Original Article

Thai Journal of Pharmaceutical Sciences

# Anti-inflammatory effect of *Centella asiatica* extract on prevented aortic intima-media thickening in diabetic rats

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**Received:** Oct 15, 2017
**Accepted:** Jan 15, 2018
**Published:** Mar 15, 2018

**Keywords:**
Aortic intima-media thickness, *Centella asiatica* extract, diabetes mellitus, endothelial nitric-oxide synthase mRNA expression, macrophage polarization

## ABSTRACT

**Objectives:** The objective of this study is to explore the effect of *Centella asiatica* extract on intima-media thickness, endothelial nitric-oxide synthase (eNOS) expression, and macrophage M1/M2 ratio in diabetic rats aorta. **Materials and Methods:** Wistar rats consisted of normal group and diabetic group which divided into negative control, positive control (captopril 50 mg/kg/day), and *C. asiatica* extract (dose 250, 500, and 1000 mg/kg/day). The aorta was harvested after treatment for 8 weeks to measure intima-media thickness, eNOS mRNA expression, and macrophage M1/M2 ratio. **Results:** Dose of 500 and 1000 mg/kg/day *C. asiatica* extract prevents aortic intima-media thickening ($P < 0.05$). Then, dose of 1000 mg/kg/day prevents the increase of M1/M2 ratio ($P < 0.05$). Expression of eNOS mRNA did not give any significant results ($P > 0.05$). **Conclusion:** The extract of *C. asiatica* has anti-inflammatory effect and ability to prevent intima-media thickening on diabetic rats aorta.

## INTRODUCTION

Diabetes mellitus (DM) is a chronic disease that can lead to several complications related to vascular dysfunction.[1] Chronic hyperglycemia may disrupt the lipid metabolism which leads to accumulation of perivascular adipose tissue.[2] Imbalance of adipocyte accumulation and blood supply to adipocyte area caused limitation of oxygen diffusion to adipose tissue. This relative hypoxic condition enhances oxidative stress[3] and releases pro-inflammatory cytokines that trigger chronic inflammation.[4,5]

Chronic inflammation was caused by disregulated of macrophage polarization, in which pro-inflammatory cytokines increased and anti-inflammation cytokines decreased.[6] It may induce oxidative stress,[7] thus inhibiting endothelial nitric-oxide synthase (eNOS) to produce nitric oxide (NO).[8] The combination of these processes related to intima-media thickening that leading to vascular dysfunction.[9] Another mechanism of vascular dysfunction is the activation of renin-angiotensin-aldosteron (RAAS) system, especially angiotensin II, which has a dominant role

in insulin resistance.[10] Chronic hyperglycemia increases oxidative stress, angiotensin II, endothelial damage,[11] and inflammation response.[12]

*Centella asiatica* leaf is empirically used as a traditional medicine and was previously reported to decrease oxidative stress and inflammatory mediators.[13,14] The effect of *C. asiatica* extract in obese-diabetic rats for 4-week showed increase insulin production, decrease blood glucose, and improve lipid metabolism.[15] A study *in vitro* showed that ethanol extract of *C. asiatica* inhibits angiotensin-converting enzyme activity that related to resistance insulin.[16] This leaf contains some active substances such as madecassoside, asiaticoside, and asiatic acid.[17] Madecassoside has anti-inflammation activity which possibly associated with protection ability on myocardial ischemia-reperfusion injury.[18] Asiaticoside might has anti-inflammation effect through inhibiting overactivation of p38 MAPK pathway related to neuroprotective effect against transient cerebral ischemia and reperfusion.[19] Asiatic acid was reported to decrease inflammatory mediator on a previous study[20] and suggested that anti-hypertensive effect might be associated with its ability to alleviate RAS overactivity and downregulation of eNOS expression.[21]

The positive control group was given 50 mg/kg/day captopril. The previous study showed captopril prevent cardiovascular event in hypertensive patients with diabetes.[22] Captopril has RAAS blockade effect and improves insulin sensitivity compared to other antihypertensive treatments.[10] Some researches on animal models of diabetes prove that captopril may improve endothelial dysfunction,[23] decrease oxidative stress,[23,24] prevent changes in aortic reactivity,[25] inhibit the degeneration and early inflammation of diabetic retinopathy,[26] improve ventricular function and myocardial structure,[27] prevent diabetic nephropathy and neuropathy,[28] and decrease mean arterial blood pressure and left ventricular wall thickness.[29]

The mechanism of *C. asiatica* on vascular changes is not fully understood. Therefore, the study was aimed to explore the effect of *C. asiatica* extract on intima-media thickness, eNOS expression, and macrophage M1/M2 ratio in diabetic rats aorta.

## MATERIALS AND METHODS

### Animals

Procedures involving animals and their care were handled according to the Institutional Guideline of Medical Faculty Universitas Gadjah Mada and with approval from the Ethics Committee of Universitas Gadjah Mada (No. KE/FK/1215/EC/2016). Male Wistar rats, weighing 180 ± 20 g (10 ± 2 weeks old), were obtained from Pharmacology Animal Laboratory, Medical Faculty, Universitas Gadjah Mada. Rats were adapted for 7 days and housed under controlled environmental conditions on 12 h light/dark cycle at 24–26°C and humidity 70–80% with standard pellet diet and water *ad libitum*. Wistar rats were divided randomly into normal group (without diabetic induction) and diabetic group which consisted of negative control (receive only aquadest), positive control (captopril 50 mg/kg/day),[25] and *C. asiatica* extract (dose 250, 500, and 1000 mg/kg/day).[30] Total Wistar rats involved in the study amounted to 50 Wistar rats. During the experiment, 9 rats died and 17 rats did not develop diabetic condition. There were 24 rats survived until the end of treatment ($n$ = 4 rats pergroup).

### *C. asiatica* Extract

Standardized *C. asiatica* extracts containing madecassoside, asiatic acid, and asiaticoside as active constituents were produced by Javaplant PT, Tri Rahardja, on April 2016. Extraction was done using water solvent and handled in accordance with good industrial hygiene and safety practice. The *C. asiatica* leaves soaked in water at boiling point for 5 h while stirring with magnetic stirrer then filtered to get extract. Freeze dry method was used to make the extract became dry. The dried powder extracts were kept in dry place (desiccator with silica gel) after opened. Shortly before use, the extract was dissolved in aquadest.

### Diabetic Induction

Diabetes was induced by an intraperitoneal injection of 120 mg/kg nicotinamide in normal saline, 15 min before intraperitoneal injection of 60 mg/kg streptozotocin (STZ) in buffer citrate solution.[31] 7 days after induction, the diabetic rats with fasting blood glucose levels more than 140 mg/dl were selected for the experiment.[31] Diabetic rats were divided randomly into negative control group (receive only aquadest), positive control group (captopril 50 mg/kg/day), and *C. asiatica* extract group (dose 250, 500, and 1000 mg/kg/day). The treatment was done for 8 weeks.

### Euthanasia and Aorta Collection

The experimental animals involved in the study amounted to 50 rats. Wistar rats were divided randomly into normal group (5 rats without diabetic induction) and diabetic induction group (45 rats). 7 days after induction, 17 rats did not develop diabetes condition and were excluded from the study. The others (28 rats) develop diabetes condition and categorized as diabetic group. Diabetic group was divided randomly into negative control group (receive only aquadest), positive control (captopril 50 mg/kg/day) group, and *C. asiatica* extract (dose 250, 500, and 1000 mg/kg/day) group. During the experiment, 9 rats died and 24 rats survived until the end of treatment ($n$ = 4 rats pergroup).

All rats ($\Sigma n$ = 24) were euthanized with intraperitoneal injection of anesthesia combination cocktail (contains ketamine, xylazine, and acepromazine) 0.2 ml/200 g,[32] and aorta were collected. The thoracic aorta placed in a tube containingRNA later solution (Ambion, AM7020) before RNA extraction. The abdominal aorta placed in neutral-buffered formalin solution for histology preparation with hematoxylin-eosin (HE) staining.

The thoracic aorta is a part of aorta which located in posterior mediastinal cavity and starts after the descending aorta and runs down until the aortic hiatus in the diaphragm. The abdominal aorta is a direct continuation of the thoracic aorta which starts at the level of diaphragm, crossing it via the

aortic hiatus, and runs down the posterior wall of abdomen until the branch of next artery.

## Histopathological Analyses

The abdominal aorta was placed in neutral-buffered formalin solution, embedded in paraffin, and then stained with HE. Aortic intima-media thickness was examined by measuring the distance between intima and media tunica in HE-stained aortas image (capture at 400× magnification) using free software ImageJ. Measure value was averaged of 10 random points of each examined aorta.

## mRNA Expression

Extraction of RNA was performed using the FavorPrep™ Tissue Total RNA Purification Mini Kit (Favorgen, FATRK001-1). The RNA product is stored at −80°C before used for synthesis cDNA. Synthesis cDNA using a highcapacity cDNA reverse transcription kit (Applied Biosystems, LT-22041) and continued polymerase chain reaction (PCR) under conditions of 25°C for 10 min, 37°C for 120 min, 85°C for 5 min, and 4°C for 10 min. The cDNA product is stored at −20°C. Amplification of cDNA was performed using Go Taq® Green Master Mix (Promega, M7122) and primary for each gene.

Rat GAPDH, forward primer 5'-TGG GAA GCT GGT CAT CAA C-3', reverse primer 5'-GCA TCA CCC CAT TTG ATG TT-3'; product 78 bp (the PCR conditions were as follows 94°C for 2 min; 30 cycles of 95°C for 30 s, 55°C for 30 s, 72°C for 1 min; 72°C for 5 min; and 4°C for 10 min). Rat eNOS, forward primer 5'-CCG GCG CTA CGA AGA ATG-3 ', reverse primer 5'-AGT GCC ACG GAT AA TAT-3'; product 78 bp (the PCR conditions were as follows 94°C for 5 min; 35 cycles of 94°C for 1 min, 56°C for 1 min, 72°C for 1 min; 72°C for 10 min; and 4°C for 10 min).

Rat CD11c (M1 marker), forward primer 5'-GAA GCT CAC GTG CAT GTT GT -3 ', reverse primer 5'-GCT ACA ATT GGG ATG ATG TCG -3'; product 68 bp (the PCR conditions were as follows 94°C for 5 min; 35 cycles of 94°C for 1 min, 57°C for 1 min, 72°C for 1 min; 72°C for 10 min; and 4°C for 10 min). Rat CD206 (M2 marker), forward primer 5'-GAC AGA TA GAA CAA GCA TTC C-3', reverse primer 5'-TGA ACTA TCT GAG AGT CCT GTC-3'; product 83 bp (the PCR conditions were as follows 94°C for 5 min; 35 cycles of 94°C for 1 min, 55°C for 1 min, 72°C for 1 min; 72°C for 10 min; and 4°C for 10 min).

The PCR products were run on 2% agarose gel with a voltage 100 V for 25 min. Gel electrophoresis densitometry was analyzed using ImageJ. The eNOS mRNA expression was calculated by comparing eNOS to GAPDH (housekeeping gene) expression. Macrophage M1/M2 ratio was the comparison between macrophage type 1 (M1, expressed by CD11c gene) and macrophage type 2 (M2, expressed by CD206 gene).

## Data Analyses

Data normality test was done using the Shapiro–Wilk test. The mean of the data was analyzed using one-way ANOVA test or Kruskal–Wallis test depending on the normality of the data. Differences with $P < 0.05$ were considered statistically significant.

# RESULTS

## Aortic Intima-media Thickness

Aortic intima-media thickness was calculated by measuring the distance between intima and media tunica in images of HE-stained aorta (capture at 400× magnification) using free software ImageJ. Measure value was averaged of 10 random points of each examined aorta and four rats per group were analyzed. After treatment for 8 weeks, aortic intima-media thickness on extract dose 500 and 1000 mg/kg/day groups was lower than negative control and did not give any significant result compared with positive control [Figure 1].

## The mRNA Expression of eNOS

The eNOS mRNA expressed by eNOS was compared to GAPDH mRNA expression based on calculation of the PCR product. The eNOS mRNA expression did not give any significant results ($P > 0.05$) among all groups [Figure 2].

## Macrophage M1/M2 Ratio

Macrophage M1/M2 ratio was a comparison between macrophage type 1 (M1, expressed by CD11c gene) and macrophage type 2 (M2, expressed by CD206 gene). After treatment for 8 weeks, M1/M2 ratio on extract dose 1000 mg/kg/day group was lower than negative control and did not give any significant result compared with positive control [Figure 3].

# DISCUSSION

The result in the present study shows that diabetic condition for 8 weeks induces intima-media thickening. It is supported by some previous study that proves intima-media thickening process which involves collagen deposition[33] and reduced elastin fiber,[34] perivascular fibrosis,[35] extracellular matrix accumulation,[36] smooth muscle cell proliferation, and intima hyperplasia.[37] Aortic intima-media thickness on positive control, extract dose at 500 and 1000 mg/kg/day, was significantly different than negative control. This is in accordance with the results of several previous studies. Captopril may provide protection against vascular morphology changes and endothelial function,[38] prevent perivascular fibrosis,[39] and reduce recruitment of proatherogenic immune cells.[40] The previous study on cultured fibroblast cell showed that the extract of *C. asiatica* has antifibrosis effect possibly due to its ability to decrease the expression of transforming growth factor β1 (TGF-β1), collagen 1 type 2, and type 3 collagen 1.[41] Furthermore, other study on madecassoside which is one of the active compounds of *C. asiatica* showed antifibrotic effect by preventing deposition of extracellular matrix in a model of pulmonary fibrosis by inhibiting TGF-β1.[42]

Other variable examined in the study was eNOS mRNA expression. Chait and Bornfeldt explain that hyperglycemic conditions in diabetes trigger accumulation of diacylglycerol and activation of protein kinase C that increased nuclear factor κB (NF-κB) activation, inflammatory response, and reduced NO production.[43] Chronic inflammation triggers the

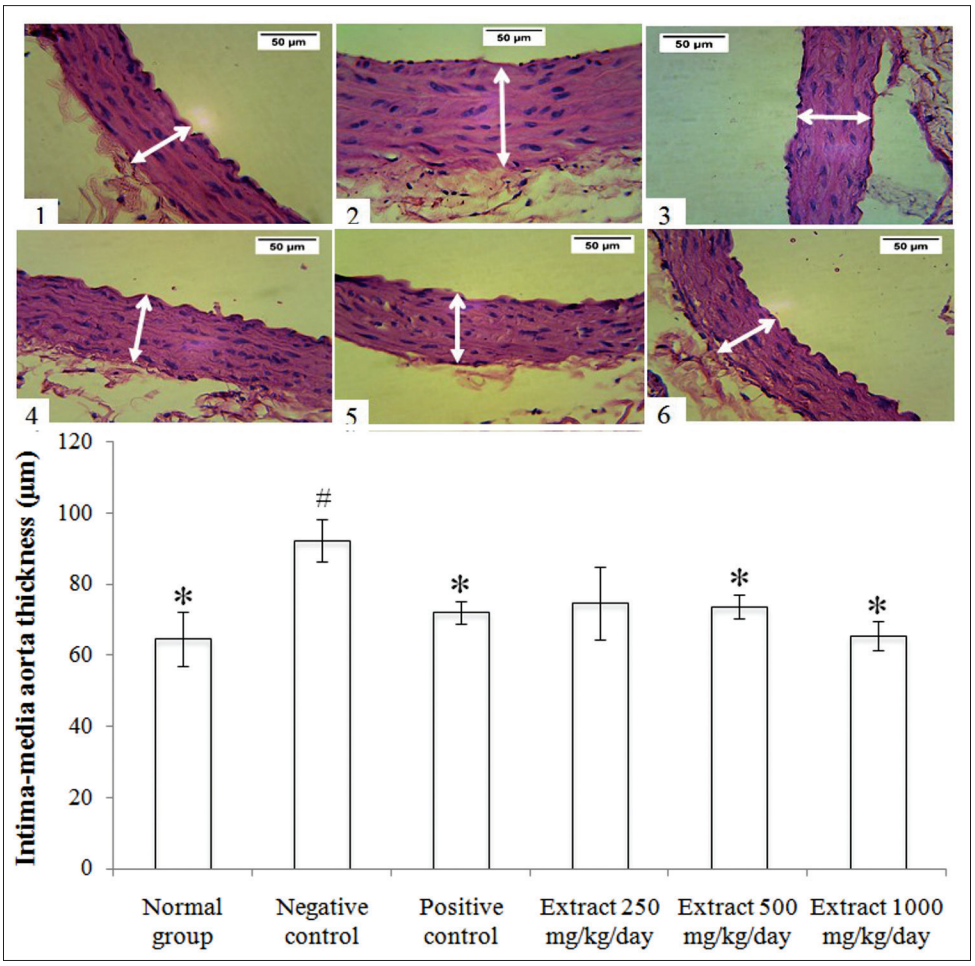


**Figure 1:** Representative picture of histology aorta with hematoxylin-eosin staining ×400 magnification on (1) normal group, (2) negative control, (3) positive control, (4) extract 250 mg/kg/day, (5) extract 500 mg/kg/day, (6) extract 1000 mg/kg/day. White arrows indicate intima-media thickness. The aortic intima-media thickness after treatment for 8 weeks. Values are expressed as means ± standard deviation, $n = 4$ rats pergroup. One-way ANOVA test ($P < 0.05$) followed by Tamhane *post-hoc* test. *$P < 0.05$ versus negative control; #$P < 0.05$ versus positive control

formation of oxidative stress.[7] Oxidative stress inhibits eNOS which plays a role in producing NO.[8]

The extract of *C. asiatica* in this study did not give any significant result in eNOS mRNA expression. However, not only on *C. asiatica* extract group but also the expression of eNOS mRNA showed no significant result between all groups. The result differs from other studies which show that administration of asiatic acid may increase the expression of eNOS protein on aorta[44] and mesenteric arteries.[20] We suggest that, in our study, the eNOS mRNA expression was not disturbed; however, the eNOS function might be affected. Previous studies have suggested that in STZ-induced DM, Wistar rat occurs uncoupling eNOS phenomenon in which the expression of eNOS was normal despite its function is impaired.[45-47] The eNOS uncoupling is a functional impairment phenomenon that eNOS being the source of $O_2^-$ production.[48] It may due to the electron transfer process to molecular oxygen at uncoupled eNOS.[46]

The last variable, result of macrophage M1/M2 ratio proved that there was a significant difference in normal group compared to negative control group. This result is in accordance with the results of previous research. Increased expression of CD11c (marker M1) and CD11c/CD206 ratio (marker M1/M2) is associated with insulin resistance in DM.[49] Increased M1/M2 ratio proves an imbalance between pro-inflammatory and anti-inflammatory mediators leading to the dominance of inflammatory response.[50] Inflammation and macrophage polarization involves in the progression of cardiovascular disease.[51] The macrophage M1/M2 ratio in positive control and extract dose 1000 mg/kg/day are lower than the negative control. The previous study in hypertensive model rats showed that captopril may decrease the inflammatory mediator by inhibition of NF-κB.[52] In experimental autoimmune myocarditis (EAM) model rats, captopril may inhibit EAM progression and decrease the cell-mediated inflammatory response.[53]

The extract of dose 1000 mg/kg/day showed no significant result based on the macrophage M1/M2 ratio compared with the positive control. This proves that the effect of *C. asiatica* dose 1000 mg/kBB/day may be prevented the increase of macrophage M1/M2 ratio. Previous research proves that madecassoside which is one of the active compounds of *C. asiatica* has a cardioprotective effect by suppressing inflammatory mediators.[18] While asiatic acid may inhibit the expression of inducible nitric oxide synthase,

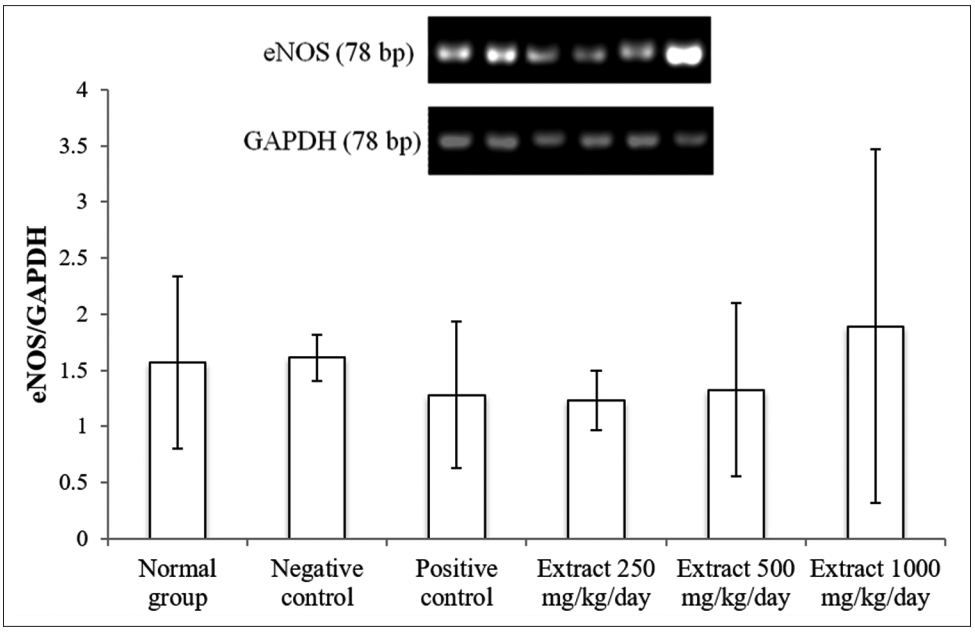


**Figure 2:** Representative picture of endothelial nitric-oxide synthase mRNA expression. Values are expressed as means ± standard deviation, $n = 4$ rats per group. Kruskal–Wallis test ($P > 0.05$)

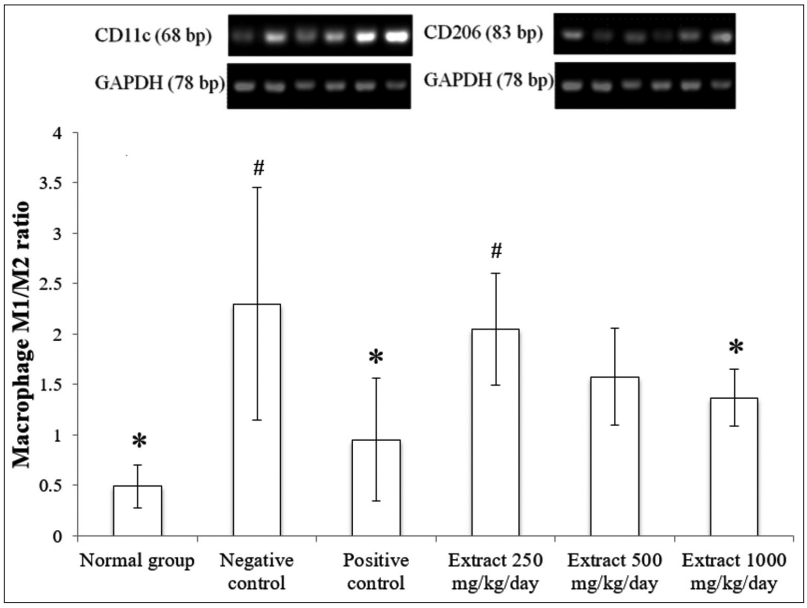


**Figure 3:** Representative picture of M1/M2 ratio. Values are expressed as means ± standard deviation, $n = 4$ rats per group. Kruskal–Wallis test ($P < 0.05$), followed by Mann–Whitney *post hoc* test. $^{*}P < 0.05$ versus negative control; $^{\#}P < 0.05$ versus positive control

cyclooxygenase-2, interleukin (IL)-6, IL-1β, and tumor necrosis factor alpha (TNF-α) which are pro-inflammatory agents by inhibition of NF-κB activation.[54]

Dose of *C. asiatica* extract required to prevent thickening of the aortic intima-media differs from dose to prevent an increase in the macrophage M1/M2 ratio. Dose of 500 mg/kg/day has an effect to prevented intima-media thickening, whereas a dose of 1000 mg/kg/day is the necessary dose to prevent an increase in macrophage M1/M2 ratio. We suggest that other mechanisms other than inflammation might play a role in intima-media thickening. These mechanisms might include oxidative stress, angiotensin II, endothelial damage, proliferation and smooth muscle cell migration, increased collagen fibers, intima tunica proliferation,[9] and extracellular matrix accumulation[8] as have been explained by the previous study.

## CONCLUSIONS

We conclude that administration of *C. asiatica* extract dose of 500 and 1000 mg/kg/day may prevent aortic intima-media thickening. Dose of 1000 mg/kg/day may prevent the increased of macrophage M1/M2 ratio in diabetic rats aorta. Those results suggest that *C. asiatica* extract has anti-inflammatory effect on prevented aortic intima-media thickening on diabetic

rats. However, more studies have to be done to be able to use *C. asiatica* as a vasculoprotective agent in DM.

## ACKNOWLEDGMENTS

We thank to Mr. Nurhadi, Mr. Suroso, and Mr. Wagimin for the technical assistance. This study was supported by a grant from research fund of Indonesia higher education ministry.

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