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Dietary Ethanolic Extract of *Mangosteen pericarp* Reduces VCAM-1, Perivascular Adipose Tissue and Aortic Intimal Medial Thickness in Hypercholesterolemic Rat Model

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Keywords: Atherosclerosis; Dyslipidemia; Mangosteen Pericarp Ethanolic Extract; VCAM-1; PVAT; IMT

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Abstract

BACKGROUND: High-fat diet (HFD) is associated with dyslipidemia which is a risk factor for atherosclerosis. Dyslipidemia causes oxidative stress which induces vascular cell adhesion molecule-1 (VCAM-1). Oxidative stress also triggers the thickening of tunica intima-media (IMT) and Perivascular Adipose Tissue (PVAT). Xanthone compound in ethanolic extract of *Mangosteen pericarp* (EEMP) has an antioxidant property to overcome the oxidative stress.

AIM: The objective of this study is to investigate the effect of dietary EEMP administration on the expression of VCAM-1 and thickness of PVAT and IMT in atherosclerotic rat model fed with HFD.

METHODS: This experimental laboratory study uses 25 Wistar strain *Rattus norvegicus* which were divided into 5 study groups. Negative Control group (GT1) was given a normal diet, Positive Control group (GT2) was treated with HFD, and three treatment groups were each treated with HFD with *Mangosteen pericarp* extract of 200 mg/kg BW (GT3), 400 mg/kg BW (GT4), and 800 mg/kg BW (GT5). Measurements of VCAM-1 expression were performed using immunofluorescence. PVAT and IMT measurements were performed on rat aortic preparations.

RESULTS: One-way ANOVA test showed the addition of dietary EEMP significantly ($p < 0.05$) decreased the expression of VCAM-1 and decreased the thickness of PVAT and IMT in treatment groups as compared with both negative and positive controls. Tukey HSD test showed a dose of 800 mg/kg BW was the most effective dose for decreasing VCAM-1 level, PVAT and IMT.

CONCLUSION: Dietary EEMP significantly decreases the expression of VCAM-1, as well as the thickness of PVAT and IMT in Wistar strain *Rattus norvegicus* treated with HFD.

Introduction

Cardiovascular disease such as hypertension, stroke, coronary heart disease and heart failure, is the leading cause of global death. In 2015, it was estimated that 17.7 million people or about 31% of the world's deaths were due to this disease [1]. Coronary heart disease develops from the atherosclerotic process. The exact cause of atherosclerosis still unknown, but research suggests that atherosclerosis starts when certain factors damage the inner layers of the arteries [2].

Atherosclerosis is an irreversible process, causing disease by the slow development of narrowing of the arterial lumen, mainly located in the intima of middle-large size arteries [3], [4]. Atherosclerosis is progressive and leads to the formation of atherosclerotic plaques that causes obstruct in blood vessels. Atherosclerotic plaque formation begins from chronic inflammation which triggers oxidative stress. Oxidative stress is a state of

reactive oxygen species (ROS) and anti-oxidants imbalance [5]. Physiologically; ROS acts as a regulator in various processes of defence mechanisms, differentiation, proliferation, and migration of body cells. Conversely, an excess of ROS levels in the body is a sign of a pathological condition characterised by excessive inflammation and contributes to the pathogenesis of atherosclerosis [6], [7].

High ROS production can increase the production of PVAT and thickened IMT in dyslipidemia [8]. ROS can activate peroxisome proliferator-activated receptor-ɣ (PPAR-ɣ), which is a master regulator of adipogenesis, thus causing expansion and dysfunction of PVAT [9]. ROS also contribute to endothelial dysfunction characterised by increased expression of adhesion molecules such as vascular cell adhesion molecule 1 (VCAM-1) [10].

Garcinia mangostana L., or commonly known as mangosteen, is rich in phenol components, such as xanthones, tannins, and anthocyanins [11]. These components have been shown to have many biological activities, and in particular, mangosteen pericarp extract has anti-inflammatory, anti-cancer, anti-microbial, and anti-oxidant effects both *in vivo* and *in vitro [12]*. Xanthone has a role as a scavenger antioxidant capable of inhibiting ROS [13]. Based on these facts, ethanolic extract of mangosteen pericarp (EEMP) is expected to inhibit VCAM-1 expression and decrease PVAT and IMT thickness in the aorta of atherosclerotic rat model treated with high-fat diet (HFD).

Material and Methods

Laboratory Animals

This research used 8-week-old male Wistar strain *Rattus norvegicus* weighing about 1.5-2 kg which was kept in Pharmacology Laboratory of Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia. Before the treatment, the rats underwent an acclimatization process for 2 weeks, and later were divided into 5 treatment groups (n = 5), i.e. Negative Control with normal diet (G1), Positive Control with HFD diet (G2), the group with HFD and 200 mg/kg BW EEMP administration (G3), the group with HFD and 400 mg/kg BW EEMP administration (G4), and the group with HFD and 800 mg/kg BW EEMP administration (G5).

Feeding and Creating Dyslipidemic Rat Model

Rats were fed according to their respective treatment groups. The normal group was given a normal diet of PARS 62%. Groups with HFD were given 2% cholesterol, 0.2% cholic acid, and 5% lard oil supplements, 30 grams daily, *ad libitum [1]*.

EEMP (Ethanolic extract of mangosteen pericarp) Process

The EEMP was obtained through drying, extraction, and evaporation processes. *Mangosteen pericarps* were washed and then dried in an oven at 80 degrees until dry or free of water content. The dried mangosteen pericarp was refined evenly into 100 grams of dried sample, and placed into a 1-litre Erlenmeyer flask. It was soaked in 70% ethanol until the volume reached 1000 mL, shaken for 30 minutes, and settled for 1 night until a sediment form. The next step was the evaporation process using *Rotary Evaporator*. The evaporation process is completed when the ethanol solution is separated from the active ingredient. The EEMP were administered to the research animals through a feeding tube with doses of 200 mg/kg BW, 400 mg/kg BW and 800 mg/kg BW respectively.

Rat Dissection

Dissection was performed 2 months subsequent of EEMP administration. Surgery was performed after the animal had been given ketamine as an anaesthetic agent. Later, the aorta was retrieved and preserved using 10% formalin for subsequent aortic preparation with haematoxylin-eosin staining.

Measurement of IMT and PVAT Thickness

Measurement of IMT and PVAT thickness was conducted at Anatomy Pathology Laboratory, Faculty of Medicine, Brawijaya University, Malang, East Java, Indonesia. Aortic preparation was done using paraffin block and *HE*. The measurements were made by drawing perpendicular vertical lines of the intima tunica and outline of the media tunica. Measurements were made in 5 clockwise zones and then averaged. The measurement of PVAT thickness was performed by measuring the mean of the lowest, medium, and highest thickness of PVAT on aortic preparations. Measurements with 400 x magnification and were made using *scan dot slide Olyvia* software.

VCAM-1 Measurement

Aortic tissue was fixated with PHEMO buffer (Pipes 0.068 M, HEPES 0.0025 M, EGTA 0.005 M, MgCl 0.003 M, 10% DMSO, PH 6.8), which contained 3.7% formaldehyde, 0.05% glutaraldehyde, 0.5 triton x-100, for 10 minutes at room temperature. Coverslip was blocked by 10% goat serum/PBS for 10 minutes.

VCAM-1 expression on aortic tissue was identified through double-staining immunofluorescence using anti-rat VCAM-1 with rhodamine as the secondary antibody, and α-actin was coloured by fluorescein isothiocyanate as the secondary antibody, and then when observed with Confocal Laser Scanning Microscope (CLSM), the double-stained result would show VCAM-1 expression in smooth muscle cells. Lastly, the collected data were quantitatively analysed with Olympus fluoview 1.7A software.

Ethics

The medical research ethics committee of the Faculty of Medicine of Brawijaya University has stated that this research is ethically detailed with No.211 / EC / KEPK-S1-PD / 06 / 2017

Statistical Analysis

Statistical analysis was performed using SPSS version 16 software with significance level of 0.05 (p = 0.05) and 95% confidence interval (α = 0.05). This research used One Way ANOVA Parametric Test to determine the effect of EEMP in 5 groups of an atherosclerotic rat model. Data analysis was further conducted using *Tukey HSD* Test to understand the difference between groups.

The presentation of ANOVA and Post Hoc Tukey HSD data tables refer to Seng *et al.,* (2018) [15].

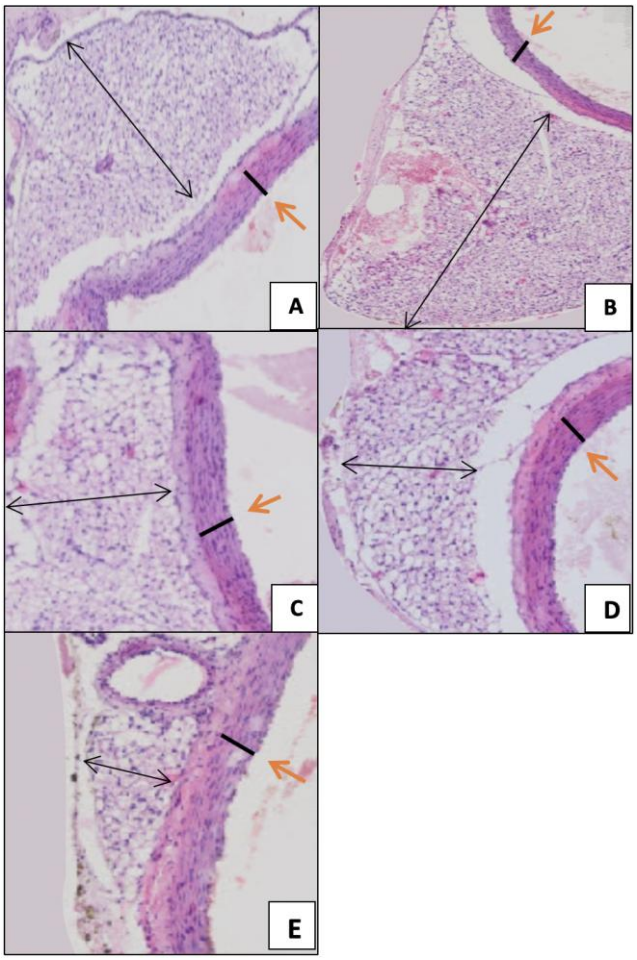

Figure 1: Images of aortic preparation with tunica intima-media and PVAT thickness measurement on each research animal treatment group; A) Normal diet group (GT1); B) HFD group (GT2); C) the group with HFD and 200 mg/kg BW EEMP administration (GT3); D) the group with HFD and 400 mg/kg BW EEMP administration (GT4); and (E) the group with HFD and 800 mg/kg BW EEMP administration (GT5)

Results

Histopathological Findings of IMT and PVAT

Measurements were done with 400 x magnification and were made using *scan dot slide Olyvia* software. Blackline that is pointed by red arrow shows the tunica intima-media thickness and black arrow of each image shows the PVAT thickness. Histopathological features of the aorta between 5 groups show a clear difference in tunica intima-media and PVAT thickness. Intima-media of picture D has a similar thickness to intima-media of picture A as the negative control. Tukey HSD test shows that the dosage of EEMP 200 mg/kg BW can lower the BMI, with the mean dosage value closest to negative control is 400 mg/kg BW. The thickness of PVAT in figure E is thinner than others, shown as the length of the black arrow. Tukey HSD Test shows that PVAT thickness in 800 mg/kg BW EEMP group was significantly different compared to the HFD group.

Immunofluorescence Findings of VCAM-1

On this study, statistically significant differences were seen in all of the parameters; IMT ($p < 0.001$), PVAT ($p = 0.008$), VCAM-1 ($p = 0.032$), HDL ($p = 0.010$), LDL ($p < 0.001$), TG ($p = 0.007$), and TC ($p < 0.001$. From the data presented in Table 1, it appears that there was a worsening of lipid profiles in the group of rats with HFD administration.

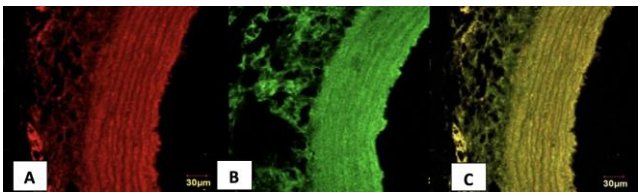

Figure 2: A) VCAM-1; B) α-actin expression; C) expression of VCAM-1 in Smooth Muscle Cell (SMC); Immunofluorescence findings of VCAM-1 in Smooth Muscle Cell (SMC), labelled with α-actin; A) VCAM-1 – rhodamine; B) α-actin – fluorescein isothiocyanate (FITC). The colour green shows α-actin expression (SMC marker); C) Double staining of VCAM-1 and α-actin showed the expression of VCAM-1 in smooth muscle cells of aortic tissue. The colour yellow shows expression of VCAM-1 in SMC

Then with EEMP treatment, there was a significant improvement of lipid profile from ONEWAY ANOVA test. Not only improved lipid profiles, but EEMP administration can also reduce IMT, VCAM-1, and PVAT thickness with significant ONE-WAY ANOVA test results.

Table 1: IMT, PVAT thickness, and VCAM-1 Expression Calculation, and Lipid Profile variables at Groups of Treatment (GT1-GT5) with ANOVA and Tukey HSD results

Variables	Groups of Treatment	Mean ± SD	F-statistics (df)	*P*	Tukey HSD	95% CI		adj p
IMT (μm)	GT1	61.05 ± 2.01	13.998 (24)	<0.001	GT1-GT2	0.98	3.21	< 0.001
	GT2	78.19 ± 6.33			GT1-GT3	-1.02	3.35	0.128
	GT3	62.76 ± 6.71			GT1-GT4	-0.67	1.59	0.468
	GT4	61.10 ± 3.35			GT1-GT5	-0.47	0.85	0.263
	GT5	60.59 ± 1.31			GT2-GT3	1.68	3.27	< 0.001
					GT2-GT4	1.22	3.14	< 0.001
					GT2-GT5	0.54	3.06	< 0.001
					GT3-GT4	-1.15	2.93	0.206
					GT3-GT5	-0.83	2.25	0.068
					GT4-GT5	-0.33	1.76	0.104
PVAT	GT1	547.48 ± 152.10	4.608 (24)	0.008	GT1-GT2	3.42	6.28	0.006
(μm)	GT2	744.24 ± 115.00			GT1-GT3	1.68	3.76	0.012
	GT3	737.00 ± 58.27			GT1-GT4	2.82	4.08	0.020
	GT4	711.64 ± 112.11			GT1-GT5	-4.42	1.36	0.075
	GT5	554.77 ± 33.95			GT2-GT3	-3.84	0.87	0.187
					GT2-GT4	-4.86	2.48	0.245
					GT2-GT5	1.27	5.08	0.046
					GT3-GT4	-2.63	1.34	0.591
					GT3-GT5	2.74	4.92	0.024
					GT4-GT5	1.04	3.86	0.012
VCAM-1	GT1	477.01 ± 81.35	3.542 (24)	0.032	GT1-GT2	0.38	2.74	0.026
(AU)	GT2	628.51 ± 18.00			GT1-GT3	-3.26	1.82	0.143
	GT3	497.52 ± 64.70			GT1-GT4	-2.84	2.49	0.281
	GT4	451.26 ± 100.84			GT1-GT5	-3.04	1.86	0.309
	GT5	447.71 ± 101.30			GT2-GT3	2.32	4.68	0.027
					GT2-GT4	2.28	5.86	0.018
					GT2-GT5	1.96	4.70	0.009
					GT3-GT4	-2.46	1.68	0.074
					GT3-GT5	-3.86	2.92	0.065
					GT4-GT5	-3.58	3.28	0.073
HDL	GT1	30.00 ± 7.52	4,865 (24)	0.010	GT1-GT2	-2.38	3.31	0.086
(mg/dL)								
	GT2	22.25 ± 6.50			GT1-GT3	-3.26	3.98	0.078
	GT3	28.75 ± 7.50			GT1-GT4	-3.53	3.74	0.056
	GT4	37.25 ± 7.13			GT1-GT5	1.64	2.32	0.035
	GT5	41.50 ± 5.25			GT2-GT3	-2.75	3.87	0.062
					GT2-GT4	2.51	3.04	0.020
					GT2-GT5	1.79	3.35	0.012
					GT3-GT4	-2.92	4.02	0.073
					GT3-GT5	1.81	3.69	0.009
					GT4-GT5	-2.86	3.63	0.086
LDL	GT1	60.25 ± 16.19	15.474 (24)	<0.001	GT1-GT2	1.44	4.82	< 0.001
(mg/dL)								
	GT2	126.25 ± 17.03			GT1-GT3	0.86	5.18	< 0.001
	GT3	89.50 ± 9.84			GT1-GT4	-0.98	4.32	0.012
	GT4	78.75 ± 12.94			GT1-GT5	-2.45	1.72	0.087
	GT5	65.75 ± 8.26			GT2-GT3	1.36	4.27	< 0.001
					GT2-GT4	0.46	2.62	< 0.001
					GT2-GT5	2.38	3.86	< 0.001
					GT3-GT4	1.26	2.76	< 0.001
					GT3-GT5	0.26	2.39	< 0.001
					GT4-GT5	-1.41	3.84	0.065
TG	GT1	101.75 ± 6.80	5,331 (24)	0.007	GT1-GT2	0.97	4.05	0.013
(mg/dL)	GT2	146.25 ± 33.38			GT1-GT3	-2.25	5.72	0.067
	GT3	102.50 ± 12.17			GT1-GT4	-1.36	3.84	0.248
	GT4	101.75 ± 11.70			GT1-GT5	-1.72	3.24	0.357
	GT5	97.50 ± 10.66			GT2-GT3	4.84	8.63	0.004
					GT2-GT4	4.81	8.97	0.002
					GT2-GT5	5.94	9.08	0.001
					GT3-GT4	-0.45	4.64	0.492
					GT3-GT5	-2.76	3.86	0.074
					GT4-GT5	-3.46	4.06	0.062
TC	GT1	107.75 ± 10.53	18.442 (24)	< 0.001	GT1-GT2	2.76	5.87	< 0.001
(mg/dL)	GT2	181.25 ± 23.72			GT1-GT3	-4.64	1.08	0.083
	GT3	113.25 ± 12.55			GT1-GT4	-5.26	2.21	0.074
	GT4	104.00 ± 18.31			GT1-GT5	-5.46	1.98	0.059
	GT5	102.50 ± 7.18			GT2-GT3	2.43	4.66	0.001
					GT2-GT4	1.24	3.57	< 0.001
					GT2-GT5	0.69	2.74	0.001
					GT3-GT4	-4.79	3.27	0.056
					GT3-GT5	-4.02	1.85	0.086
					GT4-GT5	-2.52	0.82	0.074

Footnote: GT1: Negative control with normal diet; GT2: Positive control with HFD diet; GT3: the group with HFD and 200 mg/kg BW EEMP administration; GT4: the group with HFD and 400 mg/kg BW EEMP administration; GT5: the group with HFD and 800 mg/kg BW EEMP administration. Abbreviation: HFD, High Fatty Diet; IMT, Intima-Media Thickness; PVAT, Peri Vascular Adipocyte Tissue; VCAM-1, Vascular Cell Adhesion Molecule, HDL, High-Density Lipoprotein, LDL, Low-Density Lipoprotein; TG, Triglyceride; TC, Total Cholesterol.

Tukey HSD test was performed on VCAM-1, IMT and PVAT showed that a dose of 400 mg/kg BW was a significant dose to decrease IMT and VCAM-1 thickness because approached with normal diet group and a dose of 800 mg/kg BW was a significant dose to decrease PVAT thickness approached with normal diet group.

Discussion

The peel of mangosteen fruit has long been used by residents in various countries as an ingredient in traditional medicine. Several experimental studies have proved that EEMP could be used as anti-tumour, anti-inflammatory, anti-bacterial, anti-viral, and anti-oxidant agents. In previous studies, the ethanolic extract of mangosteen pericarp was mentioned to have the highest antioxidant effect [14]. The EEMP as a source of antioxidants works by releasing electrons to free radicals to form stable products to prevent the generation of chain reactions [15]. Xanthone compound in the pericarp of mangosteen is needed by the body for the balance of pro-oxidants. Xanthone has binding properties towards unstable free oxygen. This free oxygen acts as free radical and destroying body cells; therefore, xanthone can inhibit the degeneration process of cell damage [15]. Several *in vitro* studies have shown that mangosteen pericarp extract can absorb free radicals [16]. Xanthones are divided into α-mangosteen, β-mangosteen, γ-mangosteen, and methoxy-betamangosteen, but the most common form is α-mangosteen [17]. The α-mangosteen antioxidant may increase lipoprotein lipase enzyme activity and improve very-low-density lipoprotein (VLDL) catabolism. As a result, the total cholesterol, triglyceride, and LDL levels fall, and HDL or good cholesterol level rises [18].

In Table, HFD administration has been shown to worsen the lipid profiles of research animals significantly. This worsening of lipid profile shows the presence of dyslipidemia. Dyslipidemia is one of the risk factors for atherosclerosis [17]. HFD causes dyslipidemia, and manifestations are elevated LDL level in the body; in a condition of excessive fat intake, LDL may accumulate in IMT and PVAT of the blood vessels. On the other hand, dyslipidemia will cause adipocyte cells hypertrophy. Adipocyte growth is associated with an increase of pro-oxidant enzymes, such as NADPH oxidase, and a decrease of antioxidant enzymes, such as catalase [19. These two conditions will increase the H_2O_2 production. H_2O_2 reacts with Fe_2^+ to release hydroxide (OH^-) via Fenton reaction. OH^- is a free radical that oxidises LDL accumulated in tunica intima; this process is called lipid peroxidation, and it will produce oxidised LDL (oxLDL); from this stage, the atherosclerosis process begins. OxLDL will later lead to an increase of proinflammatory cytokines, such as *Tumor Necrosis Factor-α* (TNF-α) [17]. Increased proinflammatory cytokines will trigger an increase in VCAM-1 expression [20]. Increased expression of VCAM-1 causes accumulation of monocytes in IMT. The adhesion of monocyte-endothelial cells to atherosclerotic lesions is affected by intercellular oxidative stress in Mononuclear Cells (MNC) and causes the thickening of IMT [21]. The administration of HFD which may cause thickening of PVAT will also

lead to an increase in PVAT proinflammatory property that is characterised by decreased expression of adiponectin and increased expression of IL-6, IL-8, and monocyte chemoattractant protein-1 (MCP-1). These parameters (VCAM-1, PVAT, and IMT) play a role in atherosclerosis pathogenesis.

VCAM-1 is also a molecule that plays a role in atherogenesis. One component in atheroma plaque is accumulated leukocytes. Adhesion molecules, such as VCAM-1, activates endothelial cell signal transduction, which in turn alters the form of endothelial cells to pave the way for leukocyte migration [22]. Increased expression of VCAM-1 in HFD group showed a positive tendency. This result is accordance with previous research by Huang *et al.,* reported that after treating rats with HFD for 12 weeks, VCAM-1 expression in the aorta and the levels of sVCAM-1 were increased significantly [23]. After EEMP administration, VCAM-1 expression displayed a significant decrease. Aside from being an anti-oxidant, xanthone also has anti-inflammatory properties. In an experiment conducted by Wihastuti *et al*., the administration of EEMP exhibit the ability to lower TNF-α and IL-1 levels in high-cholesterol diet-induced rats compared to the positive control group. The two cytokines are known to be related to the expression of VCAM-1 [24]. These findings will contribute to further improving our understanding of the underlying mechanism regulating adhesion molecule levels by exploring the effects of EEMP on the pathogenesis of atherosclerosis.

The thickness of the research animal's PVAT in the HFD group (seen in Table) experienced a significantly different increase, as evidenced by Tukey HSD *post-hoc* test results. HFD administration may induce a pro-inflammatory phenotype in PVAT with low adiponectin expression and high IL-6, IL-8, and MCP-1 expression [25]. This thickening of PVAT illustrates a condition of inflammation and endothelial dysfunction as a result of an increase in ROS [25]. Consistent with our studies, others reported that PVAT plays a role in the pathogenesis of vascular lesion formation. Ketonen *et al*. reported that obesity-induced endothelial dysfunction is caused by increased oxidative stress and enhanced expression of inflammatory cytokine in PVAT [26]. In this research, the administration of EEMP that contains xanthone as an anti-oxidant agent was proved by ANOVA parametric test to be able to decrease the thickness of PVAT significantly. The EEMP dose that significantly lowered PVAT thickness was 800 mg. This EEMP dose could decrease PVAT thickness almost until the level of normal group rats. PVAT thickness in 800 mg/kg BW EEMP group was significantly different compared to the HFD group. The increase of free radical and oxidative stress conditions in atherosclerosis leads to the thickening of adipocyte tissue that produces proinflammatory and anti-inflammatory cytokines. This event is characterised by an increase in PVAT thickness observed using histopathologic imaging. In addition to PVAT, oxidative stress is also evident from the increasing number of foam cells and narrowing of the lumen diameter of the blood vessel due to atherosclerotic plaque deposition [27].

Based on results, it can be concluded that administration of mangosteen pericarp ethanolic extract (*Garcinia mangostana Linn*) may decrease the expression of VCAM-1, IMT and PVAT thickness in research animals with the high-fat diet group. 400 mg/kg BW of EEMP was proved to exhibit IMT and VCAM-1 expression value, and 800 mg/kg BW of EEMP was able to exhibit the value of PVAT thickness which was equivalent to a normal physiological condition in research animal groups. The results of this study were similar to other studies which proved that the administration of anti-oxidants might reduce oxidative stress, therefore inhibiting atherosclerosis process by inhibiting LDL oxidation and decreasing ROS in endothelial cells [9].

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