicotine modifies lipid and lipoprotein metabolism in several ways.[15–17] Some of them are lipid oxidation, changing in lipoprotein composition, plasma-associated and lipoprotein-associated lipid transfer enzymes' activity, alternating fatty acid metabolism, lipid postprandial and RCT (Reverse Cholesterol Transport).[18]

Nicotine significantly decreases several hormones and lipid metabolic enzymes' activity. The activation of nAchRs promotes the activity of epinephrine and catecholamine hormones which exaggerate lipolysis and free fatty acid release. Nicotine impaired the activity of CETP (Cholesteryl Ester Transfer Protein) and decreases LCAT (Lecithin Cholesterol Acyl Transferase), HL (Hepatic Lipase) and LL (Lipoprotein Lipase).[6–8,18,19] CETP and LCAT involved in RCT while CETP works on interlipoprotein exchange and LCAT works on maturating HDL. The inhibition effect on these hormones leads to VLDL overproduction.[20,21] On the other hand, nicotine elevates HMG Co-A reductase's activity, which catabolize cholesterol production through mevalonate pathway.[8,18,22,23]

Furthermore, nicotine worsens negative emotional stress which promotes the HPA axis to produce more ACTH (Adrenocorticotropin Hormone). ACTH affects adrenal to increases cortisol levels which associated with lipid profiles (significantly increase of total cholesterol and triglyceride-rich lipoprotein, decrease HDL) and central adiposity.[19] Altered lipid profiles and high LDL cholesterol level worsen atherogenicity.[9,24–26]

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This study supports all of the theory mentioned by reporting that the exposure of 4 rods cigarette smoke every day for 30 days increases significantly LDL cholesterol level serum.

Rosella has its hypotriglyceridemic effect by having HCA (hibiscus acid/hydroxicytrate acid) which inhibits lipolysis and citrate lyase enzyme activity. Its main component, anthocyanin, also can inhibit CETP enzyme activity. The inhibition of CETP promotes cholesterol clearance from peripheral to liver, excreted to bile so that total cholesterol diminished.[27] This synergizes with the effect of pectin (fiber) as an absorbent in the gastrointestinal tract so that bile acids are prevented from returning to the liver through enterohepatic cycle. The liver needs cholesterol as the raw material of bile acids so that total blood cholesterol levels decrease. Pectin and PCA (Procathecuic acid) are also having an inhibitory effect on lipase and HMG Ko-A reductase which inhibits lipolysis.[25,28]

This study is in line with the study performed by Dinayanti, et al. which states that the administration of dried rose calyx reduces cholesterol levels significantly in accordance with increasing doses.[28] This is supported by another study from Zainalabidin, et al. saying that the administration of rosella flower extract increases HDL levels and reduce total cholesterol and LDL.[29] This study is in accordance with Chen, Chang Che, et al. that stated whether *Hibiscus sabdariffa* extract, macerates with hot water, could significantly reduce serum triglycerides, total cholesterol, and LDL cholesterol levels, also not significantly increase HDL cholesterol level in rabbits fed high cholesterol.[30] This research was in line with Artini et. al's study which states that the administration of rosella tea 2 grams once a day combined with 330 g of avocado and 10 mg of simvastatin can significantly reduce total cholesterol levels in hypercholesterolemia.[31] Another study from Hirunpanich, et al. also saying similar things that *Hibiscus sabdariffa* calyx extract at the dose of 500 and 1000 mg/kg could significantly reduce total cholesterol, triglycerides and LDL cholesterol serum in hypercholesterolemic rats, but not significantly reduces $CuSO_4$ induced ox-LDL.[32]

Overall, the result of this study supports previous studies about the antihyperlipidemic effect of rosella. Rosella infusion could significantly reduces LDL cholesterol serum level on male *Sprague dawley* rats exposed to cigarette smoke in a dose-dependent manner. This means that the greater dose of rosella infusion given, there is greater LDL cholesterol serum decline although rosella infusion at the dose of 1000 mg/kg doesn't show a sharp drop in LDL cholesterol serum level compared to the lower doses of rosella infusion. Further research needs to be carried out to find the optimal dose, proper frequency, and duration of administration of rosella infusion to achieve normal LDL cholesterol serum level in rats.

CONCLUSION

Our study found that cigarette smoke significantly increased LDL cholesterol levels in male *Sprague dawley* rats. On top of that, we found that rosella infusion at various doses could significantly lower LDL cholesterol levels in male *Sprague dawley* rats exposed to cigarette smoke. The reduction of serum LDL cholesterol levels in male Sprague dawley rats given rosella flower infusion was comparable to an increase in the therapeutic dose when compared to those exposed only to cigarette smoke. Interestingly, this study suggested that the effective dose of rosella flower infusion to reduce LDL cholesterol levels in male Sprague Dawley rats exposed to cigarette smoke was in the range of 500 mg/dL, as evidenced by the extreme decrease in LDL levels at these levels. However, further research to find the accurate dose is certainly still needed.

CONFLICT OF INTEREST

No potential conflict of interest relevant to this article was reported.

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