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Effect of *Piper crocatum* leaves extract on atherosclerosis in diabetic rats

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ABSTRACT

Atherosclerosis is caused by an inflammatory process in the endothelium due to an imbalance between oxidant and antioxidant agents that often occurs in Diabetes mellitus (DM) patients. Antioxidants that function to neutralize superoxide can be used to prevent atherosclerosis in DM. *Piper crocatum* leaves shows an unknown antioxidant effect on the atherosclerosis process. The aim of this study was to determine the effect of *Piper crocatum* leaves extract on the severity of atherosclerosis in diabetic rats. Thirty Sprague Dawley rats were randomly distributed into 6 groups such as HC (healthy control), NC (diabetic-no therapy), PC (metformin 45 mg/kg), PC200, PC300, and PC400 (*Piper crocatum* leaves extract 200, 300, and 400 mg/kg/day). Blood sampling and histopathological analysis were performed after 14 days of treatment. MDA was measured using the TBARS method. The severity of atherosclerosis of the rat carotid artery was observed from tissue stained with haematoxylin eosin (HE) staining. Dose dependent treatment of *Piper crocatum* leaves extract decreased the blood glucose and MDA levels in the PC200, PC300, and PC400 groups compared to group NC while the body weight was increased. The study showed a decrease in the severity of atherosclerosis in groups PC200, PC300, PC400 compared to groups NC. In conclusion, *Piper crocatum* leaves extract may influence in decreasing blood glucose, MDA, and severity of atherosclerosis in diabetic rats.

INTRODUCTION

Diabetes mellitus (DM) is categorized as a chronic disease that can cause macrovascular complications associated with high mortality [1,2]. Theoretically, the pathway that causes chronic complications in patients with DM is through the formation of oxidative stress [3]. The increase in free radicals causes continuous lipid peroxidation by free radicals and produces peroxyl radicals. Consequently, this situation increases the levels of oxidative stress markers such as malondialdehyde (MDA) which triggers atherosclerosis [4,5].

Diabetes patients have higher triglyceride and LDL levels, whereas HDL levels are lower. This suggests that people with diabetes are more susceptible to cardiovascular disease because the process of lipid peroxidation is speeding the development of atherosclerosis [6,7]. Atherosclerosis is caused by an inflammatory process in the endothelium due to an imbalance between oxidant and antioxidant agents. Reactive oxygen species (ROS) cause cellular degeneration when they interact with other substances in the body such as nucleic acids lipids, proteins, and lipids [8]. Therefore,



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¹antioxidants that function to neutralize superoxide can be used to prevent atherosclerosis in DM patients [9].

In a previous study, ⁶Chung Hun Wha Dam Tang, a traditional herbal remedy, was found to have suppressive effects on oxidative stress and inflammation in vitro in various cellular models and in vivo in HFD-induced atherosclerosis in ApoE^{-/-} mice. This suggests that herbal antioxidants may have a positive impact on the improvement of cardiovascular disease [10]. One important source of antioxidants available in Indonesia is *Piper crocatum* leaves. Phytochemical screening shows that *Piper crocatum* leaves contains flavonoids, saponins, alkaloids and tannins that have been shown to influence health. Flavonoids have been shown to have antioxidant properties [11,12]. Previous research has demonstrated that flavonoids also have Vaso-protective properties by targeting the Nrf2-HO-1 signaling pathway, which is crucial in the development of cardiovascular disease and other vascular-related diseases [13,14].

Research shows that the antioxidant activity of ethanol extract of *Piper crocatum* leaves is very strong with an IC₅₀ level of 47.55 ppm [15]. Several studies have shown antidiabetogenic and antihyperlipidemic effects of *Piper crocatum* leaves [11]. The compounds such as flavonoids, polyphenols, terpenoids, and alkaloids are thought to have antidiabetic activities. Research that has proven the effect of *Piper crocatum* leaves on the improvement of atherosclerosis under DM has never been explored. Therefore, the current study aimed to examine the effects of *Piper crocatum* leaves in these conditions.

MATERIALS AND METHODS

Experimental animals

Thirty male Sprague Dawley rats, 2-3 months old, weighing 182.63±10.91 grams were used in this study. Before the tests began, rats were acclimated for 5 days and given water and food ad libitum. ⁵Rats were divided into 6 groups (5 rats per group) such as healthy control (HC), normal rats received a single intraperitoneal injection of buffer citrate; negative control (NC), streptozotocin (STZ)-induced diabetic rats; PC, PC200, PC300, PC400, diabetic rats received metformin (45 mg/kg; p.o.), *Piper crocatum* leaves extract (200/300/400 mg/kg; p.o.), respectively, for 2 weeks after induction periods. All groups were weighted by digital scale. Rats were sacrificed on day 29 to be carried out with blood taking and carotid artery tissue collection. All the study's research participants were located, and the study's activities were carried out in facilities that complied with the standards for human and animal health research. The ethical committee that oversees the topic granted approval for animal experimentation (No. 43/EC/H/FK-UNDIP/V/2022).

Induction of diabetes

⁵After 5 days adaptation period, the rats were randomly divided into two groups: the healthy control group (HC, n=5) and the STZ induced diabetic group (n=25). The rats were given a single intraperitoneal injection of 35 mg/kg STZ in a citrate buffer (pH 4) after being fasted overnight prior to injection, and the HC rats received only the citrate buffer. After being induced, the rats were given 30% sucrose solution ad libitum for 3 days, to prevent hypoglycemia. Blood samples were obtained under anesthesia from the orbital vein in 12 h-fasted rats three days after induction. Blood sugar levels were checked using the GOD-PAP method, if fasting blood sugar levels > 126 mg/dl rats

were considered diabetic. The rats were allocated into two dietary regimens, HC were fed on normal pellet diet and STZ induce diabetic group were fed on high fat diet ad libitum, for an initial period of two weeks.

***Piper crocatum* leaves extract administration**

Extract from *Piper crocatum* leaves was received from the Center of Traditional Medicine, Universitas Diponegoro, and was made in accordance with the procedure described in a study by Prayitno *et al.* [15]. Briefly, the red betel leaves were dried in the sun, then grinded to make simplicial powder. Simplicia powder was soaked using 70% ethanol solution with a ratio of simplicia powder and solvent 1: 3 for 3 x 24 h with stirring every 6 h. The maceration results were filtered using filter paper, then the macerated filtrate separated from the solvent using water bath at 40°C until become thick. *Piper crocatum* leaves extract was given 2 weeks after 2 weeks of diabetes initial period, based on the treatment group. The intervention was administered once daily orally for 14 days in a row.

Total flavonoid measurement

The total flavonoid content of ethanol extract of *Piper crocatum* leaves was examined using the reduction method using quercetin as standard. 0.1 g of red betel leaf extract dissolved in 10 ml of ethanol. Then add 1 ml of AlCl₃ and 1 ml of potassium acetate into 1 ml of that solution. The solution then incubated at room temperature for 1h. After that the solution is read using a UV-vis spectrophotometer at a wavelength of 435 nm in triple to determine the absorbance. Finally the absorbance was compared with the standard curve readings on quercetin [15].

Blood MDA and blood glucose level analysis

At day 29, under anesthesia, blood samples were collected from the orbital vein of 12 h-fasted rats. To extract plasma, the blood samples were centrifuged at 3500g for 10 minutes after being stored in an EDTA tube at 4 °C for 2 hours. The thiobarbituric acid reactive substance (TBARS) method was used to assess the plasma MDA level (in units of mmol/l) and read on a 532 nm wavelength UV-vis spectrophotometer. The quantitative enzymatic colorimetric test of Glucose Oxidase Phenol 4-Aminoantipyrine was used to measure the fasting blood glucose (FBG) level (GOD-PAP).

Carotid artery atherosclerosis analysis

Sections of tissue from the carotid artery were fixed in 10% buffered formalin. The material was fixed, then processed to create 5 m thick paraffin slices by washing it under running water. Carotid artery section was stained with Hematoxylin Eosin (HE). A binocular microscope with a 400x magnification was used to perform a qualitative assessment of the severity of carotid artery atherosclerosis. An accredited pathologist performed the preparation and histological analysis.

Statistical analysis

The statistical analysis used the SPSS version 26 (SPSS Inc., Chicago, Ill., USA). The Shapiro-Wilk test was used to check whether the data were normal. The blood MDA data had a normal distribution; thus, the One-Way ANOVA test was run, and the Post Hoc Games Howell test was performed once a significant difference was discovered. The data for the severity of the carotid artery were not normally distributed, so the Kruskal-Wallis test was used before the Man-Whitney test. In terms of statistics, the p-value of 0.05 was significant.

RESULTS

Effect of *Piper crocatum* leaves extract on total flavonoids

The average total flavonoid content obtained in the ethanol extract of *Piper crocatum* leaves in this study was 134.67±9.07 mg QE/g. Flavonoid was measured to investigate the amount of flavonoid that suspected to have the antidiabetic and anti-atherosclerotic effects.

Effect of *Piper crocatum* leaves extract on body weight

The Shapiro Wilk test findings for body weight revealed significance in all groups ($p>0.05$). This demonstrates that the data had a normal distribution and that the ANOVA's criteria had been met. The Levene's test results indicated that the population variance was homogeneous with a significance of $p=0.495$ ($p>0.05$). The ANOVA test findings revealed a significance of $p=0.001$ ($p<0.05$), indicating that the treatment of the different groups differed significantly from one another. A post hoc LSD test was used to determine which groups were statistically different. Figure 1 displays the outcomes of the pos hoc LSD test. A significant difference was found in the post hoc LSD test between the HC group and the groups NC, PC, PC200, PC300, and PC400; NC against the groups PC, PC200, PC300, and PC400; PC200 against PC400; and PC300 against PC400.

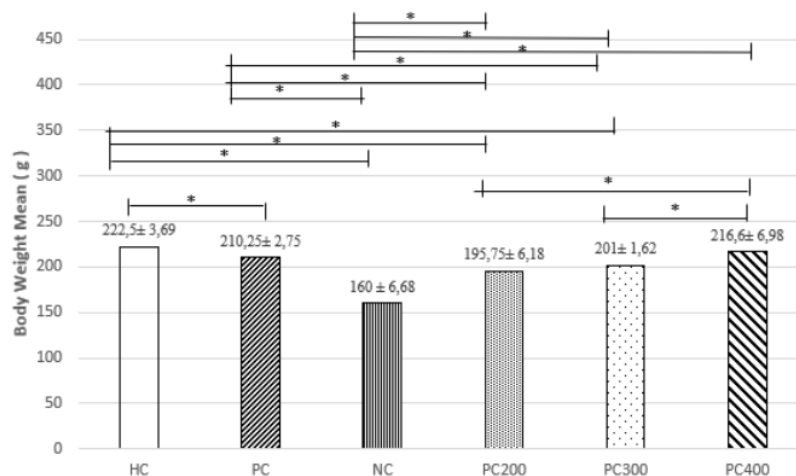


Figure 1. Effect of *Piper crocatum* leaves extract on body weight in rats. Results from post-hoc LSD test are indicated on the graph in case significant body weight differences were found. HC (nondiabetic), NC (diabetic-no therapy), PC (metformin 45 mg/kg), PC200, PC300, and PC400 (*Piper crocatum* leaves extract 200/300/400 mg/kg). * Significant ($p < 0.05$).

Effect of *Piper crocatum* leaves extract on blood glucose levels

All groups except for PC200 group ($p = 0.021$) demonstrated significance of $p > 0.05$ for the Shapiro Wilk test for the blood glucose level. This shows that the data were not normally distributed, and as a result, the ANOVA requirements were not satisfied. The Kruskal-Wallis test was used to continue the research. The Kruskal-Wallis test findings revealed a significance of $p = 0.001$ ($p < 0.05$), indicating that there was a significant difference in the way the groups were treated. Mann-Whitney test was run to determine which group had significant differences. Figure 2 displays the Mann-Whitney test findings. In the Post Hoc Mann-Whitney test, there was a significant difference between the HC group against NC, PC, PC200, PC300 and PC400; PC against NC, PC200 and PC300; Group NC against PC200, PC300 and PC400; PC200 against PC400; PC300 against PC400.

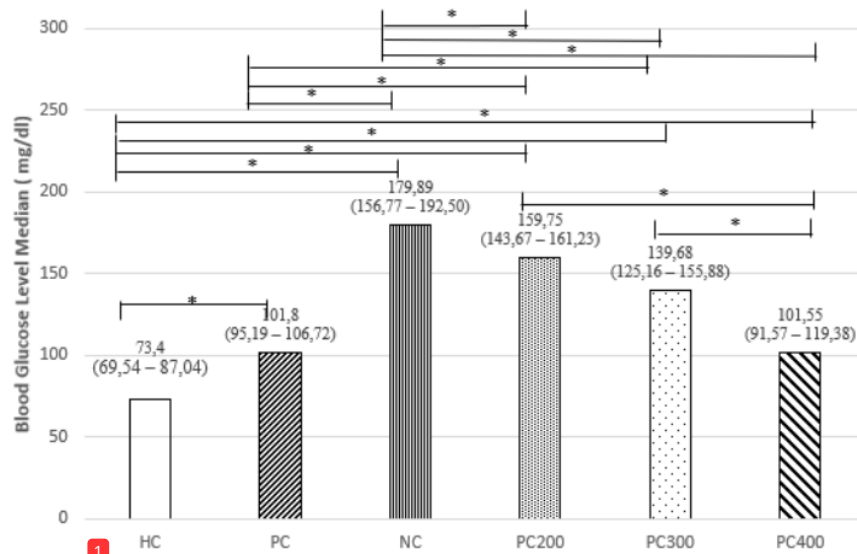


Figure 2. Effect of *Piper crocatum* leaves extract on blood glucose levels in rats. Results from post-hoc Mann-Whitney test are indicated on the graph in case significant blood glucose differences were found. HC (nondiabetic), NC (diabetic-no therapy), PC (metformin 45 mg/kg), PC200, PC300, and PC400 (*Piper crocatum* leaves extract 200/300/400 mg/kg). * Significant ($p < 0.05$).

Effect of *Piper crocatum* leaves extract on blood MDA levels

According to Shapiro Wilk Test all groups outcomes for blood MDA levels were significant ($p > 0.05$). This demonstrates that the data had a normal distribution and that the ANOVA's criteria had been met. To confirm the homogeneity of the sample variances, the Levene's test was used after the normalcy test. The results of the Levene's test indicated that the population variance was not homogeneous with a significance of $p = 0.017$ ($p < 0.05$). The ANOVA Welch test findings revealed a significance of $p = 0.001$ ($p < 0.05$), indicating that the treatment of the different groups differed significantly from one another. A post hoc Games Howell test was used to determine which groups were statistically distinct. Figure 3 displays the outcomes of the post hoc Games-Howell test. There was a significant difference in the pos hoc Games Howell test between the HC group against PC, NC, PC200 and PC300; PC against NC and PC200; NC against PC300 and PC400; and PC200 against PC400.

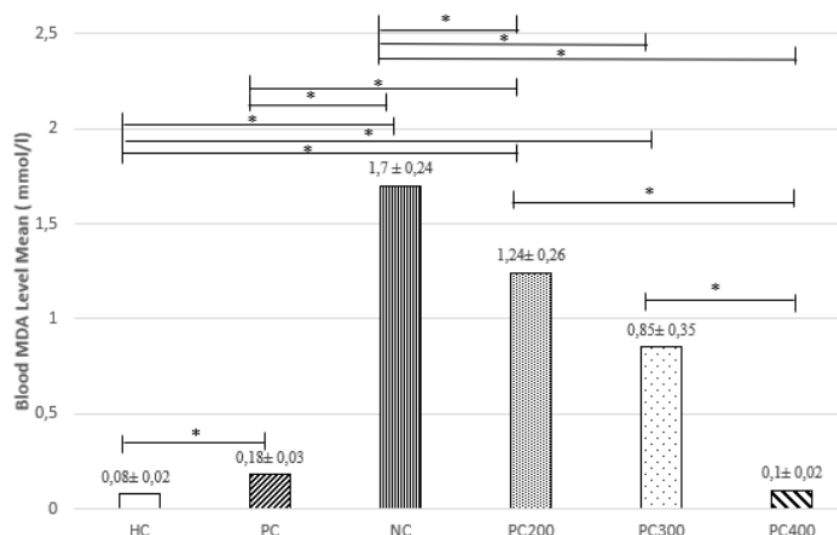


Figure 3. Effect of *Piper crocatum* leaves extract on blood MDA levels in rats. Results from post-hoc Games Howell test are indicated on the graph in case significant blood MDA differences were found. HC (nondiabetic), NC (diabetic-no therapy), PC (metformin 45 mg/kg), PC200, PC300, and PC400 (*Piper crocatum* leaves extract 200/300/400 mg/kg). * Significant ($p < 0.05$).

Effect of *Piper crocatum* leaves extract on atherosclerosis severity in carotid artery

The chi square test with the substitute Kruskal Wallis test demonstrated a significance of $p=0.004$ ($p<0.05$), indicating that the treatment of the groups differed significantly (Table 1). The Mann Whitney post hoc test was used to continue identifying differences across groups. Table 2 revealed a significant difference between the HC group against PC, NC, PC200 and PC300 groups in the Post Hoc Mann-Whitney test. The findings also revealed that the NC group had significantly more severe atherosclerosis than the PC, PC200, PC300, and PC400 groups. Results were obtained as shown in Figure 4 based on histological assessment of the degree of atherosclerosis from carotid artery tissue cuts in experimental animals.

Table 1. Carotid artery atherosclerosis severity among experimental and control groups.

Group	Carotid Artery Atherosclerosis Severity				P value
	Normal	Mild	Moderate	Severe	
HC	5 (100%)	0 (0%)	0 (0%)	0 (0%)	0.004*
PC	0 (0%)	3 (75%)	1 (25%)	0 (0%)	
NC	0 (0%)	0 (0%)	4 (100%)	0 (0%)	
PC200	0 (0%)	3 (75%)	1 (25%)	0 (0%)	
PC300	1 (25%)	3 (75%)	0 (0%)	0 (0%)	
PC400	2 (40%)	3 (60%)	0 (0%)	0 (0%)	

*Significant ($p<0.05$)

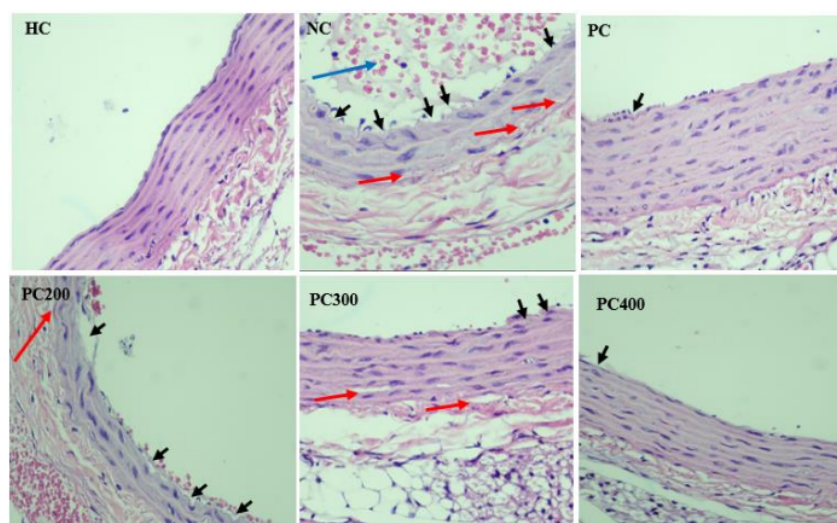


Figure 4. Effect of *Piper crocatum* leaves extract on carotid artery (400x magnification). Cross section of carotid artery showing lipid deposits in tunica media (red arrows), foam cell (black arrow), thrombosis (blue arrow). HC (nondiabetic), NC (diabetic-no therapy), PC (metformin 45 mg/kg), PC200, PC300, and PC400 (*Piper crocatum* leaves extract 200/300/400 mg/kg).

Table 2. Comparison of carotid artery atherosclerosis severity between groups.

	HC	NC	PC	PC200	PC300	PC400
HC	-	0,011*	0,008*	0,011*	0,040*	0,074
NC	-	-	0,040*	0,040*	0,011*	0,009*
PC	-	-	-	1,000	0,186	0,107
PC200	-	-	-	-	0,186	0,107
PC300	-	-	-	-	-	0,655
PC400	-	-	-	-	-	-

*Significant (p<0,05)

DISCUSSION

Total flavonoid content obtained in 70% ethanol extract of *Piper crocatum* leaves in this study was 134.67 mg QE/g. Another study found that the total flavonoid content in extract ethyl acetate fraction of 96% ethanol was 133.84 mg QE/g [16]. Other examination of 70% ethanol extract of *Piper crocatum* leaves total flavonoid content, showed a higher level of 168.33 mg QE/g [17]. There was no large difference in total flavonoid content, compared to previous studies, that show the extraction process has been carried out properly.

Oral administration of *Piper crocatum* leaves extract for 14 days showed a significant decrease in blood glucose levels of diabetic male rats. The results showed that administration of *Piper crocatum* leaves extract at doses of 200, 300, and 400 mg/kg/day in diabetic male rats could reduce blood glucose levels. *Piper crocatum* leaves extract contains antioxidants that can reduce blood glucose levels through two main mechanisms such as intrapancreatic mechanism and extra-pancreatic mechanism. The intrapancreatic mechanism works by protecting cells from damage, regenerating damaged pancreatic cells, and stimulating insulin secretion. Flavonoids produce an increase in intracellular Ca^{2+} ion concentration and inhibit the KATP ATP-sensitive potassium channel in the pancreatic islets of Langerhans [13-15]. By blocking glucose

absorption in the intestine, increasing blood glucose transit,⁵ promoting glycogen synthesis, and limiting glucose synthesis by blocking the enzymes glucose 6-phosphatase and fructose 1,6-bisphosphatase, flavonoids lower blood sugar levels extra-pancreatic [15-17].

The lowest glucose levels were found in the HC group, followed by the PC and PC400 groups. The administration of *Piper crocatum* leaves extract in the PC400 group with a dose of 400 mg/day showed no difference compared to PC group which was given metformin 45 mg/kg. This indicates that *Piper crocatum* leaves extract at a dose of 400 mg/Kg has the ability to lower glucose levels which is comparable to metformin at a dose of 45 mg/Kg. This is in accordance with the benefits of metformin used as a DM therapy by increasing peripheral glucose absorption through increasing cell sensitivity to insulin, reducing fatty acids, suppressing glucose production by the liver, and increasing the use of glucose in the intestine through a non-oxidative process [18-20].

The results showed that there was a significant decrease in blood MDA levels of mice in groups PC200, PC300, PC400, and PC when compared to groups NC. This shows that the administration of *Piper crocatum* leaves extract in graded doses of 200, 300, 400 mg/kg/day and metformin 45 mg/kg in hyperglycemic male rats can reduce blood MDA levels. The decrease in blood MDA levels may be due to the presence of flavonoid and phenolic compounds in the *Piper crocatum* leaves extract. The results of phytochemical screening proved that the ethanolic extract of *Piper crocatum* leaves contains flavonoid and phenolic compounds [21]. Based on previous research, the phenol and flavonoid groups are antioxidant compounds that are more effective than vitamin C, vitamin E and carotenoids. This is in accordance with Ramadhan *et al.* and Prayitno *et al.* that the administration of *Piper crocatum* ethanol extract with doses of 100, 200, and 400 mg can increase levels of glutathione peroxidase which is an antioxidant, and decrease free radical products such as MDA [22,23]. Previous research has also stated that flavonoids are exogenous antioxidant compounds which is able to reduce MDA levels [12,24].

This study showed that the administration of *Piper crocatum* leaves extract at doses of 200, 300, and 400 mg/kg/day and metformin 45 mg/kg could increase the body weight of diabetic rats. Based on previous research, the flavonoid content in *Piper crocatum* extract can improve pancreatic function and increase cell sensitivity to insulin. Increasing insulin levels and cell sensitivity to insulin will increase the process of glycogenesis and reduce the lipolysis process, so that the body weight increase in rats [11,12,25].

The results showed that there was a significant decrease in the degree of atherosclerosis in the administration of *Piper crocatum* extract at doses of 200, 300, and 400 mg/kg to the NC group. The administration of *Piper crocatum* extract significantly reduced the severity of atherosclerosis in diabetic rats. This is supported by several previous studies that showed flavonoids would prevent atherosclerosis by reducing free radical levels, glucose levels and cholesterol levels [22,26,27]. By inhibiting the production of 3-hydroxy-3-methylglutaryl coenzyme A reductase (HMG CoA reductase), a crucial enzyme in the production of cholesterol, flavonoids reduce hypercholesterolemia [28,29]. Flavonoid also reduce the need for NADPH for the synthesis of fatty acids and cholesterol. Improvement of hypercholesterolemia can also be caused by the ability of flavonoids to modify lipoprotein metabolism by increasing LDL uptake through increased LDL receptors and/or increased activity of cholesterol acyl transferase (LCAT). LCAT has a key role in combining free cholesterol into HDL [22,25]. Flavonoids are natural antioxidants present in *Piper crocatum* that inhibit free radicals. By inhibiting LDL oxidation in macrophages and lowering the attraction of Ox-LDL by

macrophage receptors, these substances have antioxidant effects in atherosclerosis [30]. Previous research has demonstrated that *Piper crocatum* reduces TNF-alpha and IL-6 in a mouse model of atherosclerosis. Pinocembrin and pinostrobin, two flavonoids that are widely present in the leaves of *Piper crocatum*, have antioxidant activities and are thought to slow the progression of atherosclerosis. Reduced TNF-alpha causes Selectin and VCAM-1 production decrease in endothelial cells, which in turn reduces monocyte adherence. These cytokines were produced as a result of the interaction between CD40 and CD40 ligand in the intima of arteries, the uptake of oxLDL by the oxLDL receptor-1 (LOX-1), and the signaling protease-activated receptor's creation of CRP protein in response to IL-6 [31].

Flavonoids also improve endothelial function and lower blood pressure by increasing the vasorelaxation ability of blood vessels. The reactivity of platelets in aggregating can also be inhibited by flavonoids. As anti-inflammatory substances, flavonoids regulate transcription of nuclear factor kappa B (NF-kB) to express TNF- α . This regulation causes inhibition of the secretion of the proinflammatory cytokine TNF- α [26]. Inhibition of the COX and lipoxygenase pathways prevents the accumulation of leukocytes causing a decrease in the secretion of proinflammatory cytokines. As primary antioxidants, flavonoids provide hydrogen ions so that free radical ions become stable [15,18].

According to the previous study, there are three techniques to assess the degree of atherosclerosis in an atherosclerotic mouse model. Three cross sections were stained: 1) elastic van Gieson staining to identify the location of the atherosclerotic lesion; 2) HE for histological analysis; and 3) Oil Red O and immunohistochemical staining for quantitative investigation of the cellular components within the lesions [32]. New methods to analyze and quantify atherosclerotic lesions in mouse studies have also been developed because of advances in three-dimensional imaging. But for the time being, their reliance on pricey specialized equipment will probably restrict their accessibility to the majority of researchers [33]. Although HE observation is sufficient for determining the severity of atherosclerosis, further tests, like those mentioned above, would have provided a more accurate description of the atherosclerosis. However, those tests were not conducted due to a lack of facilities and costs. It is necessary to carry out a longer atherosclerosis induction period to show the severity of severe atherosclerosis and the effect of red betel extract on severe atherosclerosis, since this research shown no severe atherosclerosis in all groups.

Differentiating the dose of *Piper crocatum* leaves is intended to find the minimum and maximum effective dose. The maximum effective dose is defined the dose "beyond which additional benefit would be unlikely to occur". In contrary, the minimum effective dose is defined as the lowest dose level that yields a therapeutic benefit to subjects [34]. In this study, the maximum and minimum effective dose hasn't seen yet, so a wider range of *piper crocatum* leaf extract of doses is needed in further study to determine the minimum and maximum effective doses. As with most studies, the design of the current study is subject to limitations. In this research due to limited research cost, we didn't evaluate the effect on liver and renal function of the rats as compared with the healthy group. Previous study shown that therapeutic doses of red betel vine leaves ethanolic extract (50/100/200/400 mg/kg) on the liver and kidneys of DDY mice appear tolerable in both acute and sub chronic toxicity studies [35]. This study proves that the administration of *Piper crocatum* leaves extract inhibit atherosclerotic plaques progression. For clinicians, this study provides information that *Piper crocatum* leaf extract has the potential effect as an adjuvant therapy to prevent the progression of atherosclerosis in DM patients. However, further clinical trials are

needed to prove the anti-atherosclerosis effect of *Piper crocatum* leaf extract in patients with DM.

CONCLUSIONS

The administration of *Piper crocatum* leaves extract in graded doses of 200, 300, and 400 mg/kg for 14 days was able to reduce blood glucose level, blood MDA level, and the severity of atherosclerosis of the carotid artery in diabetic Sprague-Dawley rats. This study provides information that *Piper crocatum* leaf extract has the potential effect as an adjuvant therapy to prevent the progression of atherosclerosis in DM patients. Also, the maximum and minimum effective dose hasn't seen yet, so a wider range of *Piper crocatum* leaf extract doses is needed in further study to determine the minimum and maximum effective doses. New more accurate methods to analyse and quantify atherosclerotic lesions by using three-dimensional imaging in mouse studies have also been developed, but due to a lack of facilities and costs, this study only use HE staining to determine the severity of atherosclerosis.

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AUTHOR CONTRIBUTIONS

The study's chief investigator, MW Adiansyah, conceptualised and designed it, wrote the manuscript's first draught, and oversaw data collecting; Endang M. evaluated the text, provided advice on the interpretation and analysis of the data. ; H Istiadi analyze and interpret carotid artery severity preparations. The following people reviewed the manuscript: FEP Mundhofir; N Maharani; Y Nindita.

CONFLICTS OF INTEREST

There is no conflict of interest among the authors.

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