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Ethanolic Extract of *Moringa oleifera* Leaves Reduces the Expression of ICAM-1 in Aortic Tissues of Metabolic Syndrome Induced *Rattus norvegicus*

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ABSTRACT

Introduction: *Metabolic syndrome poses a risk for the development of atherosclerotic lesions, which are associated with various cardiovascular diseases. Moringa leaves are known for their antioxidant and anti-inflammatory properties. ICAM-1 serves as an early marker of atherosclerosis. This study aims to investigate how the dosage of ethanolic extract from Moringa leaves affects the expression of ICAM-1 in the aortic tissue of Wistar rats with metabolic syndrome.*
Methods: *This study employed a laboratory experimental design with a posttest only control group. The research involved 30 Wistar rats, divided into five groups: the normal control group (G1), the positive control group (G2), and three experimental groups (G3, G4, and G5). The experimental groups (G3, G4, and G5) were induced with metabolic syndrome and administered ethanolic extract of Moringa leaves at doses of 150 mg/kg, 250 mg/kg, and 350 mg/kg, respectively. The expression of aortic ICAM-1 was evaluated using the Intensity Distribution Score (IDS) and analyzed using one-way ANOVA and Post-hoc Tukey HSD test.*
Results: *Increasing the dosage of ethanolic leaf extract of Moringa oleifera reduces the IDS value of ICAM-1 in the aortic tissue of Wistar rats with a significance of $p<0.05$..*
Conclusion: *The higher dosage of ethanolic leaf extract from Moringa oleifera Lamk has the potential to decrease ICAM-1 expression in the aortic tissue of Wistar rats with metabolic syndrome. The author hopes that Moringa leaf extract can be utilized as a supplement to prevent the formation of atherosclerotic plaques and cardiovascular diseases resulting from atherosclerotic plaques, such as thromboembolism and stroke*.

Keywords: *moringa oleifera* leaves; ICAM-1; metabolic syndrome; *rattus norvegicus*

INTRODUCTION

Metabolic syndrome (MetS) is a group of symptoms that includes hyperglycemia, increased blood pressure, elevated triglyceride levels, low HDL cholesterol levels, and central obesity (Xu et al., 2019). This syndrome increases the risk of Atherosclerosis. Atherosclerosis is a diffuse and slow-developing disease that can affect most arteries in the body, leading to a wide range of clinical manifestations depending on the arteries involved. Rupture of the fibrous cap in atherosclerotic lesions can form thrombosis that may detach and further block the arteries. This can cause severe organ damage, intestinal perforation, stroke, and limb loss. Meanwhile, atheroemboli originate from cholesterol crystals in the lipid core of atherosclerosis, which are carried by the bloodstream and can obstruct distal arterioles mechanically. Atherosclerotic plaques in the abdominal aorta weaken its walls, posing a high risk of aortic aneurysm and aortic rupture (Guembe et al., 2020; Molisse et al., 2007).

The formation of atherosclerotic lesions is initiated by the deposition of low-density lipoprotein (LDL) molecules in the intima layer, which can then be oxidized through the reactive oxygen species pathway. According to Leiva et al. (2015), oxidized LDL can induce the expression of intercellular

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adhesion molecule-1 (ICAM-1) and vascular-cell adhesion molecule-1 (VCAM-1) through the same pathway as the pro-inflammatory cytokine interleukin-1β (IL-1β). The increased expression of ICAM-1 enhances leukocyte adhesion to endothelial cells and their migration towards the intima layer of arteries, thereby accelerating the development of atherosclerotic lesions (Libby, 2021). The level of ICAM-1 expression can serve as a biomarker in the progression of atherosclerosion (Bui et al., 2020).

Moringa oleifera Lamk, also known as *Moringa* or drumstick tree, is a plant whose leaves have the potential as a source of antioxidants (Rani et al., 2018). Some of the antioxidants found in *Moringa oleifera* include saponins, alkaloids, phytosterols, tannins, phenolics, and flavonoids. *Moringa* leaves are also known to possess anti-inflammatory activity (Islam et al., 2021). Research on the health benefits of *Moringa* has been widely reported. A study by Putri (2017) demonstrated that *Moringa* seed extract can significantly reduce ICAM-1 expression in the carotid tissue of Wistar rats. The primary objective of this study is to examine the impact of *Moringa oleifera* Lamk leaf extract on the expression of ICAM-1 in the aortic tissue of Wistar rats (*Rattus norvegicus*) with metabolic syndrome.

METHODS

The study conducted was an experimental laboratory research employing a post-test only control group design. The study was carried out at the Food and Nutrition Study Center Laboratory, Gadjah Mada University, Yogyakarta, and at the Anatomy Pathology Laboratory, Faculty of Medicine, Sebelas Maret University, Surakarta.

Animal subject used were 30 male Wistar rats weighing between 150-200 grams and aged 2 months. These rats were randomly divided into five groups: the Negative Control Group (G1) consisted of rats not induced with metabolic syndrome and not given *Moringa* leaf extract; the Positive Control Group (G2) comprised rats induced with metabolic syndrome but not given *Moringa* leaf extract; Treatment Group I (G3) consisted of rats induced with metabolic syndrome and administered *Moringa* leaf extract at a dosage of 150 mg/kg/day; Treatment Group II (G4) included rats induced with metabolic syndrome and given *Moringa* leaf extract at a dosage of 250 mg/kg/day; and lastly, Treatment Group III (G5) comprised rats induced with metabolic syndrome and given *Moringa* leaf extract at a dosage of 350 mg/kg/day. The development of MetS rat model was achieved with the combination of High-Fat Diet and STZ Induction.

Histopathological observations of the aortic tissue specimens were conducted using a light microscope, specifically the Olympus CX21 model. The expression of ICAM-1 was assessed by counting the number of ICAM-1 expressions on endothelial cells in nine microscopic fields of the rat aortic tissue using the Image Raster application at a magnification of 400x. The measurement for calculating ICAM-1 expression was done semi-quantitatively using the Intensity Distribution Score (IDS) formula, which is calculated as follows: (% strongly positive stained cells x 3) + (% moderately positive stained cells x 2) + (% weakly positive stained cells x 1) + (% negatively stained cells x 0). The maximum score possible is 300.

For result interpretation, a strong positive interpretation is assigned when the staining appears as dark brown, a moderate positive interpretation for a faint brown color, a weak positive interpretation for a very faint brown color, and a negative interpretation when the staining shows a blue color in the cytoplasm.

Analysis of ICAM-1 endothelial cell expression data in aortic tissue after the administration of *Moringa* leaf ethanolic extract in all experimental groups was conducted using the Shapiro-Wilk test because the sample size was less than 50. Subsequently, a one-way ANOVA test followed by post-hoc analysis with the Tukey HSD (Honest Significant Differences) test was performed.

This study has obtained ethical clearance from the research ethics committee of Dr. Moewardi General Hospital with the number 1.524/XII/HREC/2022, issued on 22 December 2022.

RESULTS

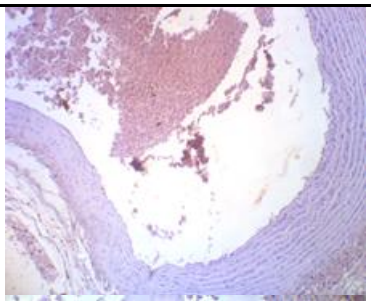
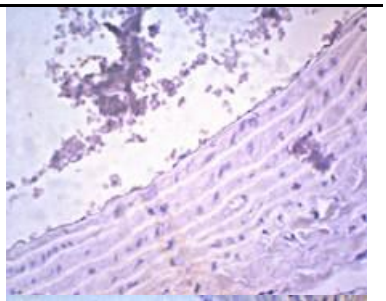
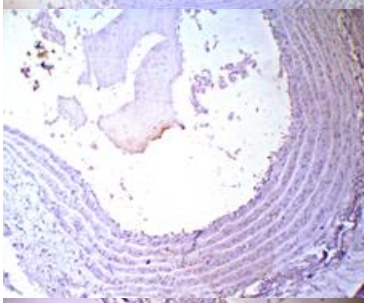
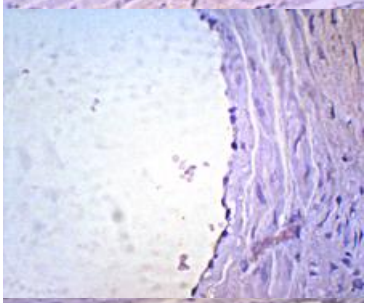
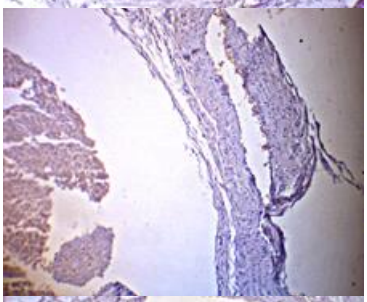
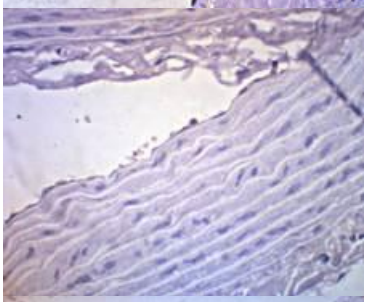
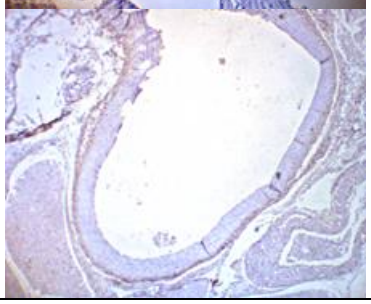
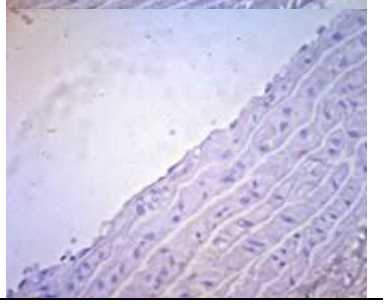
Development of Rat Metabolic Syndrome Models

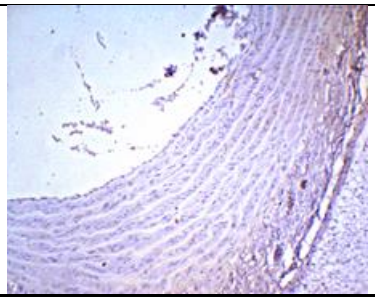
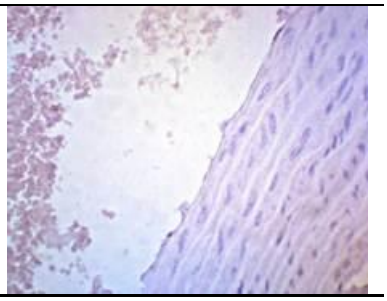
The parameters monitored to determine the success of inducing Metabolic Syndrome are body weight, fasting blood glucose, postprandial blood glucose, blood HDL, blood LDL, blood triglycerides, and systolic blood pressure. Each rat in G2, G3, G4, and G5 experiences an increase in body weight of more than 8%, fasting blood glucose and postprandial blood glucose levels exceeding 250mg/dL, LDL levels exceeding 35mg/dL, triglyceride levels exceeding 150mg/dL, and a decrease in HDL levels less than 35mg/dL after the induction of metabolic syndrome.

The Expression of ICAM-1 in the Aortic Tissue

The aortic organ was harvested from euthanized rats, and tissue sections were obtained using a microtome with a thickness of 4-5 µm. Subsequently, immunohistochemical staining was performed on the preparations, and they were observed under a light microscope. The histopathological images of the preparations are presented in Table 1.

Table 1. Histopathology of the aortic tissue.

Group	100x Magnification	400x Magnification
G1		
G2		
G3		
G4		

Group	100x Magnification	400x Magnification
G5		

ICAM-1 expression was observed by examining the color of the endothelial cell cytoplasm in the aortic tissue and evaluated using the IDS method. The average ICAM-1 expression results are shown in Image 1. The G2 group of rats exhibited the highest ICAM-1 expression, while the G1 group showed the lowest ICAM-1 expression.

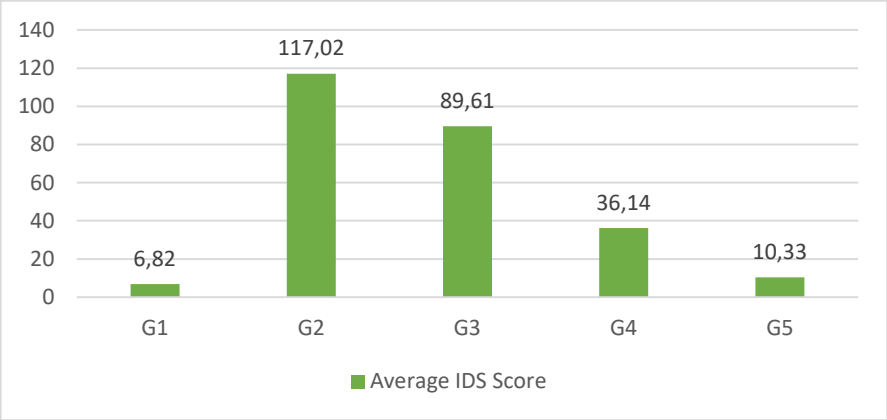


Image 1. Average IDS expression in each group.

The data analysis results were obtained using the Shapiro-Wilk normality test through the SPSS 23.00 for Windows application. The authors used the normality test to determine the normal distribution of data. Based on the table below, the Shapiro-Wilk normality test results show p>0.05, indicating that the data distribution in each group is normal, allowing for parametric One-Way ANOVA test.

Table 2. One-Way ANOVA test Result.

Group	Sample size	*P* value
G1	6	0.380
G2	6	0.653
G3	6	0.861
G4	6	0.728
G5	6	0.060

The parametric One-Way ANOVA test was conducted to determine the differences in ICAM-1 expression levels in the aortic tissue among each group of rats. The obtained p-value is 0.000 (p<0.05), indicating that there are significant differences in ICAM-1 expression levels among the rat groups. The result of p<0.05 indicates that the Tukey-HSD (Honest Significant Difference) test can be performed, which is used to determine the significant differences in ICAM-1 expression levels in the aortic tissue of each group. The results of the Tukey-HSD test can be seen in Table 3.

Table 2. Tukey-HSD test Result.

Group	Group	Mean Difference	Sig.
G1	G2	-110.203108	.000
	G3	-82.790701	.000
	G4	-29.323071	.026
	G5	-3.505725	.995
G2	G3	27.412407	.042
	G4	80.880036	.000
	G5	106.697383	.000
G3	G4	53.467629	.000
	G5	79.284976	.000
G4	G5	25.817346	.061

DISCUSSION

Development of Rat Metabolic Syndrome Model

The combination of a high-fat diet and STZ induction can create a type 2 diabetes mellitus model that resembles human diabetes mellitus. The high-fat diet induces insulin resistance in peripheral tissues due to lipotoxicity, while a low dose of STZ (25-30 mg/kg) leads to a mild impairment in insulin secretion. A high-fat diet leads to insulin resistance through the activation of protein kinase C caused by elevated levels of free fatty acids, which disrupt the activation of insulin receptor substrate. The combination diet contributes to dyslipidemia by increasing free fatty acids, leading to inflammation and higher metabolism of triglycerides and cholesterol. Moreover, hypertension arises from heightened peripheral resistance and activation of the renin-angiotensin-aldosterone system (Gunawan et al., 2021).

The Expression of ICAM-1 in Metabolic Syndrome Rat Models versus Healthy Rats

Based on the Tukey HSD test results conducted between G1 and G2, a p-value of 0.000 or $p<0.05$ was obtained, indicating a significant difference in the level of ICAM-1 expression in the aortic tissue of Wistar rats between the G1 and G2 groups. The IDS value of ICAM-1 expression in the aortic tissue of Wistar rats in the G2 group was higher compared to the G1 group. This indicates that the high-fat diet and STZ-NA injection administered to the G2 rats had an impact on increasing the IDS value of ICAM-1 expression in the aortic tissue of Wistar rats.

In the condition of metabolic syndrome, hypertriglyceridemia, hypertension, and abdominal obesity increase the production of superoxide anions through the NADPH oxidase pathway. Hyperglycemia enhances the formation of reactive oxygen species (ROS) through the processes of glycosylation and glucose autooxidation (Kwaifa et al., 2020). The generation of ROS can induce the activation of NFκB. NFκB is a key transcription factor in the expression of various pro-inflammatory cytokines such as TNF-α, IL-1, IL-6, IL-8, and COX-2. NFκB is mainly located in the cytoplasm and is regulated by IκB (Inhibitor Kappa B). IκB binds to and retains NFκB in the cytoplasm. External stimuli, such as the presence of ROS, lead to the phosphorylation and ubiquitination of IκB. As a result, the 26S proteasome degrades, leading to the release of the NFκB dimer (p50-p65) from the NFκB-IκB cytoplasmic complex and translocation to the nucleus. NFκB then binds to target genes and induces the transcription of pro-inflammatory genes. This process contributes to the increased expression of ICAM-1 in the context of metabolic syndrome (Bui et al., 2020).

The results of the research and literature study are consistent, indicating a significant increase in ICAM-1 expression in the aortic tissue of Wistar rats with metabolic syndrome compared to healthy rats.

The Effect of *Moringa* Leaf Extract on ICAM-1 Expression.

The results of the Tukey HSD test for the G2 group compared to G3, G4, and G5 are 0.042, 0.000, and 0.000, respectively. All p-values are less than 0.05, indicating a significant difference in ICAM-1 expression levels in the aortic tissue of Wistar rats between the G2 group and G3, G4, and G5 groups. The mean IDS value of ICAM-1 expression in the G2 group is higher than in G3, G4, and G5, suggesting that the administration of ethanolic leaf extract in G3, G4, and G5 can significantly reduce ICAM-1 expression in the aortic tissue of Wistar rats.

The reduction in ICAM-1 expression in the G3, G4, and G5 groups is believed to be due to the antioxidant activity of secondary metabolites present in *Moringa oleifera* leaves. Some of the bioactive components found in the ethanolic leaf extract of *Moringa* include phenolic acids, flavonoids, tannins, and saponins (Anzano et al., 2021).

The flavonoid content, particularly quercetin, in *Moringa* leaf extract can reduce the activity of NFκB. When NFκB is unable to bind to target genes, it does not induce the transcription of adhesion molecules such as ICAM-1 and VCAM-1. Rutin, which is a secondary metabolite of flavonoids, can enhance the regulation of IκB-α expression and reduce the regulation of NFκB expression (Imam et al., 2017). Experimental results conducted on Wistar rats by Suchal et al., (2016) showed that kaempferol has anti-inflammatory activity by inhibiting the expression of NFκB protein.

The phenolic acid tannin present in *Moringa* leaves can enhance the activity of antioxidant enzymes such as superoxide dismutase, catalase, and glutathione peroxidase, which neutralize free radicals (Hossain et al., 2020). Additionally, flavonoids can balance ROS by reacting with reactive components of ROS and neutralizing them. Research conducted on rats induced with oxidative stress by Cui et al., (2020) demonstrated that saponins can reduce free radicals.

ICAM-1 is an adhesion molecule expressed by endothelial cells in areas predisposed to atherosclerosis. ICAM-1 plays a role in attracting neutrophils in the atherogenesis process. Increased ICAM-1 expression leads to monocyte adhesion and transendothelial migration, contributing to the formation of mature atherosclerotic lesions (Chistiakov et al., 2017). When ICAM-1 expression decreases, the progression of atherosclerotic lesion formation is hindered, but the initial formation of foam cell lesions remains unaffected. Low ICAM-1 expression has been shown to reduce the size of atherosclerotic lesions by 25% (Bui et al., 2020).

Based on the literature study, it can be concluded that the research results are consistent with the existing literature, which suggests that the decrease in ICAM-1 expression in the aortic tissue of Wistar rats with metabolic syndrome is influenced by the ethanolic leaf extract of *Moringa*.

Increasing the dosage of ethanolic leaf extract of *Moringa* can significantly reduce ICAM-1 expression, approaching normal levels. There is a significant decrease in ICAM-1 expression between the dosage of 150mg/Kg and 250mg/Kg, as evident from the significance value of the G3 group compared to G4 group being less than 0.05 (p=0.05). However, even with the dosage of 150mg/Kg and 250mg/Kg, the ICAM-1 expression has not yet reached normal levels, as there is a significant difference in significance values between the G1 group and G3 and G4 groups, with p-values of 0.000 and 0.026, respectively. The dosage of 350mg/Kg can reduce ICAM-1 expression close to normal, as indicated by the significance value of the G5 group compared to G1 being greater than 0.05 (p=0.995). Therefore, the administration of 350mg/Kg dosage of ethanolic leaf extract of *Moringa* is the most optimal dose for reducing ICAM-1 expression compared to the dosages of 250mg/Kg and 150mg/Kg.

CONCLUSION

The dosage of ethanolic *Moringa oleifera* Lamk leaf extract has an effect on reducing ICAM-1 expression in the aorta of Wistar rats with metabolic syndrome. The ethanolic leaf extract at a dosage of 350 mg/kg can significantly decrease ICAM-1 expression in the aorta of Wistar rats with metabolic

syndrome, approaching normal levels. The authors hope that the administration of *Moringa* leaf extract can be utilized as a supplement to prevent the formation of atherosclerotic plaques and cardiovascular diseases resulting from atherosclerotic plaques, such as thromboembolism and stroke.

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