Pharmacogn J. 2021; 13(1): 172-178
A Multifaceted Journal in the field of Natural Products and Pharmacognosy
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Research Article

Analysis of Several Inflammatory Markers Expression in Obese Rats given *Plectranthus amboinicus* (Lour.) Spreng Ethanol Extract

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ABSTRACT

Introduction: Oxidative stress is one of the inflammatory events caused by obesity. This condition is characterized by an increase in various inflammatory markers, such as intercellular adhesion molecule 1 (ICAM-1), vascular cell adhesion molecule 1 (VCAM-1), and a cluster of differentiation 40 (CD40). This study aimed to analyze the effect of Plectranthus amboinicus (Lour.) Spreng ethanol extract on ICAM-1, VCAM-1, and CD40 in obese rats. **Methods:** The study used a pure experimental method with a completely randomized design. There were 6 groups, namely, C– (negative control), C+ (positive control), CMC (soluble control), EE300 (P. amboinicus ethanol extract, 300 mg/kg body weight [BW]), EE600 (P. amboinicus ethanol extract, 600 mg/kg BW) and EE900 groups (P. amboinicus ethanol extract, 900 mg/kg BW). **Results:** The results showed low levels of ICAM-1 and VCAM-1 in the blood plasma, especially in the EE900 group, but the difference was not substantial. The same trend also occurred in the expression of CD40 in the tunica intima layer of the rat aorta.
Conclusions. Thus, the administration of 900 mg/kg BW P. amboinicus ethanol extract for 45 days has the potential to treat obesity in rats through the suppression of oxidative stress and inflammatory markers (ICAM-1, VCAM-1 and CD40).
Key words: Rats, Enzyme-linked immunosorbent assay, Immunohistochemistry, Plant extract, Obese.

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History

- Submission Date: 23-10-2020;
- Review completed: 18-11-2020;
- Accepted Date: 23-11-2020;

DOI : 10.5530/pj.2021.13.24

Article Available online

http://www.phcogj.com/v13/i1

INTRODUCTION

Obesity can cause various diseases, such as atherosclerosis. It has fatal effects and can even cause death if left untreated. The markers of atherosclerosis include molecule 1 VCAM-1.[1,2] ICAM-1 and cluster of differentiation 40 (CD40).[3,4] ICAM-1, also known as CD54, is an adhesion molecule with similar structure and function as VCAM-1.[2-4] VCAM-1 concentration increases remarkably after the cytokine stimulation of endothelial cells. VCAM-1 is expressed in neurons, fibroblasts, smooth muscle cells, macrophages, and endothelial cells.[3] The synthesis and secretion of ICAM-1 can be stimulated by interleukin-1 (IL-1) and tumour necrosis factor-α (TNF-α).[2-4] ICAM-1 serves as a ligand for lymphocyte function-associated antigen 1 (LFA-1).[2,4,6] Upon activation, ICAM-1 or LFA-1 binds the leukocytes to endothelial cells, allowing the cells to migrate to the tissues.

Increased ICAM-1 levels can be observed in patients with cardiovascular disease, oxidative stress and abdominal obesity.[7] ICAM-1 promotes angiogenesis and can be used as an indicator of endothelial cell activation or damage.[7,8] Adhesion molecules mediate cell interactions with the extracellular matrix, as well as other cells. Thus, adhesion molecules and extracellular matrix molecules are used as markers of endothelial dysfunction (ED) and are involved in the pathogenesis of atherosclerosis.[9] Proinflammatory cytokines lead to the increased ICAM-1 expression in the vascular endothelium and the activation of leukocyte integrins, which result in the leukocytes adhesion to endothelial cells and their migration to the sites of inflammation.[10] VCAM-1 expression in small blood vessels increases after the cytokine stimulation of endothelial cells, and patients with coronary heart disease have higher levels of VCAM-1 compared with normal epicardial coronary arteries.[2-6] People with higher basal levels of ICAM-1 have a double risk of developing cardiovascular disease. Thus, ICAM-1 is also a biomarker of ED.[2-6] These two markers of plasma ED are closely associated with total mortality from cardiovascular disease. Both types of adhesion molecules were expressed in the aortic endothelium and in areas prone to atherosclerosis under experimental conditions.[9]

Plectranthus amboinicus (Lour.) Spreng. is an herb that has the therapeutic and nutritional properties of its multipurpose phytochemicals for a variety of biomedical applications.[11]The essential oil of this herb has significant antioxidant properties against the stress-causing factors of lung cancer, Cardiovascular Disorders, Analgesic Activity, Activity against Genitourinary Diseases, Anti-Inflammatory Activities and Antitumorigenic Activities.[11-14] This study aimed to analyse the effect of *Plectranthus amboinicus* (Lour.) Spreng ethanol extract on ICAM-1, VCAM-1 and CD40 in obese rats.

Cite this article: Harefa K, Sulastri D, Nasrul E, Ilyas S. Analysis of Several Inflammatory Markers Expression in Obese Rats given *Plectranthus amboinicus* (Lour.) Spreng Ethanol Extract. Pharmacog J. 2021;13(1): 172-8.

MATERIALS AND METHODS

Ethanol extract of *P. amboinicus* (Lour.) Spreng

The extract was made at the Pharmacy Laboratory of the University of Sumatera Utara, *P. amboinicus* (Lour.) leaves were collected, cleaned of dirt and dried in an oven at 40 °C. About 1000 g of dried leaves was mashed in a blender. The drying process is carried out based on drying andaliman (*Zanthoxylum acanthopodium),* so that the phytochemical content and bioactive compounds in the plant are not lost due to heat.[15-18] The simplicia powder formed was placed in a macerator with 10.000 mL of 70% ethanol for 6 hours. The mixture was stirred occasionally for up to 18 hours. The leaf powder was separated using Whatman paper. Then, 5000 mL of 70% ethanol was added again (half of the previous volume). The meserate was separated after 18 hours by filtering it with Whatman paper, and the extract was concentrated at 30–50 °C in a rotary evaporator. A water bath with a temperature of ±40 °C was used to remove water from the extract and obtain a constant weight. The process of making this extract is based on the research method by Ilyas.[19,20]

Animal handling

Twenty-four male Wistar rats (*Rattus norvegicus* L.) were obtained from the animal pen of the Biology Department, Faculty of Mathematics and Natural Sciences, Universitas Sumatera Utara, Medan, Indonesia. Rats aged 8–11 months and weighed 180–200 g were given a standard diet of CP511 with the addition of a high-fat diet, which comprised 10% goat fat, 1% cholesterol, 0.2% cholic acid, 10% quail egg yolk and standard feed, for 21 weeks. The rats were housed in a 30×15×40 cm^3 cage, which was covered with husks and cleaned every day.

Research design

Each treatment group consisted of six Wistar rats with four replications and was given tap water ad libitum and food for 45 days. There are six groups: the negative control group (C−), positive control group (C+), CMC group, and three groups of *P. amboinicus* (Lour.) Spreng extract (300 mg/kg BW = EE300, 600 mg/kg BW = EE600 and 900 mg/kg BW = EE900). The treatments were administered daily using an oral swab. Group C+ was given cholesterol feed, the CMC group was given cholesterol feed and CMC, EE300 was given cholesterol feed and 300 mg/kg BW of *P. amboinicus* ethanol extract for 45 days, EE600 was given cholesterol and 600 mg/kg BW of *P. amboinicus* for 45 days, and EE900 was given cholesterol feed and 900 mg/kg BW of *P. amboinicus* for 45 days. This study was approved by the animal health research ethics committee (No. 0492, KEPH-FMIPA, 2018).

ICAM-1 and VCAM-1 measurement

ICAM-1 and VCAM-1 contents were analysed using enzyme-linked immunosorbent assay (ELISA) kits (R&D Systems Company, Minneapolis, USA). The final absorption was assessed with an ELISA photometer (Bio-Rad; Model 680).[2-4,15]

Immunohistochemistry of the aorta

The ten percent formalin is used to fix the aortic tissue at room temperature, embedded in paraffin, sliced, attached to a glass object and stained with hematoxylin–eosin. A paraffin block containing 4 µm-thick tissue was cut for immunohistochemistry. Antigen retrieval was performed by microwave oven heating at 800 W for 20 min in 0.1 M citrate buffer (pH 6.0) for monoclonal antibody CD40 (HM40-3) at a dilution of 1:100. The detection of these CD40 was a two-stage polymer-based technique (Envision K-5007, Dako Corp., Glostrup, Denmark). Tracking dye was Diaminobenzidine, and Harris hematoxylin was the counterstain. Tris-buffered saline substituted the primary antibodies in the control groups. CD40 was noticed in the tunica intima layer. CD40 expression was evaluated on a semiquantitative scale of 0–3 (negative=0; weak intensity staining=1; medium intensity staining=2; and strong intensity staining=3). Two pathologists were undertaken 'single blind' and independently. Inter-observer variable was <5%[21]. In cases of disagreement, the slides were re-evaluated jointly until a consensus was reached.

Data analysis

Numerical data (ICAM-1 and sVCAM-1) were tested for normal distribution and homogeneity. Data that were normally distributed and homogeneous were subjected to 'one-way ANOVA' ($p<0.05$) and post hoc test (Duncan) to compare between treatments Meanwhile, ordinal data (CD40 expression) were analysed using the Kruskal–Wallis test.

RESULTS

ICAM-1 expression

Analysis of the ICAM-1 protein expression using enzyme-linked immunosorbent assay (ELISA) showed significant results. ICAM-1 expression in the blood serum of obese rats is illustrated in Figure 1 ($p<0.05$). The increasing doses of *P. amboinicus* extract decreased the ICAM-1 levels but not remarkably. A decrease in ICAM-1 indicates a reduced risk of atherosclerosis in the cardiac aorta. ICAM-1 mediates the binding of leukocytes with vascular endothelial cells. Thus, ICAM-1 levels help determine the thickening of the blood vessel walls.

VCAM-1 expression

VCAM-1 expression in the blood serum of high-fat diet rats is illustrated in Figure 2 ($p<0.05$). VCAM-1 expression decreased with increasing doses of *P. amboinicus* ethanol extract (EE300, EE600 and EE900). Thus, *P. amboinicus* ethanol extract, which also contains flavonoids, can suppress VCAM-1 expression in rat blood serum. The effect of the ethanol extract of P. amboinicus on VCAM-1 expression was more expressed than that of the C + group. Differences were observed in VCAM-1 expression between the group receiving P. amboinicus extract and the control group.

CD40 detection

The results of detection of CD40 expression in the aortic intima tunica of obese rats given several treatments ($p<0.05$). The administration of ethanol extract of P. amboinicus (Lour.) Spreng at different doses did not have a substantial effect in reducing CD40 expression in obese rats. However, increasing the dose of P. amboinicus extract showed a tendency to decrease CD40 expression in the tunica intima (Figures 3,4 and Table 1). The results of this study indicate that the phytochemical content of this plant functions as an anti-inflammatory agent to reduce CD40 expression which functions as an inflammatory mediator P.*amboinicus.*

DISCUSSION

In the membrane of leukocytes and endothelial cells, ICAM-1 is continuously present in low concentrations, and its concentration is greatly increased by cytokine stimulation. ICAM-1 can be stimulated by IL-1 and TNF-α and is expressed in the vascular endothelium, macrophages and lymphocytes.[2,4,7] ICAM-1 is a ligand for the leukocyte receptor, LFA-1 (integrin).[10] Through ICAM-1, activated leukocyte cells bind to endothelial cells, then migrate to the endothelial tissue (aorta).[1,2,7] Development of atherosclerosis correlate with inflammatory process. Hypercholesterolemia is one of the main risk factors for atherosclerosis.[9] The water extract of *Piper sarmentosum*, which contains flavonoids, plays a role in reducing inflammatory markers, such as VCAM-1,ICAM-1 and C-reactive protein.[22-24]

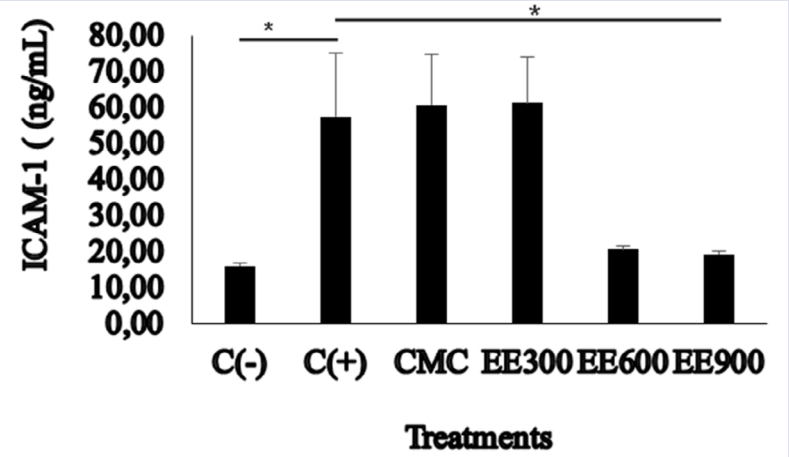

Figure 1: Average ICAM-1 levels after the administration of *P. amboinicus* (Lour.) Spreng ethanol extract in obese rats. Note: C– (negative control), C+ (positive control), CMC (soluble control), EE300 (*P. amboinicus* ethanol extract, 300 mg/kg body weight [BW]), EE600 (*P. amboinicus* ethanol extract, 600 mg/kg BW) and EE900 groups (*P. amboinicus* ethanol extract, 900 mg/kg BW).*P<0.05.

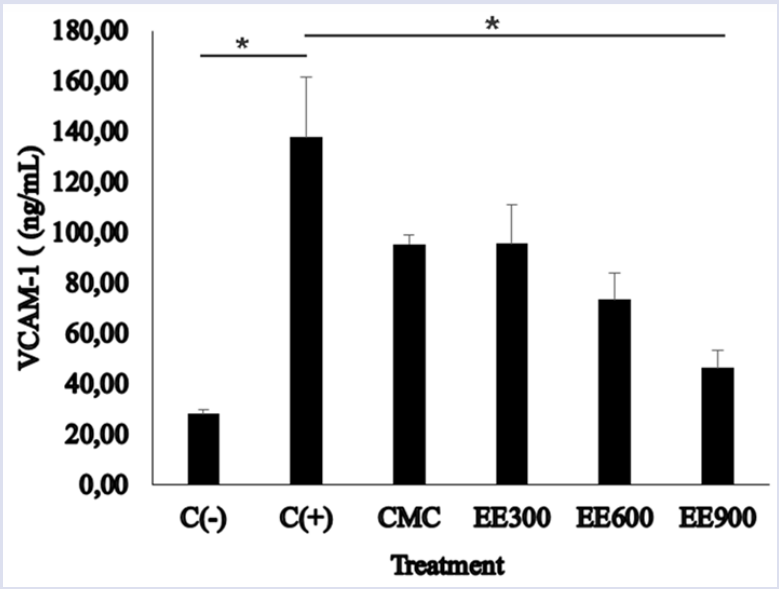

Figure 2: Average VCAM-1 level after the administration of *P. amboinicus* (Lour.) Spreng ethanol extract in obese rats. Note: C– (negative control), C+ (positive control), CMC (soluble control), EE300 (*P. amboinicus* ethanol extract, 300 mg/kg body weight [BW]), EE600 (*P. amboinicus* ethanol extract, 600 mg/kg BW) and EE900 groups (*P. amboinicus* ethanol extract, 900 mg/kg BW).

Table 1: Descriptive data of CD40 expression in the aortic intima tunica of obese rats.

Item	Mean	SD	SE	Min.	Max.	Median	95% Confidence Interval for Mean		P value
							Lower Bound	Upper Bound	
C-	3.00	3.46	1.73	0.00	6.00	3.00	-2.51	8.51	0.13 = p>0.05
C+	7.00	0.82	0.41	6.00	8.00	7.00	5.70	8.30	
CMC	6.75	0.96	0.48	6.00	8.00	6.50	5.23	8.27	
EE300	5.00	3.37	1.68	0.00	7.00	6.50	-0.36	10.36	
EE600	5.00	3.37	1.68	0.00	7.00	6.50	-0.36	10.36	
EE900	4.25	2.87	1.42	0.00	6.00	5.50	-0.32	8.82	

Note: C– (negative control), C+ (positive control), CMC (soluble control), EE300 (*P. amboinicus* ethanol extract, 300 mg/kg body weight [BW]), EE600 (*P. amboinicus* ethanol extract, 600 mg/kg BW) and EE900 groups (*P. amboinicus* ethanol extract, 900 mg/kg BW).

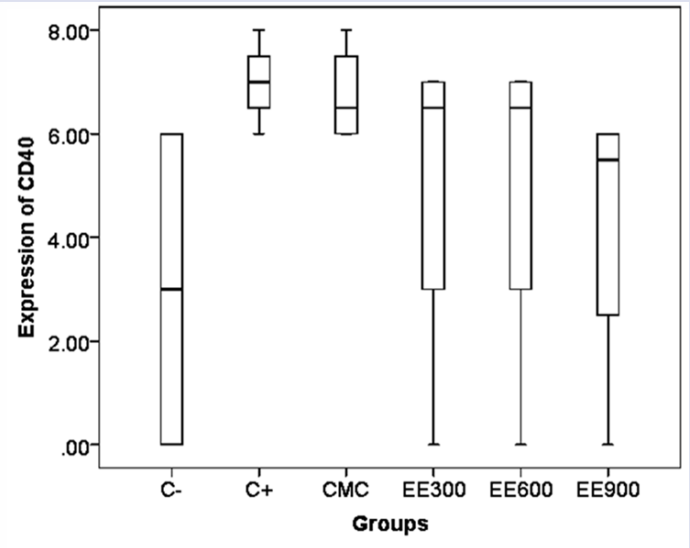

Figure 3: CD40 expression in the aortic intima tunica of obese rats after different treatments. C– (negative control), C+ (positive control), CMC (soluble control), EE300 (*P. amboinicus* ethanol extract, 300 mg/kg body weight [BW]), EE600 (*P. amboinicus* ethanol extract, 600 mg/kg BW) and EE900 groups (*P. amboinicus* ethanol extract, 900 mg/kg BW).

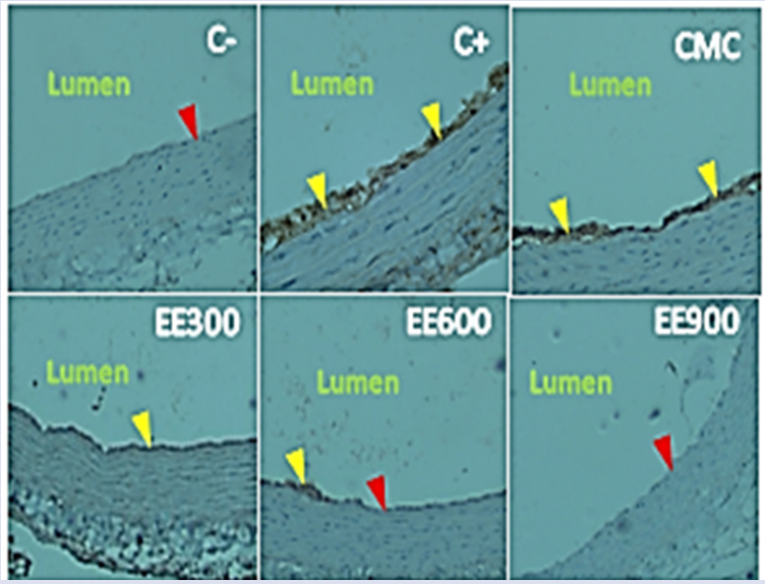

Figure 4: CD40 immunohistochemistry results in the aortic intima of obese rats. The tunica intima with CD40 is shown in yellow, and the red arrows are those without CD40. Note: C– (negative control), C+ (positive control), CMC (soluble control), EE300 (*P. amboinicus* ethanol extract, 300 mg/kg body weight [BW]), EE600 (*P. amboinicus* ethanol extract, 600 mg/kg BW) and EE900 groups (*P. amboinicus* ethanol extract, 900 mg/kg BW).

VCAM-1 expression decreased with increasing doses of *P. amboinicus* ethanol extract (EE300, EE600 and EE900). Thus, *P. amboinicus* ethanol extract, which also contains flavonoids, can suppress VCAM-1 expression in rat blood serum. Flavonoids reduce the expression of VCAM-1.[11] A decrease in VCAM-1 also occurs in the intake of citrus, which contains flavonoids and thus can protect the cardiovascular system.[25] Flavonoids work as antioxidants and anti-inflammatory agents and play a role in the process of regulating monocyte adhesion in cultured human endothelial cells, such as suppressing the expression of VCAM-1 and ICAM-1. The suppression of VCAM-1 and ICAM-1 is carried out by flavonoids through the inhibition of IL-1β.[2-4,11] Suppressing VCAM-1 expression can reduce the binding between monocytes and T lymphocytes and therefore reduce the possibility of the binding of leukocytes and endothelial cells. This bond makes way for the emergence of reactive oxygen species or free radicals from oxygen, which eventually give rise to cytotoxic oxidants and inflammatory mediators that activate the complement system.

Leukocytes move freely along the endothelium under normal conditions. During ischemia and inflammation, endothelial cells

release various mediators that cause leukocyte adhesion molecules to appear on the surface to mobilise and stimulate the leukocyte granules. The oxidants produced will cause injury to the surface of the tissue.[2,25,26]

The highest CD40 expression in the intima tunica was found in the C+ group and was not remarkably different from that of the CMC group. This result is due to the fact that obesity caused by hyperlipidaemia increases lipid peroxidation, which degrades fat and produces malondialdehyde (MDA). MDA stimulates TNF-α and increases the incidence of oxidative stress. The increase in oxidative stress triggers the oxidation of low-density lipoprotein (LDL) and results in the adhesion of monocytes to the endothelium. These monocytes eventually turn into macrophages. Oxidised LDL is phagocytosed by the macrophages and accumulates in the endothelium to form foam cells. Finally, the inflammatory mediators CD40 and TNF-α expressed in muscle cells form unstable atherosclerotic plaques (rich in macrophages and foam cells.[2]

The administration of different doses of *P. amboinicus* (Lour.) Spreng ethanol extract did not seem to have a subtantial effect on decreasing the CD40 expression of obese rats. However, increasing the dose of *P. amboinicus* extract showed a tendency to decrease CD40 expression in the tunica intima (Figure 4). This result shows that the phytochemical content of these plants, such as flavonoids, can function as an anti-inflammatory agent to decrease the expression of CD40, which functions as an inflammatory mediator., *P. amboinicus* contains several phytochemical classes, including diterpenoids, monoterepenoids, triterpenoids, phenolics, sesquiterpenoids, flavonoids and esters.[11-14]

New insights into the functions and mechanisms of *P. amboinicus* include the following: its anti-inflammatory ability reduces swelling (oedema) in carrageenan-induced rat and the levels of proinflammatory mediators (TNF-α and COX-2).[2,11,27], its antioxidant ability is indicated by increased levels of superoxide dismutase,[27-29] and glutathione reductase and decreased levels of MDA and glutathione peroxidase, as well as indomethacin (a drug used to treat the symptoms of swelling and joint pain in osteoarthritis or rheumatoid arthritis).[27-30]

CONCLUSION

The administration of 900 mg/kg BW *P. amboinicus* ethanol extract for 45 days has the potential to treat obesity in rats through the suppression of oxidative stress and inflammatory markers (ICAM-1, VCAM-1 and CD40).

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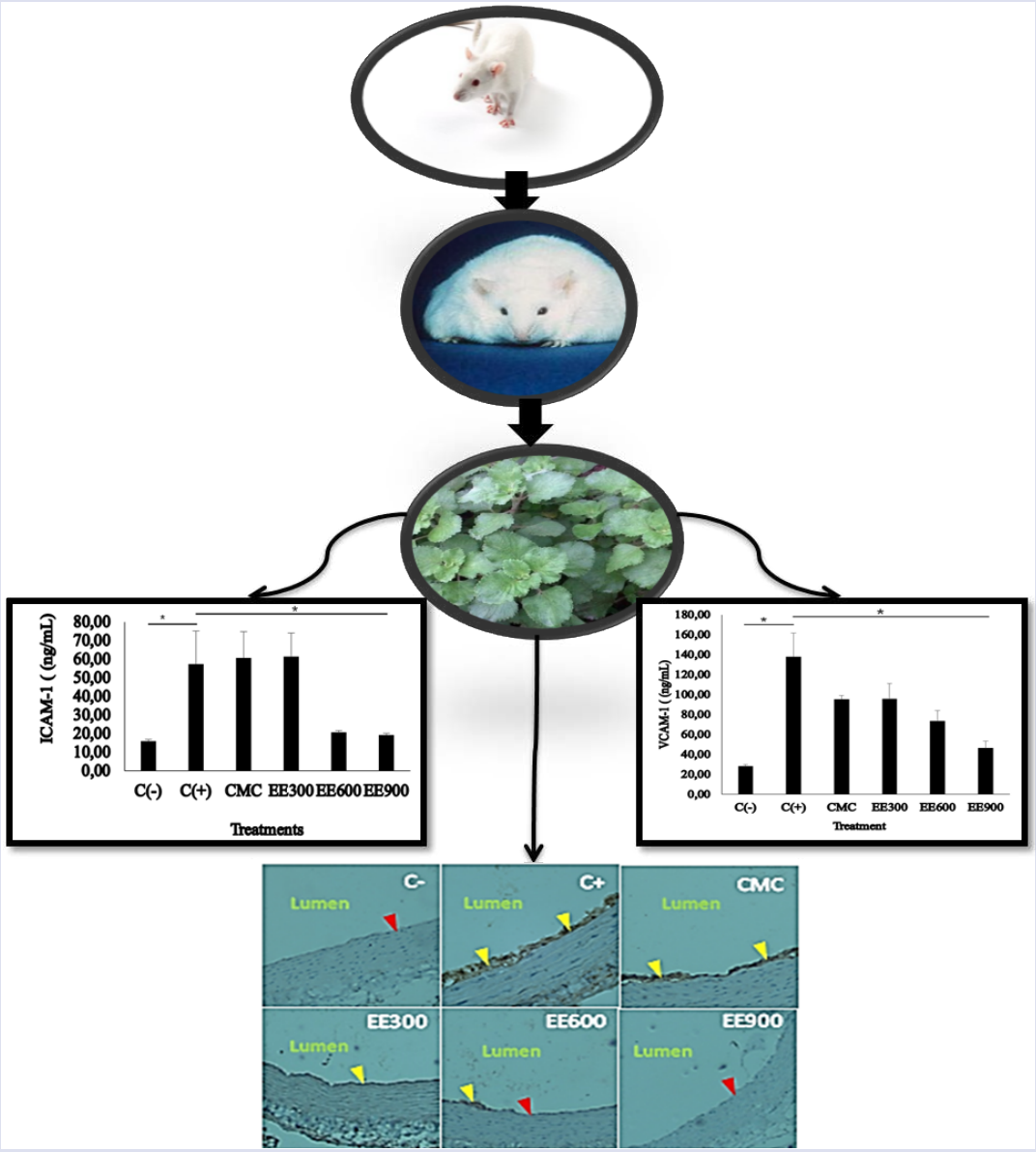
GRAPHICAL ABSTRACT



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Cite this article: Harefa K, Sulastri D, Nasrul E, Ilyas S. Analysis of Several Inflammatory Markers Expression in Obese Rats given *Plectranthus amboinicus* (Lour.) Spreng Ethanol Extract. Pharmacog J. 2021;13(1): 172-8.