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Study of the inhibition effect of ethanolic extract of mangosteen pericarp on atherogenesis in hypercholesterolemic rat

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ABSTRACT

Objective: To investigate the effect of ethanolic extract of mangosteen pericarp (EEMP) through lipid profile, H_2O_2, nuclear factor-kappa B (NF-κB), inducible nitric oxide synthase (iNOS) and endothelial nitric oxide synthase (eNOS) measurement in hypercholesterolemic rat.
Methods: A total of 20 rats were used in true laboratory experiment which were divided into 5 groups (n = 4) using posttest-only design. There were a normal diet group, a hypercholesterol diet (HCD) group, a group that was given HCD with EEMP 200 mg/kg body weight, a group that was given HCD with 400 mg/kg body weight and a group that was given HCD with 800 mg/kg body weight. The lipid profile was measured using Cobas Mira. On the other hand, H_2O_2 was analysed using colorimetric hydrogen peroxide kit. Double staining immunofluorescence was given to observe NF-κB, iNOS and eNOS by using confocal laser scanning microscopy. The result was analyzed quantitatively using Olymphus Fluoview software (version 1.7a).
Results: Lipid profile was significantly worsened in HCD and H_2O_2 level and expressions of NF-κB, iNOS and eNOS were also increased in HCD. EEMP 200 mg/kg body weight generally did not show significant results. However, high density lipoprotein level was affected by EEMP 400 mg/kg body weight, but not for other lipid profiles which reduced H_2O_2 level and NF-κB, iNOS and eNOS expressions significantly. EEMP 800 mg/kg body weight had been shown to be the most effective dose to improve lipid profile, decrease level of H_2O_2 and the expression of NF-κB and iNOS and maintain expression of eNOS.
Conclusions: EEMP is an anti-inflammatory and antioxidant agent to inhibit atherogenesis in hypercholesterolemic rat.

1. Introduction

Atherosclerosis is one of cardiovascular diseases (CVDs) that become the main cause of mortality and morbidity globally. It has been predicted that prevalence of CVDs will increase quickly in developing countries. In 2012, about 17.5 million people died because of CVDs. Its number tends to rise from year to year. Most of CVDs are influenced by human lifestyle[1]. One of risk factors of atherosclerosis that becomes lifestyle in human is hypercholesterolemic food consumption. This will lead to the increase of lipid level in blood (hyperlipidemia), induce the production of free radical, stimulate inflammatory cytokine responses and result in endothelial dysfunction which contributes to atherogenesis mechanism[2].

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Several biomolecules are involved in hypercholesterol diet (HCD) pathway to atherosclerosis. HCD produces high fatty acid which causes high low density lipoprotein (LDL) level and low high density lipoprotein (HDL) level. HCD also increases nicotinamide adenine dinucleotide phosphate that stimulates high production of H_2O_2 in macrophages[3]. Reaction among H_2O_2, myeloperoxidase and Cl^- will produce hypochlorous acid, which also uses the role of nicotinamide adenine dinucleotide phosphate to oxidize LDL[4]. The oxidized LDL (OxLDL) can trigger some redox-sensitive processes that have pro-inflammation and proatherogenic characteristics[4]. OxLDL is one of the important precursors in the nuclear factor-kappa B (NF-κB) activation. Immunochemical staining of the previous study showed that NF-κB moved from cytoplasm to nucleus which was confirmed by NF-κB p50 staining in nucleus contained OxLDL. Active NF-κB becomes the key in the inflammatory response[5].

NF-κB involves in initiation process of inducible nitric oxide synthase (iNOS) gene transcription[6]. High amount of iNOS is found

to be oxidative stress[7]. It produces high amount of nitric oxide (NO) that can react with superoxide to form peroxynitric and has toxicity effect to cells. On the contrary, endothelial nitric oxide synthase (eNOS) is an enzyme that is responsible for producing NO which protects blood vessels and smooths muscle cells from adhesion and platelet aggregation, therefore NO also prevents atherogenesis and fibrosis continuity[8]. The decreased function of endothelial NO produced by eNOS is related to cardiovascular disturbances, including atherosclerosis because of the increased degradation of NO[9].

Ethanolic extract of mangosteen pericarp (EEMP) contains xanthones (α and γ mangosteen)[10]. This substance has a protective effect in cardiovascular system because of its characteristics in several pharmacological actions[10,11]. Xanthones draw a great attention because of its electron donor activity, which plays a significant role in reactive oxygen species (ROS) scavanging[12]. Xanthones also have anti-inflammation properties. α-Mangostin and γ-mangostin can suppress the expression of pro-inflammatory genes, such as tumor necrosis factor (TNF)-α and interleukin-6[13]. Xanthone inhibits LDL to OxLDL[14], reduces NO produced by iNOS[14] and inhibits NF-κB, TNF-α and interleukin-1 activation[14]. Several studies observe the effect of EEMP in some diseases. However, studies that prove the effect of EEMP to lipid profile, H_2O_2, NF-κB, iNOS, and eNOS expressions in the context of atherosclerosis have not been practiced in abundance. Thus, the purpose of this study is to prove whether EEMP can be suggested for atherogenesis inhibition agent through measurement of lipid profile, H_2O_2, NF-κB, iNOS and eNOS in hypercholesterolemic rat.

2. Materials and methods

2.1. Animal group and study design

The animals used in this study were male *Rattus norvegicus* Wistar strain (n = 20) from Pharmacology Laboratory of Faculty of Medicine, Brawijaya University, Malang, Indonesia. Rats were eight weeks old, weighing about 1.5–2 kg. Before treatment, all rats were adapted in laboratorium, divided into 5 groups and treated by following protocols: 1) Normal diet (ND) group was given a ND for three months; 2) HCD group was given an HCD for three months; 3) EEMP 1 was given an HCD for one month, continued by giving EEMP 200 mg/kg body weight for two months; 4) EEMP 2 was given an HCD for one month, continued by giving EEMP 400 mg/kg body weight for two months; and 5) EEMP 3 was given an HCD for one month, continued by giving EEMP 800 mg/kg body weight for two months.

2.2. Preparation of animal feed

The animal diet was taken from Pharmacological Laboratory, Faculty of Medicine, Brawijaya University. ND was composed of wheat flour. However, HCD contained 2 g of yolk, 4 g of sheep oil, 0.4 g of coconut oil, 3.22 g of pig oil, 0.06 g of cholic acid and 30 g of Pars starch.

2.3. Mangosteen pericarp extract process

Extraction process was done in Pharmacological Laboratory, Faculty of Medicine, Brawijaya University. It consisted of three processes including drying, extracting and evaporation. Drying process was done by washing mangosteen pericarp cleanly, then being placed in an oven at 80 °C until drying. Extraction was done by blending pericarp until obtaining as much as 100 g of dry sample. It was soaked with ethanol (70%, 900 mL), shaken for 30 min and incubated one night until sediment was formed. Evaporation was done by taking a top layer containing a mixture of ethanol and active substance placed in an evaporation flask (1 L). EEMP was given to animal models (EEMP 200 mg/kg body weight, EEMP 400 mg/kg body weight and EEMP 800 mg/kg body weight) every day using sonde.

2.4. Blood sample and aorta isolation

After treatment for three months, sacrificing was done. The purpose of this process was to take blood and aorta was measured as samples. Taking rat blood was aimed to measure lipid profile. Aorta of the rat was fixated using PHEMO buffer (0.068 mol/L PIPES, 0.0025 mol/L HEPES, 0.005 mol/L EGTA, 0.003 mol/L $MgCl_2$, 10% DMSO, pH 6.8). After dehydrated, clearing, impragnating, imbeding and mounting were done.

2.5. Lipid profile and H_2O_2 measurement

Lipid profile was measured by Cobas Mira device (GMI Inc., Minnesota, USA).

The measurement of H_2O_2 was done using colorimetric hydrogen peroxide kit (Abcam, Cambridge, MA, USA). Standard solution I was made by dissolving 34 μL hydrogen peroxide. Standard stock with 966 μL diluent was dissolved to produce standard solution I, while standard solution II was made by dissolving 500 μL of standard I solution with 500 μL diluent. The solutions were continuously made until standard solution VI was made. Well 1 was filled with 50 μL diluent as a blanco solution. A volume of 50 μL of standard solution I, II, III and IV were filled in the next wells. About 100 μL color reagent was added to all wells and then pipetted for 1 s. Incubation was done in room temperature for 30 min. Lastly, quantification was done with ELISA reader (Abcam, Cambridge, MA, USA) on 570 nm-length wave.

2.6. NF-κB measurement

The NF-κB activity on the aortic tissue was identified through double staining immunofluorescence using anti-NF-κB antibody (BIOS Inc., Boston, Massachusetts, USA) with fluorescein isothiocyanate (FITC) as a secondary antibody (BIOS Inc., Boston, Massachusetts, USA) and anti-hypoxia inducible factor 1α (HIF-1α) antibody (BIOS Inc., Boston, Massachusetts, USA) with rhodamine as a secondary antibody (BIOS Inc., Boston, Massachusetts, USA). It then was observed with confocal laser scanning microscope (CLSM) (Olympus Corporation, Tokyo, Japan). The double staining results showed NF-κB activities in nucleus. Finally, the collected data were quantitatively analyzed with Olympus FluoView 1.7A software (Olympus corporation, Tokyo, Japan). The measurement of NF-κB was done in the Central Laboratory of Biological Sciences, Brawijaya University.

2.7. iNOS measurement

The observation of iNOS expression was done in the Central Laboratory of Biological Sciences, Brawijaya University. The expression was identified by double staining immunofluorescence using anti-iNOS antibody with α-actin antibody (BIOS Inc.), observed with CLSM and analyzed with Olympus FluoView 1.7 A software.

2.8. eNOS measurement

Similar to the method applied to iNOS, double staining immunofluorescence eNOS was done to identify anti-eNOS antibody using FITC antibody (BIOS Inc.).

2.9. Ethics

The experimental procedure was evaluated and approved by Research Ethics Committee of the Faculty of Medicine, Brawijaya

University, Malang, Indonesia.

2.10. Statistical analysis

The data were reported as mean ± SD to confirm significant effects in all groups. Data was analyzed using ANOVA followed by *post hoc* test (Duncan's test). The statistical analysis was performed using SPSS software, and the probability values of 0.05 or less were considered statistically significant.

3. Results

3.1. Lipid profile measurement

HCD administration generally caused decreasing HDL level and increasing LDL, trigliserida and cholesterol total levels. EEMP 1 showed tendency to improve lipid profile but the value did not reach that in ND. EEMP 2 attained the value similar to that in ND for HDL, trigliserida and cholesterol total although there was not any significant

Table 1
Lipid profile, H_2O_2, NF-κB, iNOS and eNOS measurement.

Parameters	ND	HCD	High fat diet + EEMP			*P* value
			EEMP 1 (200 mg/kg body weight)	EEMP 2 (400 mg/kg body weight)	EEMP 3 (800 mg/kg body weight)	
HDL (mg/dL)	30.00 ± 7.53^{ab}	22.25 ± 6.50^{a}	28.75 ± 7.50^{ab}	37.25 ± 7.14^{bc}	41.50 ± 5.26^{c}	0.010
LDL (mg/dL)	60.25 ± 16.19^{a}	126.25 ± 17.04^{c}	89.50 ± 9.85^{b}	78.75 ± 12.95^{ab}	65.75 ± 8.26^{a}	0.000
TG (mg/dL)	101.75 ± 6.80^{a}	146.25 ± 33.38^{b}	102.50 ± 12.18^{a}	101.75 ± 11.70^{a}	97.50 ± 10.66^{a}	0.007
TC (mg/dL)	107.75 ± 10.53^{a}	181.25 ± 23.73^{b}	113.25 ± 12.55^{a}	104.00 ± 18.31^{a}	102.50 ± 7.19^{a}	0.000
H_2O_2 (ng/mL)	1.07 ± 0.05^{a}	1.36 ± 0.05^{b}	1.30 ± 0.05^{c}	1.03 ± 0.06^{d}	0.06 ± 0.03^{e}	0.000
NF-κb (au)	1 655.33 ± 315.10^{ab}	1 953.21 ± 80.46^{b}	1 902.42 ± 58.81^{b}	1426.21 ± 187.92^{a}	1343.47 ± 260.71^{a}	0.010
iNOS (au)	860.46 ± 24.05^{a}	1 283.84 ± 23.50^{b}	947.11 ± 17.75^{c}	666.94 ± 37.14^{d}	581.56 ± 12.05^{e}	0.000
eNOS (au)	946.21 ± 10.85^{b}	1 117.47 ± 13.74^{c}	1 194.23 ± 22.75^{d}	763.59 ± 15.48^{a}	943.36 ± 29.62^{b}	0.000

Data were expressed as mean ± SD. Based on the Duncan statistical analysis, different letter notations showed significant differences. TG: Triglyceride; TC: Total cholesterol.

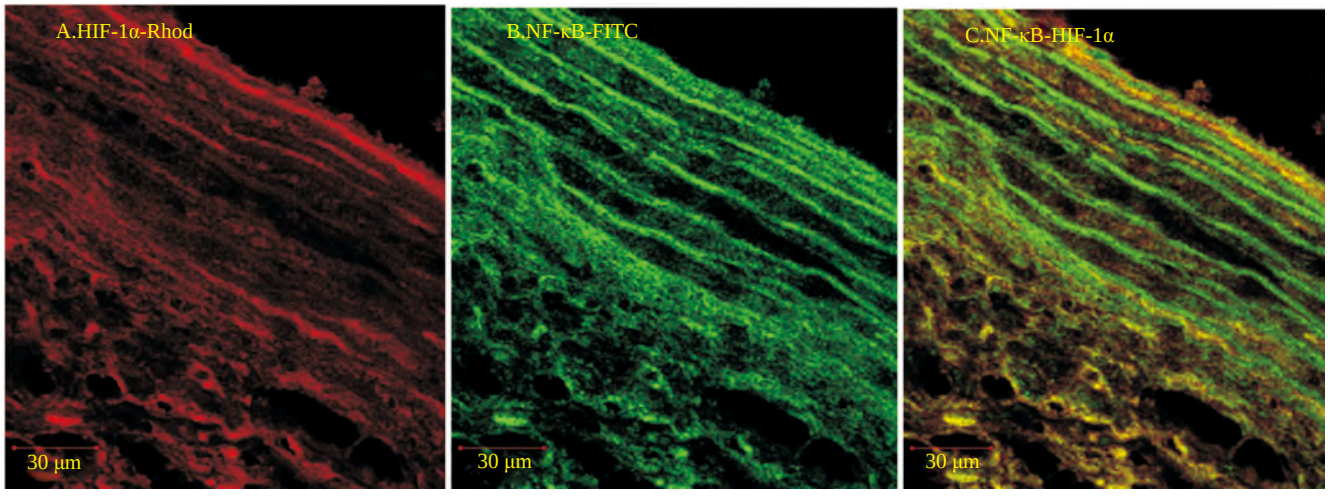

Figure 1. Qualitative observation of NF-κB activation in nucleus by double staining immunofluorescence.
A: HIF-1α with rhodamine; B: NF-κB with FITC; C: Result of merger between two staining immunofluorescence (A and B) showing NF-κB activity in nucleus.

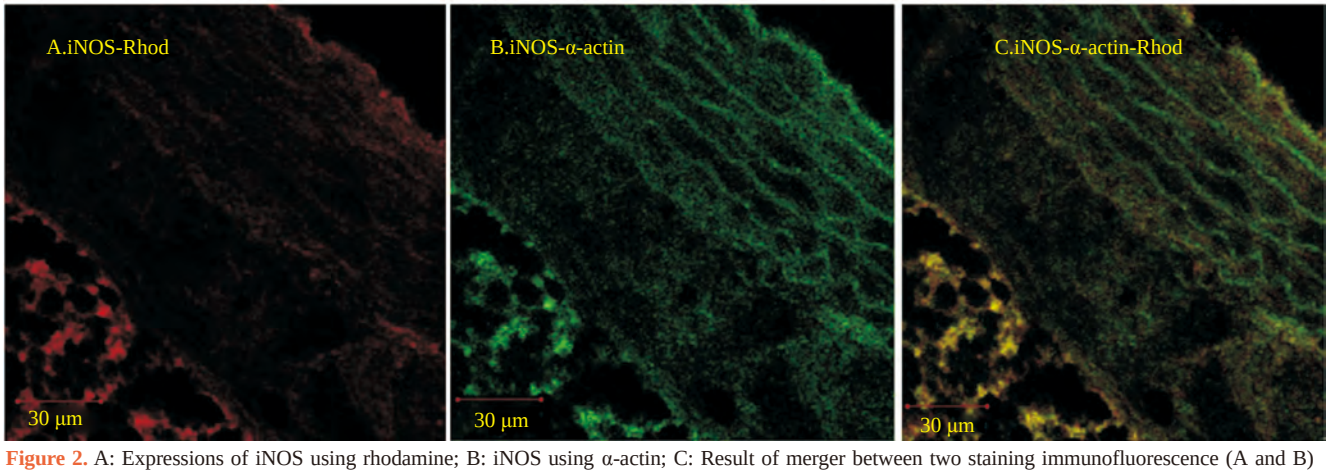

Figure 2. A: Expressions of iNOS using rhodamine; B: iNOS using α-actin; C: Result of merger between two staining immunofluorescence (A and B) showing iNOS activity in smooth muscle cells assessed by immunofluorescence method observed with CLSM.
Original magnifications: ×400.

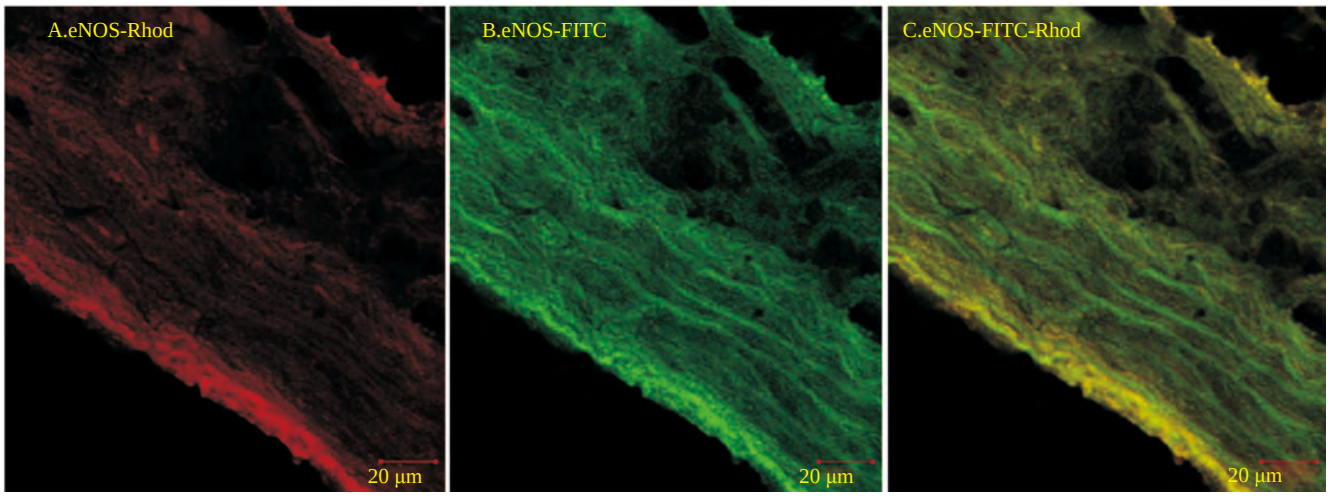

Figure 3. A: Expressions of eNOS using rhodamine; B: eNOS using FITC; C: Result of merger between two staining immunofluorescence (A and B) showing eNOS activity in smooth muscle cells assessed by immunofluorescence method observed with CLSM. Original magnifications: ×400.

difference. EEMP 3 had more improved effects of lipid profile than other doses although a significant difference was present in HDL (Table 1).

3.2. H_2O_2, NF-κB, iNOS and eNOS measurement

According to Table 1 and Olympus FluoView 1.7 A analysis result of Figures 1–3, the highest value of H_2O_2, NF-κB, iNOS, eNOS was found in the HCD group. The EEMP 1 reduced the value of them but increased the eNOS expression, compared to HCD group, but it did not reach ND value. Whereas, the EEMP 2 was seen to reduce the value of them, including eNOS. In this dose, eNOS value was the lowest among the groups ($P < 0.05$). EEMP 3 had the lowest values of all except in eNOS. eNOS expression in this dose was higher than in EEMP 2. It was almost similiar as that in ND ($P > 0.05$).

4. Discussion

Hypercholesterol is related to endothelial dysfunction which involves in atherogenesis. In this condition, lipid profile worsens and oxidative stress dominates, thereby making oxidant and antioxidant imbalanced[15]. Based on the analysis in this study, the worse lipid profile generates ROS production including H_2O_2. The OxLDL binding with LOX-1 as main receptor of LDL, promote endothelial dysfunction by generating excessive ROS production[16]. Besides oxLDL, H_2O_2 was proved as modulator agent for NF-κB production. Moderate dose of H_2O_2 led to increase NF-κB p65 subunit translocation[17]. The result of this study showed that the increase of NF-κB expression was more significantly present in HCD than in ND after the lipid profile measurement. The high level of H_2O_2 was also found, so was NF-κB as an inflammatory cytokine regulator seen in HCD. The NF-κB activation could be observed by using double staining with HIF-1α that showed the location of NF-κB in the nucleus[18]. NO produced by eNOS acted as a vascular protector, while ROS production generated iNOS to produce high amount of radical NO. When eNOS did not produce NO well, its activity was low, leading to atherosclerosis progressivity. Oxidative stress involved in down-regulation of eNOS and up-regulation of iNOS led to endothelial dysfunction[19]. Compensatory mechanism to fulfill NO demand was conducted by increasing eNOS expression as proved by the previous study[20]. The comparison between ND and HCD groups, showed significant differences between each lipid profile level and both enzymes producing NO (iNOS and eNOS) seen in *post hoc* Duncan test.

Antioxidant consumption is needed to reduce oxidative stress, preventing progressivity of disease. EEMP is used as the plant that has several pharmacological functions including antioxidant, which in this study, contributed as a therapeutic agent in atherogenesis. Based on the finding in this study, administrations of EEMP 200 mg/kg body wight, 400 mg/kg body weight and 800 mg/kg body weight were seen to improve lipid profile by increasing the level of HDL and decreasing others, compared to HCD. On the other hand, the analysis of comparison between EEMP groups and ND group had to be done to obtain the effective dose. The analysis result showed that dose of EEMP 200 mg/kg body weight was not effective to improve lipid profile compared to the sample of physiologic condition (ND) because the lipid consumption effect was more dominating than the antioxidant effect. Moreover, dose of 400 mg/kg body weight had therapeutic effect in lipid profile by lowering cholesterol total and raising HDL. The most effective dose however, was present in 800 mg/kg body weight. It is supported by the previous study showing that cholesterol formation process was inhibited by antioxidant of EEMP[21]. This shows that EEMP has a benefit effect in lipid profile improvement.

The impact of EEMP administration was also found in the H_2O_2 level. EEMP decreased H_2O_2 as it did to LDL. Therapeutic dose started at 400 mg/kg body weight. In line with that, Adiputro *et al*. proved that EEMP inhibited production of oxLDL by binding the scavenger A receptor in macrophages. The scavenging effect in EEMP has been known to reduce the level of H_2O_2 from the EEMP modulation effect on catalase activity[22]. Based on the result, giving EEMP influenced the NF-κB activation, oxLDL formation and H_2O_2 production. It was also found that the NF-κB lowering effect was line with H_2O_2 and LDL lowering effects. The previous study proved that α- and γ-mangostin significantly reduced the NF-κB activation during adipogenesis[23]. Similar to that, this study also found that EEMP in 400 mg/kg body weight and 800 mg/kg body weight were seen as effective doses to reduce the NF-κB expression.

Xanthone from EEMP affected the alteration of both iNOS and eNOS expression. Pretreatment with α-mangostin decreased eNOS expression and NO level in isoproterenol-induced to myocardial necrosis rat[24] while α- and γ-mangostin treatment showed the decrease of iNOS and COX-2 mRNA expressions[25]. From this study result, iNOS expression was line with NF-κB expression. It was known that there is involvement of NF-κB in iNOS transcription[6]. EEMP affected the suppresion of iNOS as well as NF-κB. Therapeutic effect was found in 400 mg/kg body weight and 800 mg/kg body weight. Study also proved that EEMP (200 mg/kg body weight, 400 mg/kg body

weight and 800 mg/kg body weight) significantly decreased NO level produced by iNOS, with the best dose approaching normal level found in 800 mg/kg body weight[14]. Buelna-Chonta *et al.* in their study proved that EEMP reduced asymmetric dimethylarginine concentration via increasing dimethylarginine dimethylaminohydrolase[11]. It indicated that EEMP maintained eNOS production in normal condition. However, highest eNOS expression in EEMP 200 mg/kg body weight was found which might be a result from the compensatory mechanism to supply NO in tissue. Nevertheless, this study still found domination of oxidative stress in this group. Previous study stated that high H_2O_2 induced eNOS phosphorilation in Ser-1177 and activated Akt to maintain NO bioactivity in oxidative stress[26]. Lowest eNOS value was found in 400 mg/kg body weight indicating that NO supply was sufficient to normal condition. The sufficient eNOS may affect the hypocholesterolemic effect of this dose as the consequence of the antiatherogenic NO to decrease LDL, H_2O_2, NF-κB and iNOS. EEMP 800 mg/kg body weight influenced the production of eNOS. The value was almost similar to that of ND group which produced eNOS. The value of eNOS was found higher in this dose than in 400 mg/kg body weight. It may be an ideal dose to maintain NO as in physiologic condition. It confirmed the result of eNOS comparing to other parameters such us lipid profile, H_2O_2, NF-κB and iNOS after this dose was applied.

Administration of EEMP (*Garcinia mangostana* Linn) in hypercholesterolemic rat was shown to improve the lipid profile, decrease H_2O_2 level, reduce NF-κB and iNOS expressions and maintan eNOS at 800 mg/kg body weight as the effective dose. Active compound in EEMP has a role as antiatherogenesis that can be measured from those parameters. To sum up, 800 mg/kg body weight dose of EEMP is the most effective antiatherogenesis agent.

Conflict of interest statement

We declare that we have no conflict of interest.

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