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Short Communication

Immunoexpression of aortic endothelial P-selectin and serum apolipoprotein A-1 levels after administration of arabica (*Coffea arabica*) and robusta (*Coffea canephora*) coffee bean extracts: In vivo study in atherosclerosis rat model

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Abstract

Atherosclerosis is a leading cause of cardiovascular disease-related death worldwide. Some studies suggested that the natural ingredients in coffee may negatively affect cardiovascular diseases, while other studies indicated that coffee contains anti-inflammatory compounds that are beneficial for cardiovascular diseases. The aim of this study was to measure the expression of P-selectin in aortic endothelial cells and the level of serum apolipoprotein A-1 (ApoA-1) in an atherosclerosis rat model after the administration of arabica and robusta coffee bean extracts at mild-moderate and high doses. An experimental study was conducted with a complete randomized design using 36 adult male white rats (*Rattus norvegicus*) divided into six groups: negative control (NC), positive control (PC), arabica mild-moderate dose (A1), arabica high dose (A2), robusta mild-moderate dose (R1), and robusta high dose (R2). Animals were induced atherosclerosis with atherogenic feed and then were treated with arabica and robusta coffee bean extracts at two different doses for four weeks. The results showed that the expression of P-selectin in the group of rats treated with robusta coffee bean extract was lower than arabica coffee bean extract group. Rats with robusta coffee bean extract mild-moderate dose had the highest ApoA-1 levels compared to other groups significantly ($p<0.05$). The level of ApoA-1 was higher in both mild-moderate and high dose of robusta coffee groups compared to the negative control group (both with $p<0.001$). In conclusion, mild-moderate intake of robusta coffee bean extract could reduce aortic P-selectin immunoexpression and increase serum ApoA-1 levels in an atherosclerosis rat model.

Keywords: ApoA-1, atherosclerotic biomarker, P-selectin expression, cell adhesion molecules, immunohistochemistry

Introduction

Atherosclerosis is a leading cause of cardiovascular disease-related death worldwide. Its pathogenesis involves several important inflammatory components [1-3], including various interrelated cell types such as macrophages, endothelial cells, vascular smooth muscle cells, and

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immune cells [4]. Several anti-inflammatory markers are often measured to elucidate the pathogenesis of atherosclerosis, including P-selectin and apolipoprotein A-1 (ApoA-1) [5,6]. P-selectin is a cell adhesion molecule expressed by activated platelets and endothelial cells. It plays an important role in mediating leukocyte-endothelial interactions and platelet activation, thus contributing to hemostasis and inflammation [7]. Platelet activation and leukocyte adhesion are two important processes in the pathogenesis of atherosclerosis [8]. The development of early lesions of atherosclerosis involves the process of leukocyte and monocyte adhesion to the vascular endothelium at the site of injury [9,10]. Previous studies have shown that soluble P-selectin in plasma has a positive correlation with the progressivity of atherosclerotic plaque formation, and therefore, P-selectin can be used as a biomarker to assess the incidence of atherosclerosis [5,6]. In contrast, ApoA-1 is an antiatherogenic and antioxidant protein that could inhibit atherosclerotic plaque formation [8,11].

Despite many modern treatments and interventions to reduce the incidence of atherosclerosis, the prevalence of cardiovascular disease has continued to increase in recent decades [12,13]. Therefore, nowadays, various natural products have attracted worldwide attention to be developed for the prevention and therapeutics of cardiovascular diseases through the bioactive compounds contained in natural products [4]. Natural products are thought to prevent atherosclerosis through the mechanisms of regulating lipid metabolism, improving inflammation, stabilizing plaque, and remodeling gut microbiota, making some natural products used in clinical therapy [14]. One such natural product is coffee. Bioactive compounds found in coffee are still debated regarding their ability to protect the cardiovascular system [2,15]. Some studies found that the active ingredients of coffee beans have a negative impact on the cardiovascular system [2,16]. Meanwhile, other studies provide different results reporting that coffee beans contain anti-inflammatory compounds that play a role in the pathogenesis of cardiovascular diseases [16-19].

The tradition of drinking coffee has now become a culture of global society that cannot be abandoned [20,21]. The most commonly consumed types of coffee beans are arabica and robusta [22]. Previous studies found that both arabica and robusta coffee beans have a high antioxidant content that may indicate an anti-inflammatory process and other potential health benefits [15,16]. Cohort studies have shown that some types of coffee have the ability to inhibit the incidence of cardiovascular diseases and reduce mortality [23,24]. Bioactive compounds contained in coffee beans are hypothesized to act as inhibitors of P-selectin expression. Therefore, platelet and endothelial cell activation does not occur, making coffee an effective agent in reducing the incidence of atherosclerosis. However, studies assessing the direct effect of coffee on atherosclerosis are unavailable. The aim of this study was to determine the effect of the administration of arabica and robusta coffee bean extracts with various concentrations on the early process of atherosclerosis by assessing the expression of P-selectin in aortic tissues and the level of plasma ApoA-1.

Methods

Study design and setting

An experimental study with a completely randomized design was conducted using 36 male white rats (*Rattus norvegicus*) aged four weeks with a body weight of 200 grams. The rats were divided into six treatment groups and each group consisted of six rats. The treatment groups were negative control (NC), positive control (PC), arabica mild-moderate dose (A1), arabica high dose (A2), robusta mild-moderate dose (R1), and robusta high dose (R2). To induce atherosclerosis, the atherogenic diet was fed for eight weeks, after which two doses (mild-moderate or high dose) of arabica or robusta coffee bean extracts were administered for four weeks. The outcomes (aortic endothelial P-selection expression and serum ApoA-1 level) were then assessed.

Extraction and preparation of arabica and robusta coffee bean extracts

Arabica and robusta coffee beans were collected from Bener Meriah Regency, Aceh Province, Indonesia. The coffee beans were cleaned, washed thoroughly, drained, and dried. The dried coffee beans were pulverized using a blender and then sieved to obtain a fine and uniform powder.

The extraction of arabica and robusta coffee beans was carried out using the maceration method. A total of 120 grams of coffee bean simplisia powder was soaked with 225 mL of 96% ethanol for five days with occasional stirring. Then, it was filtered using filter paper to produce filtrate and pulp. They were then macerated with a 96% ethanol solution of 75 mL, covered with aluminum foil, and left for two days while occasionally stirring. Re-maceration was done twice. Furthermore, the macerate was made into a thick extract with a rotary evaporator with the appropriate pressure and temperature. The resulting thick extract was left at room temperature until the entire ethanol solvent evaporated. The extract was weighed and stored in a closed glass container and phytochemical tests were carried out before being used for testing [17,25].

Induction of rat atherosclerosis model and administration of coffee bean extract

The rats were acclimatized for seven days with a standard diet and *ad libitum* drinking. On day 8, rats in the negative control group were provided with a standard diet, while rats of the other groups were provided with an atherogenic diet consisting of 0.2% cholic acid, 2% egg yolk, 5% goat fat, and 92.8% corn rice to induce atherosclerosis [21]. The atherogenic diet was fed for eight weeks (56 days) *ad libitum*. On week 9, the treatment was carried out by administering arabica and robusta coffee bean extracts for four weeks (28 days) to each group. Arabica and robusta coffee bean extracts were provided in two doses: mild-moderate (0.18 mg per day) and high doses (4.5 mg per day) given orally once daily.

Collection of blood and aortic tissue samples

After the treatment for four weeks, all rats were euthanized using 50 mg/kg ketamine and 5 mg/kg xylazine intraperitoneally. A total of 3 mL of blood sample was collected from the heart and centrifuged using microcentrifugation for 10 mins at 3,000 rpm to obtain plasma. The plasma was then immediately stored at -80°C for examination of ApoA-1 levels. Aortic tissue samples were collected and stored in 10% neutral buffered formalin (NBF) fixation solution for three days, processed into histological preparations and stained with an immunohistochemical method to determine the expression of P-selectin.

Immunohistochemical staining of P-selectin expression

Expression of P-selectin in aortic tissue was identified through immunohistochemistry (IHC) staining. The IHC staining process employed the avidin-biotin complex method using a Mouse and Rabbit Specific HRP/DAB Detection IHC kit following the manufacturer's protocol (Cat. No. ab64264, Abcam, Cambridge, UK) with slight modifications [22]. Briefly, after deparaffinization and rehydration, the tissues were rinsed. The process of blocking nonspecific proteins was done using a protein block solution for 10 min at room temperature and the tissues were incubated with primary antibody P-selectin (Cat No. sc-8419, Santa Cruz Biotechnology, Santa Cruz, USA) for one night at 4°C and 0.1% Triton-X followed with biotinylated goat antipolyvalent (secondary antibody). Streptavidin peroxidase was added and incubated for 10 min before 3,3'-diaminobenzidine (DAB) was applied for 1–10 minutes while controlled under a microscope. Then counterstained with hematoxylin, dehydrated, and mounted. Assessment of P-selectin expression based on the intensity of the brown color of the IHC results was carried out using a four-point scale based on the IHC intensity of the cells: negative (0), mild positivity (1+), moderate positivity (2+), and strong positivity (3+) [26].

Enzyme-linked immunosorbent assay of ApoA-1 level

ApoA-1 levels were measured using the enzyme-linked immunosorbent assay (ELISA) method. The procedure for measuring ApoA-1 levels was conducted based on the manufacturer's instruction (Cat. No. BZ-22185651-EB, Bioenzy, Jakarta, Indonesia). Briefly, 100 µL of plasma was tested for each sample, of which 100 µL of anti-ApoA-1 antibody biotin and 100 µL of streptavidin-HRP were used with 90 µL of tetramethylbenzidine (TMB) substrate and 50 µL of stop solution. The absorbance was measured in a spectrophotometer with a light wavelength of 450 nm.

Statistical analysis

P-selectin expression data were analyzed descriptively by comparing between study groups. The comparison of ApoA-1 level data between study groups was analyzed using a one-way analysis of variance (ANOVA) test followed by Duncan's further test. Statistical analysis was performed using SPSS 25 (IBM, New York, USA).

Results

Effect of arabica and robusta coffee on P-selectin immunoexpression in aortic endothelium of atherosclerosis model rats

Our data suggested that the administration of coffee bean extract affected the expression of P-selectin in the aortic endothelium (**Figure 1**). The expression of P-selectin in the untreated atherogenic group (PC) and the group given a high dose of arabica coffee bean extract (A2) had a higher P-selectin expression compared to the other groups (NC, A1, R1, and R2).

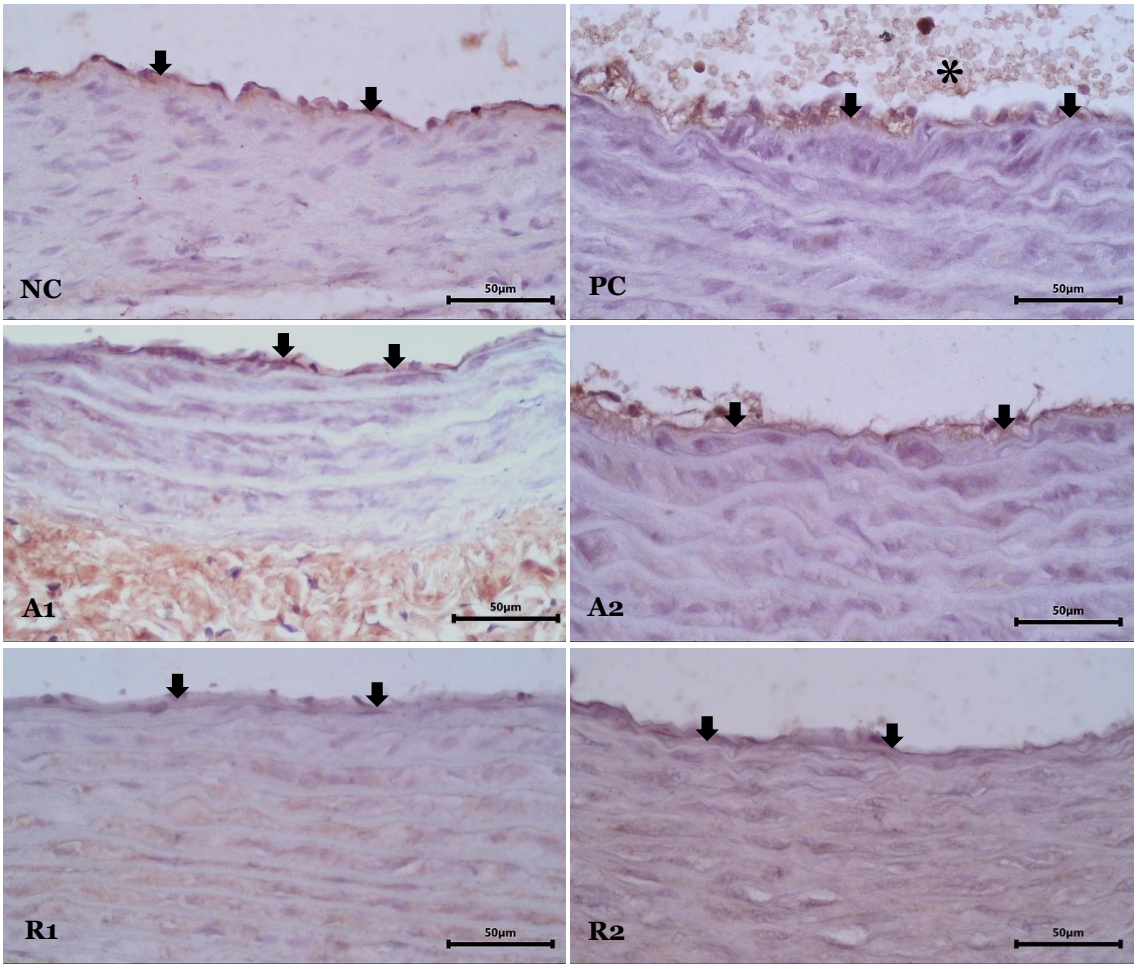

Figure 1. Immunohistochemical staining indicating the expression of P-selectin on the surface of aortic endothelial cells (arrows) of rats after administration of arabica and robusta coffee bean extracts. The expression of P-selectin is indicated by the brown color of the endothelium. Coffee bean extract was able to reduce P-selectin expression in aortic endothelial cells (A1-R2) and leukocyte accumulation (*). However, robusta coffee bean extract has better activity in reducing P-selectin expression in white rat aortic endothelium. NC: healthy untreated group; PC: atherosclerosis rats without administration of coffee bean extract; A1: atherosclerosis rats with mild-moderate dose of arabica coffee bean extract; A2: atherosclerosis rats with high dose of arabica coffee; R1: atherosclerosis rats with mild-moderate dose of robusta coffee; and R2: atherosclerosis rats with high dose of robusta coffee bean extract. Bar: 50 µm.

The untreated atherogenic group (PC) showed the initial process of atherosclerotic plaque formation on the surface of the aortic blood vessels through high expression of P-selectin and also

the appearance of leukocytes in the lumen of the aorta (**Figure 1**). However, by giving coffee bean extract, both arabica and robusta with mild-moderate intake were able to reduce the expression of P-selectin and block the presence of leukocytes in the injury area due to atherosclerosis (**Figure 1A1**, **R1**, and **R2**). In the group of rats given a high intake of arabica coffee bean extract, it was only able to inhibit the presence of leukocytes but not the expression of P-selectin (**Figure 1A2**).

Effect of arabica and robusta coffee on serum apolipoprotein A-1 (ApoA-1) levels of atherosclerosis rat model

Our data indicated that the mean of ApoA-1 levels in the healthy group (NC) was 6.28±1.19 ng/mL while the untreated atherosclerosis model rat group (PC) was 10.18±1.41 ng/mL (**Table 1**). Animals received mild-moderate (A1) and high dose (A2) of arabica coffee bean extract had mean ApoA-1 levels of 7.44±1.41 ng/mL and 6.30±1.45 ng/mL, respectively, while the mild-moderate (R1) and high dose (R2) of robusta coffee group had 17.25±1.63 ng/mL and 12.26±3.32 ng/mL, respectively (**Table 1**). The lowest ApoA-1 levels were seen in the healthy group, while the highest ApoA-1 levels were found in the mild-moderate dose of robusta coffee. The mean of ApoA-1 levels in mild-moderate dose of robusta was higher by 178 ng/mL, 68% compared to the negative control. The results of statistical analysis indicated that the mean of ApoA-1 levels among study groups were significantly different (p<0.001) (**Table 1**).

Table 1. Levels of plasma ApoA-1 in atherosclerosis rat model after administration of arabica and robusta coffee bean extracts

Groups	Plasma ApoA-1 levels (ng/mL)	p-value
	Mean±SD	
Negative control (NC), healthy untreated group	6.28±1.19[a]	<0.001
Positive control (PC), atherosclerosis rats without administration of coffee bean extract	10.18±1.41[b]	
Atherosclerosis rats with mild-moderate dose of arabica coffee (A1)	7.44±1.41[a]	
Atherosclerosis rats with high dose of arabica coffee (A2)	6.30±1.45[a]	
Atherosclerosis rats with mild-moderate dose of robusta coffee (R1)	17.25±1.63[c]	
Atherosclerosis rats with high dose of robusta coffee (R2)	12.26±3.32[b]	

Different superscripts (a, b, and c) on the histogram indicate significant differences (p<0.05)

The post-hoc analysis indicated that the mean of ApoA-1 level was significantly higher in untreated atherosclerosis rats compared to the healthy animal group (p=0.003) (**Figure 2**). The level of ApoA-1 in both mild-moderate and high dose of arabica coffee groups were not significantly different from the healthy control group (p=0.337 and p=0.984, respectively). However, both of them had lower ApoA-1 levels compared to untreated atherosclerosis rat group (p=0.03 and p=0.003, respectively). Both mild-moderate and high dose of robusta coffee groups had higher ApoA-1 levels compared to the negative control group (p<0.001 and p<0.001, respectively). Administration of arabica coffee bean extract with mild-moderate and high intake had no effect on increasing ApoA-1 levels (p=0.374). However, the administration of robusta coffee bean extract with mild-moderate and high intake was able to increase the serum ApoA-1 levels of atherosclerosis rats significantly and even exceeded the control group (**Figure 2**). In addition, it is known that robusta coffee bean extract with a mild-moderate intake provided the best results in increasing serum ApoA-1 levels of atherosclerosis rats.

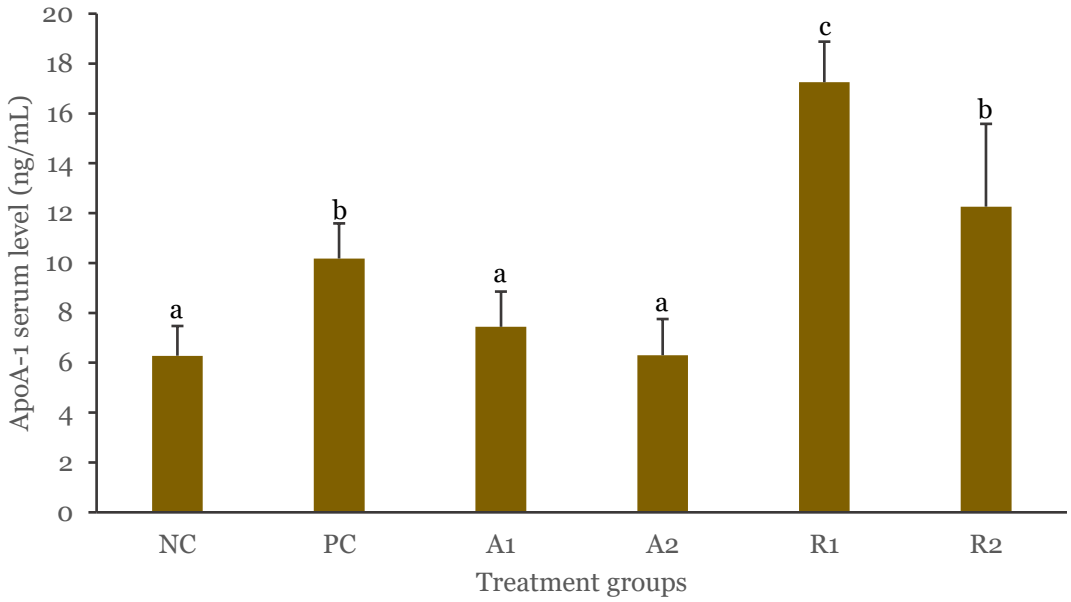

Figure 2. Comparison of serum ApoA-1 levels in atherosclerosis model after administration of arabica and robusta coffee bean extracts at mild-moderate and high dose. NC: healthy untreated group; PC: atherosclerosis rats without administration of coffee bean extract; A1: atherosclerosis rats with mild-moderate dose of arabica coffee bean extract; A2: atherosclerosis rats with high dose of arabica coffee; R1: atherosclerosis rats with mild-moderate dose of robusta coffee; and R2: atherosclerosis rats with high dose of robusta coffee bean extract. Different superscripts (a, b, and c) on the histogram indicate significant differences ($p<0.05$).

Discussion

This study was conducted to assess the effect of arabica and robusta coffee bean extracts on P-selectin expression in aortic tissues and the level of plasma ApoA-1, both as markers of the early process of atherosclerosis. Our study suggested that the expression of P-selectin in aortic endothelial cells, as shown by the results of immunohistochemical staining, was affected by the type and dose of coffee bean extracts. In the group of rats administered a mild-moderate dose of arabica coffee extract, a lower level of P-selectin expression was observed compared to the group receiving a high intake of arabica coffee extract. However, contrasting results emerged in the group of rats with robusta coffee extract diet, regardless of intensity, where both groups exhibited lower levels of P-selectin immunoexpression.

Previous studies have highlighted the impact of caffeine and chlorogenic acid, the main components of coffee, on P-selectin levels in the bloodstream [7,21]. Platelet activation triggers the translocation of P-selectin from intracellular granules to the outer membrane, while fibrinogen induces platelet aggregation by bridging GPIIb/IIIa between adjacent platelets [15,16]. Studies have demonstrated that chlorogenic acid significantly decreased P-selectin expression on platelets [14,27]. Notably, the content of chlorogenic acid (an important biologically active dietary polyphenol) is higher in robusta coffee beans compared to arabica coffee [14]. This distinction in polyphenol content might explain why in the group of rats administered robusta coffee extract, P-selectin expression was lower than in the group given arabica coffee extract, as found in the present study. The findings underscore the potential influence of coffee components, particularly chlorogenic acid, in modulating P-selectin levels.

Overexpression of P-selectin can lead to endothelial dysfunction, platelet activation and leukocyte adhesion that trigger plaque formation on the endothelial surface [6,8]. One potential therapeutic approach is to decrease or inhibit P-selectin expression using P-selectin inhibitors. These agents could be derived from active compounds in coffee beans that might interfere with P-selectin binding to ligands, inhibiting leukocyte adhesion and platelet activation. By blocking P-selectin-mediated interactions between activated endothelial cells and leukocytes, P-selectin inhibitors could reduce inflammation and thrombosis in patients with atherosclerosis [6].

Our data found that the rats subjected to an atherogenic diet along with varying intensities of arabica coffee bean extract had no significant differences in plasma ApoA-1 levels. These findings align with a previous study which explored the impact of arabica coffee consumption across a range of intensities on plasma ApoA-1 levels and found no significant effect [28]. However, another study investigating the influence of arabica coffee on a group of 70 healthy individuals found an increase in high-density lipoprotein (HDL) in the blood [29]. The high-density lipoprotein (HDL) was highly correlated with the level of ApoA-1, indicating a protective effect against cardiovascular incidents [29]. In contrast, distinct outcomes were noted in the group of rats administered robusta coffee bean extract, both at mild-moderate and high intake coffee consumption. Our study revealed that robusta coffee bean extract, irrespective of intensity, was more effective in increasing plasma ApoA-1 levels in an atherosclerosis rat model. This effect could be attributed to the caffeine content in coffee, which has antioxidant and anti-inflammatory properties that are beneficial for cardiovascular health. According to a previous study, the caffeine content of robusta coffee beans is twice as high as that of arabica coffee, presumably contributing to the more pronounced protective effect [30]. The heightened antioxidant activity of coffee beans, influenced by polyphenol components, especially chlorogenic acid, further supports this outcome. Robusta coffee contains more chlorogenic acid than arabica coffee, and previous studies have demonstrated the ability of chlorogenic acid to reduce concentrations of lipids, cholesterol, triglycerides, and various lipoproteins, which directly impacting plasma ApoA-1 levels [16,23,31]. This collective evidence underscores the potential cardiovascular benefits associated with the administration of robusta coffee bean extract in the context of atherosclerosis.

Conclusion

Mild-moderate intake of robusta coffee bean extract could reduce aortic P-selectin immunoexpression and increase serum ApoA-1 levels in an atherosclerosis rat model. Bioactive compounds in coffee beans have the potential effect to prevent atherosclerosis in the early stages.

Ethics approval

This study has obtained ethical approval to use rats as experimental animals from the Ethics Committee for the Use of Experimental Animals of the Faculty of Veterinary Medicine, Universitas Syiah Kuala, Aceh, Indonesia, with No.244/KEPH/VII/2023.

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Competing interests

All the authors declare that there are no conflicts of interest.

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Underlying data

Derived data supporting the findings of this study are available from the corresponding author on request.

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