



# Atherosclerotic Biomarkers (Interleukin-6 and CD40) and Tunica Intima Thickness in Obese Rats after the Administration of *Plectranthus amboinicus* (Lour.) Spreng Ethanol Extract

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## Abstract

**BACKGROUND:** Obesity can increase oxidative stress, thereby increasing the inflammatory markers interleukin (IL)-6. These markers are characterized by various biomarkers, such as cluster of differentiation 40 (CD40) and IL-6, and thickness of the tunica intima.

**AIM:** This study aims to analyze the effect of the *Plectranthus amboinicus* (Lour.) Spreng ethanol extract on IL-6, CD40, and thickening of the tunica intima of obese rats.

**METHODS:** The pure experimental method with complete random design consisted of several research groups: C- (negative control), C+ (positive control/cholesterol feed), positive control (cholesterol feed + CMC), T1\_Chol\_300 (cholesterol feed and administration of 300 mg/kg body weight [BW] *P. amboinicus* for 45 days), T2\_Chol\_600 (cholesterol feed and administration of 600 mg/kg BW *P. amboinicus* for 45 days), and T3\_chol\_900 (cholesterol feed and administration of 900 mg/kg BW *P. amboinicus* for 45 days).

**RESULTS:** Results showed increased CD40 levels in the blood plasma of obese rats and decreased CD40 levels after the administration of the ethanol extract of *P. amboinicus* leaves. A significantly increased ( $p < 0.05$ ) level of IL-6 in the obese mice group and a significant decrease ( $p < 0.05$ ) in IL-6 after the administration of the ethanol extract of *P. amboinicus* leaves were observed. Tunica intima thickness significantly occurred ( $p < 0.05$ ) in the group of obese rats, and normal tunica intima thickness occurred in the group administered with the ethanol extract of *P. amboinicus* leaves.

**CONCLUSION:** In conclusion, the ethanol extract of *P. amboinicus* leaves (900 mg/kg BW) had the potential to normalize obese rats.

**Edited by:** Sinisa Stojanoski  
**Citation:** Harefa K, Sulastri D, Nasrul E, Ilyas S. Atherosclerotic Biomarkers (Interleukin-6 and CD40) and Tunica Intima Thickness in Obese Rats after the Administration of *Plectranthus amboinicus* (Lour.) Spreng Ethanol Extract. Open Access Maced J Med Sci. 2020 Oct 26; 8(A):852-857. https://doi.org/10.3889/oamjms.2020.4349  
**Keywords:** *Plectranthus amboinicus*; Interleukin-6; Cluster of differentiation 40; Tunica intima  
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E-mail: syafruddin6@usu.ac.id  
**Received:** 26-Jan-2020  
**Revised:** 12-Oct-2020  
**Accepted:** 16-Oct-2020  
**Copyright:** © 2020 Karnirius Harefa, Delmi Sulastri, Ellyza Nasrul, Syafruddin Ilyas  
**Funding:** This research did not receive any financial support  
**Competing Interests:** The authors have declared that no competing interests exist  
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## Introduction

The changes in the mode of transportation, increased urbanization, and mechanization that occurs in most countries are associated with obesity. An increasing number of people who are less active and change in the diet, particularly in eating high-fat foods, are observed [1]. Asians have a high incidence of obesity due to their less physical activity compared with Europeans. Asians tend to have central obesity and a higher fat percentage than body mass index (BMI) measurements proportional to the increase in cardiovascular disease based on the declaration of agreement by the pacific regional office of WHO with overweight (BMI: 23.0–24.9) and obese (BMI > 25.0) definitions for the Asian population [2].

Recently, many studies have explained that obesity has a special relationship with the occurrence of endothelial dysfunction. In a cross-sectional study of 194 patients with overweight and obesity, 50% of the population has experienced more than average thickening of the intimal carotid arteries and more

stiffness in the obese artery wall [3]. Arterial damage in patients with obesity and atherosclerosis is more clearly seen in the coronary arteries than in other arteries [4].

Certain types of plants contain high antioxidative and anti-inflammatory compounds. The bangun-bangun (*Plectranthus amboinicus* [Lour.] Spreng.) plant is widely spread in Indonesia and believed to be a nutritious plant. This plant has a significant association with anti-inflammatory [5], [6], [7], [8], [9], [10], [11] and antioxidative [12], [13], [14] effects.

The primary contents of *P. amboinicus* (Lour.) Spreng that acts against inflammation are fitol, caryophyllene oxide [14], and thymoquinone. These active compounds suppress the expression of pro-inflammatory cytokines for tumor necrosis factor-alpha (TNF- $\alpha$ ), interleukin (IL)-6, and IL-1 $\beta$  [6]. In mice, the water extract from the leaves of *P. amboinicus* (Lour.) Spreng functions as an anti-inflammatory agent by inhibiting the pro-inflammatory mediators and blocking the activity of NF-kB [8].

The primary antioxidants [15], [16], [17], [18] present in *P. amboinicus* (Lour.) Spreng are squalene,

durohydroquinone, timol [19], and carvacrol [14]. The quality and quantity of these compounds vary depending on the region of growth [20], [21], [22], [23]. *P. amboinicus* (Lour.) Spreng, as an antioxidant, increases the activities of the antioxidant enzyme catalase, superoxide dismutase, glutathione peroxidase, lipid peroxidation, and nitric oxide and decreases the activity of glutathione [12].

Diterpenoids, phytol, eugenol, linalool, caryophyllene oxide, and caryophyllene also function against inflammation. Moreover, eugenol, hexadecanoic acid, beta-tocopherol, tetradecanoic acid, and perillidic acid have antioxidant activities [14].

Based on the aforementioned description, the researchers aim to analyze the effect of the administration of *P. amboinicus* (Lour.) Spreng ethanol extract on cluster of differentiation (CD40, IL-6, and thickening of the aortic tunica intima in obese mice.

## Materials and Methods

### **Preparation of *P. amboinicus* (Lour.) Spreng ethanol extract**

The ethanol extract of *P. amboinicus* (Lour.) leaves was cleaned and dried using an oven maintained at 40°C. The dried simplicia was mashed using a blender until smooth. The dried simplicia powder (1000 g) was placed into the macerator and added with 10,000 mL of 70% ethanol. The marinade was left for 6 h, stirred occasionally, and left for the next 18 h. The leaf immersion macerate was separated using a Whatman filter paper. The search for leaves was repeated by replacing the new 70% ethanol, but the amount of solvent was half the previous volume of 5000 mL. The process was continued until the macerate was separated. The macerate was collected, concentrated using a rotary evaporator at 30°C–50°C, and dried using a water bath to obtain the thick ethanol extract of *P. amboinicus* (Lour.) Spreng. The thick extract was suspended with 1% Na–CMC solution [24], [25], [26], [27], [28], [29].

### **Preparation of animal experiments**

Two-month-old Wistar rats (*Rattus norvegicus*) were kept in a cage with a daily dark-light cycle for a week for acclimatization. The temperature and humidity of the room were left in the natural range. The twenty-four rats (four rats each group) were fattened using high-fat foods for 13 weeks until they were declared obese. The obese criteria in Wistar rat were Lee index >300 (cube root of body weight [BW] (g)/nose-to-anus length [cm]) [30]. There are six groups and then food and tap water were given *ad libitum*. Furthermore, the Wistar rats

were given the same food for 45 days [6]. *P. amboinicus* (Lour.) Spreng extract (300, 600, and 900 mg/kg BW) was given to the experimental group each day [9]. The extract was given daily to rats using an oral sonde with the predetermined dose. The treatment groups were C– (negative control), C+ (positive control/cholesterol feed), positive control (cholesterol feed + CMC), T1\_Chol\_300 (cholesterol feed and administration of 300 mg/kg BW *P. amboinicus* for 45 days), T2\_Chol\_600 (cholesterol feed and administration of 600 mg/kg BW *P. amboinicus* for 45 days), and T3\_chol\_900 (cholesterol feed and administration of 900 mg/kg BW *P. amboinicus* for 45 days). This study was approved by the health research ethics committee (No. 0492/KEPH-FMIPA/2018).

### **Atherosclerosis biomarkers**

IL-6. Definition: IL-6 is a cytokine secreted in the blood plasma, particularly the acute or chronic phase, and induces an inflammatory response and we measured it with enzyme-linked immunosorbent assay (ELISA). Measuring Instrument: ELISA Reader. Measuring scale: Ratio. Measuring results: ng/mL [31].

CD40. Definition: CD40 is a costimulant protein in antigen-presenting cells in the aortic membrane of mice and we measured it with ELISA. Measuring instrument: ELISA Reader. Measuring scale: Ratio. Measuring results: ng/mL [32].

Tunica intima thickness. Definition: Tunica intima thickness is the cross-sectional diameter of the aortic blood vessels of obese female mice and we measured it with histopathology. Measurement: A microscope equipped with an ocular micrometer. Measuring scale: Ratio. Measuring results: micron [33], [34].

### **Data analysis**

The data obtained from the measurement results are expressed in mean  $\pm$  standard deviation using Microsoft Excel and analyzed using SPSS. The data did not meet normal data distribution. Therefore, the data were analyzed using the Kruskal–Wallis ANOVA nonparametric test at a confidence level of 95%. Bootstrapping and Duncan tests were also performed. A significant difference was indicated by a significance value of  $p < 0.05$ .

## Results and Discussion

On the basis of the current research, the following points were obtained.

Figure 1 shows that the highest IL-6 level was observed in the C+ group ( $86.63 \pm 67.44$  ng/mL),

which was significantly different from the C- ( $22.78 \pm 4.29$  ng/mL) and T3\_Chol\_900 ( $14.79 \pm 11.24$ ) groups but not evidently different from the positive control ( $86.73 \pm 50.64$  ng/mL), T1\_Chol\_300 and T2\_Chol\_600 groups. These results indicated that the administration of high-fat diets can cause inflammation in mice. The excess fat in mice can cause the activation of monocytes into macrophages and eventually leads to inflammation, which causes increased IL-6, inhibits the activity of lipoprotein lipase (LPL), and reduces the differentiation of preadipocytes in humans.

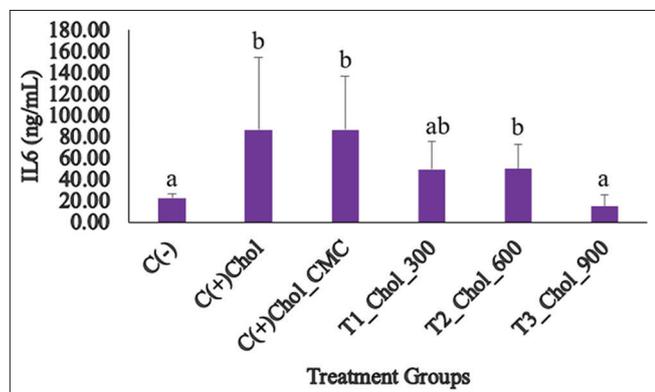


Figure 1: Average levels of interleukin-6 after the administration of the *Plectranthus amboinicus* (Lour.) Spreng ethanol extract in obese rats

Wang and Nakayama [35], Wang and Butany [36], and Manna and Jain [37] reported that two pathways, namely, stimulating pro-inflammatory activity and increasing systemic oxidative stress, can cause tunica intima dysfunction in obesity. Excess lipids, fatty acids, and cytokines in adipose tissue activate monocytes, which later turn into macrophages to produce various cytokines, such as inflammatory-related adipokines (IL-1, IL-6, and TNF- $\alpha$ ), leptin adiponectin, procoagulant (PAI-1), vasoactive substances (leptin, angiotensinogen, and endothelin), and substances that contribute to insulin resistance (FFA, TNF- $\alpha$ , and resistin). IL-1 $\beta$  stimulates NF- $\kappa$ B in the nucleus and induces the transcription of IL-6, COX-2, IL-1Ra, and iNOS.

The addition of extracts in the T3\_Chol\_900 group significantly reduced the IL-6 level ( $14.79 \pm 11.24$  ng/mL), which was significantly different from those of C+, positive control, and T1\_Chol\_300 groups. This result was caused by the content of flavonoids, fitol, caryophyllene oxide, and thymoquinone in the ethanol extract of *P. amboinicus* (Lour.) Spreng, which functioned as an anti-inflammatory agent. In this case, IL-6 prevented tunica intima dysfunction.

The primary ingredients contained in *P. amboinicus* (Lour.) Spreng as an anti-inflammatory agent is fitol, caryophyllene oxide [14], and thymoquinone. These active compounds suppress the expression of pro-inflammatory cytokines in TNF- $\alpha$ , IL-6, and IL-1 $\beta$  [6], [10]. Paniagua [38] stated that IL-6 transcription affects the regulation of C-reactive protein (CRP) and increases fibrinogen and the occurrence

of tunica intima dysfunction. Increased TNF-resi expression inhibits the differentiation of adipocyte cell maturity, whereas IL-6 inhibits the activity of LPL and reduces the differentiation of preadipocytes in humans.

As shown in Figure 2, the administration of cholesterol diets to the mice in the positive control and C+ groups for 45 days resulted in the highest CD40 antibody levels of  $63.61 \pm 89.32$  and  $44.10 \pm 20.89$  ng/mL, respectively. These results indicated that the administration of high-fat diets can increase oxidative stress, causing inflammation or rat aortic tunica intima lesions. Szmitko *et al.* [33] stated that tunica intima lesions due to inflammation and increased oxidative stress also express CD40L from the activation of T lymphocytes, platelets, monocytes, and macrophages. In addition, tunica intima lesions are expressed in the form of a solution that is an sCD40L, whose primary source is platelets. By contrast, CD40 is expressed in macrophages, tunica intima cells, vascular smooth muscle cells (VSMCs), and a little in B cells [33].

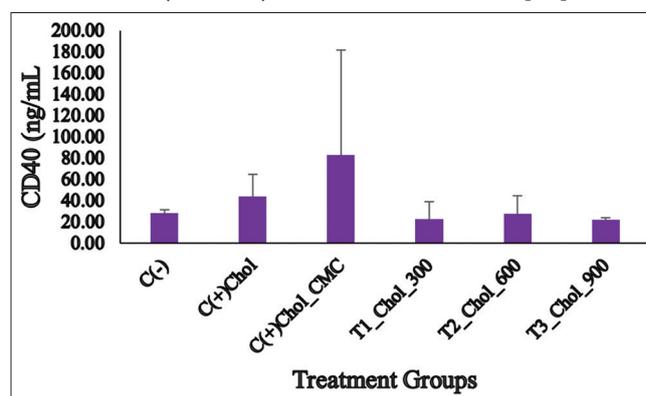


Figure 2: Average plasma CD40 levels after the administration of *Plectranthus amboinicus* (Lour.) Spreng ethanol extract in obese rats

The administration of *P. amboinicus* ethanol extract at increasing doses (300, 600, and 900 mg/kg BW) reduced the CD40 levels in blood plasma from  $22.30 \pm 16.73$  ng/mL to  $22.25 \pm 1.84$  ng/mL. This result was due to the presence of antioxidative and anti-inflammatory flavonoids and terpenoids in the extract of *P. amboinicus*. Consequently, the CD40 activity decreased and caused reduced lesions or damage to the aortic tunica intima cells. Szmitko *et al.* [33] stated that the CD40 expression stimulates inflammation. The expressions of CD40L and CD40 can occur at early and advanced atherosclerosis. Lutgens *et al.* [32] found that the inhibition of CD40L can stabilize plaque atherosclerosis by increasing the amount of collagen and VSMC and decreases the number of macrophages and T lymphocytes. CD40L can work when bound by CD40 as a receptor, resulting in marking activity on the process of atherosclerosis in the aortic tunica intima.

The highest aortic tunica intima thickness of mice (Figure 3) was observed in the C+ group ( $7.92 \pm 0.66$   $\mu$ m), which was significantly different from the C- group (without high-fat diet) ( $4.76 \pm 1.98$   $\mu$ m) and not significantly different from the positive control ( $6.44$

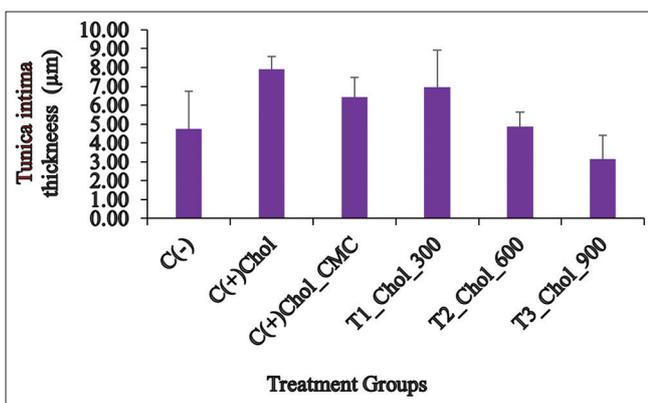


Figure 3: Average tunica intima thickness after the administration of *Plectranthus amboinicus* (Lour.) ethanol extract in obese rats

± 1.04 µm) and the T1\_Chol\_300 (6.94 ± 1.99 µm) groups. These results showed that a high-fat diet (hypercholesterolemia) can increase the thickness of the rat aortic tunica intima. Increased fat, particularly low-density lipoprotein, can cause changes in the adhesion power of leukocyte cells to aortic tunica intima cells. Oxidative stress (reactive oxygen species [ROS]) was increased through increased angiotensin II and can eventually lead to atherosclerotic lesions.

Two pathways, namely, stimulating pro-inflammatory activity and increasing systemic oxidative stress, can cause endothelial dysfunction in obesity. Excess lipids, fatty acids, and cytokines in the adipose tissue activate monocytes, which later turn into macrophages to produce various cytokines, such as inflammatory-related adipokines (interleukin-1 [IL-1], IL-6, and TNF-α), leptin adiponectin, PAI-1, vasoactive substances (leptin, angiotensinogen, endothelin), and substances that contribute to insulin resistance (FFA, TNF-α, and resistin). IL-1β stimulates NF-κB in the nucleus and induces the transcription of IL-6, COX-2, IL-1Ra, and iNOS. IL-6 transcription affects the regulation of CRP and increases fibrinogen and the occurrence of endothelial dysfunction [35], [36], [37]. Increased TNF-α expression inhibits the differentiation of adipocyte cell maturity, whereas IL-6 inhibits the activity of LPL and reduces the differentiation of preadipocytes in humans [38].

Endothelial lesions due to inflammation and increased oxidative stress express CD40 ligands (CD40L) from the activation of T lymphocytes, platelets, monocytes, and macrophages. In addition, endothelial lesions are expressed in the form of a soluble CD40L (sCD40L), whose primary source is platelets. By contrast, a CD40 is expressed in macrophages, endothelial cells, VSMCs, and a little in B cells. CD40 expression stimulates inflammation. The expression of CD40L and CD40 can occur in early and advanced atherosclerosis [33]. The inhibition of CD40L can stabilize plaque atherosclerosis by increasing the amount of collagen and VSMC and decreasing the number of macrophages and T lymphocytes [32].

Szmitko et al. [33] found that hypercholesterolemia increases the attachment of blood leukocytes to the tunica intima, the cell layer that is normally resistant to strong leukocyte adhesion. Oxidized low-density lipoprotein causes tunica intima activation and alters its biological characteristics by reducing intracellular NO concentration. Angiotensin II, a vasoconstrictor associated with hypertension, has an opposite action with NO. Angiotensin II can cause the production of ROS, increase the expression of pro-inflammatory cytokines (IL6) and monocyte chemoattractant protein-1, and increase the regulation of vascular cell adhesion molecules in ECs. Newer risk factors, such as increased levels of CRP, can also increase tunica intima dysfunction by quelling NO production and reducing its bioactivity. This tunica intima modification stimulates inflammation in the walls of blood vessels, thereby regulating the rate of initiation and development of atherosclerotic lesions.

The administration of *P. amboinicus* ethanol extract at doses of 600 and 900 mg/kg BW causes a significant reduction in the rat aortic tunica intima thickness (4.86 ± 0.77 µm; 3.14 ± 1.25 µm) compared with C- (4.76 ± 1.98 µm), C+ (7.92 ± 0.66 µm), and C+ (positive control/cholesterol feed) (6.44 ± 1.04 µm) (Figure 4).

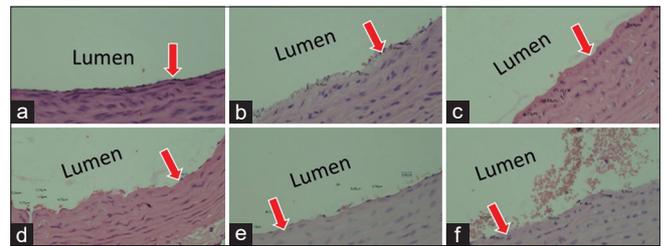


Figure 4: Histological image of the tunica intima (red arrow) of obese rats after the administration of *Plectranthus amboinicus* (Lour.) Spreng ethanol extract. (a) C-, (b) C+, (c) positive control, (d) T1\_Chol\_300, (e) T2\_Chol\_600, and (f) T3\_Chol\_900. Magnification ×400, HE

## Conclusion

The ethanol extract of *P. amboinicus* leaves can significantly decrease the level of CD40, IL-6, and the thickness of the tunica intima blood vessels that result in the group of obese rats and became normal.

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