

## Total flavonoid content and *in vivo* hypotensive effect of chloroform insoluble fraction of *Centella asiatica* leaf extract

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### Abstract

Ministry of Health of Indonesia have developed an antihypertensive Jamu formula that contains *Centella asiatica* leaf through a program namely “Evidence based Jamu Development”. Reportedly, the ethyl acetate and chloroform fractions of *C. asiatica* leaf exhibited *in vivo* antihypertensive activity. The study aimed to determine the total flavonoid content in chloroform insoluble fraction of *C. asiatica* leaf extract (CIFCA) and to evaluate the *in vivo* hypotensive effect on phenylephrine-induced hypertensive rats by non-invasive tail-cuff method. The results showed that CIFCA contained the total flavonoid of 1.19±0.01% which was equivalent to quercetin. This flavonoid fraction at dose of 50 mg/kg showed a potent *in vivo* hypotensive effect by lowering blood pressure up to 150% on phenylephrine-induced rats. The ED<sub>50</sub> values, a parameter of drug potency, of these effects on systolic, diastolic, and mean arterial blood pressure were 27.7 ± 1.52; 29.50 ± 1.61; and 27.76 ± 1.08 mg/kg, respectively. In conclusion, chloroform insoluble fraction of *C. asiatica* leaf extract is potential to develop as an antihypertensive agent.

### Keywords

*Centella asiatica*

Flavonoid

Hypotensive effect

Quercetin

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### Introduction

To date, hypertension was the top rank of global risks for mortality in the world (WHO, 2009). Recently, estimated 15 million people in Indonesia suffer from hypertension. Prevalence of hypertension in urban and rural areas range between 17-21%. Only 4% of the cases that can be controlled. Seven of ten patients were not getting good treatment, therefore several serious complications developed such as renal failure, stroke, and coronary heart disease (Rahajeng and Tuminah, 2009). Indonesia Health Profile which was reported by Health Department in 2008 showed that Case Fatality Rate for stroke, hypertension, diabetes mellitus and cancer were 72.3%, 31.7%, 7.38%, and 0.43%, respectively. This degenerative disease is estimated at 4.5% of total cases of global disease (WHO and ISH, 2003).

One of Indonesia medicinal plant was *Centella asiatica* (L.) Urban or pegagan, a perennial herb from the familia Apiaceae, has been reported to possess various pharmacological effects, including the ability to lower the blood pressure, diuretic, venous

disorders, blood cleanser, wound healing, and often referred to as a rejuvenating medicament in Ayurvedic Pharmacopoeia (Jaganath and Ng, 1999; Sudarsono *et al.*, 2002). Since 2010 Indonesia government developed a program namely “Scientification of Jamu” including antihypertensive formula that contained *C. asiatica* leaf. The major compound of *C. asiatica* was triterpenoids included asiaticoside, madecassoside, asiatic acid, and madecassic acid (James and Dubery, 2011). However flavonoid, glycosides, alkaloids, steroids, volatile and fatty oils were also found in this plant and exhibited various biological activities (Subban *et al.*, 2008).

Reportedly, *Centella asiatica* leaf consisted of high concentration of flavonoid (Zainol *et al.*, 2009). Most of the flavonoid showed *in vitro* inhibitory activity on Angiotensin-Converting Enzyme (ACE) and antihypertension effects in rats isolated-aorta (Balasuriya and Rupasinghe, 2012; Nugroho *et al.*, 2013). Chloroform and ethyl acetate fractions of *C. asiatica* leaf were also reported to have hypotensive effect in hypertensive animal models (Khuzaimah, 1997; Harwoko *et al.*, 2014). However, chloroform

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insoluble fraction of *C. asiatica* leaf (CIFCA) was also interested to be investigated for its *in vivo* hypotensive effect and its flavonoid content. Previously, CIFCA was studied for its flavonoid-rich fraction by fractionation of *C. asiatica* ethanolic extract with chloroform (Harwoko *et al.*, 2012). Determination of total flavonoid content was conducted by colorimetric assay based on the modified method of Chang *et al.* (2002). Phenylephrine was used to increase blood pressure acutely in rats as disease animal model (Rordorf *et al.*, 1997). Cardiovascular parameters such as systolic blood pressure (SBP), diastolic blood pressure (DBP), mean arterial pressure (MAP), and heart rate (HR) were measured by non-invasive tail-cuff method (Maruyama *et al.*, 2009).

## Materials and Methods

### Materials

*Centella asiatica* was collected during March 2012 from Medicinal Plant and Traditional Medicine Research and Development Centre Karanganyar, Solo Indonesia. The plant was authenticated at Department of Pharmaceutical Biology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The voucher specimen was stored in a herbarium of the department. The chemicals included 70% ethanol (Bratachem, Indonesia), methanol (Merck, Germany), chloroform (Merck, Germany), phenylephrine-HCl (Sigma, USA), captopril (Sigma, USA), quercetin (Sigma, USA), citroboric reagent (0.5 g of boric acid and 0.5 g of citric acid were dissolved in 50 mL of ethanol) and  $\text{AlCl}_3$  purchased from Merck & Co., Germany.

### Animals

Male Wistar rats weighing 200-300 g were obtained from Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. All animals were fed with standard rodent chow and water *ad libitum*. The rats were acclimatized and quarantined for at least one week before the experiment. The animal handling protocols were performed in accordance with the previous study (Harwoko *et al.*, 2014), approved by Research Ethics Committee, Integrated Research and Testing Laboratory Universitas Gadjah Mada, Indonesia (certificate number of ethic clearance: No. 120/KEC-LPPT/X/2013).

### Extraction and fractionation

Extraction and fractionation methods of *C. asiatica* leaves were based on previous study (Harwoko *et al.*; 2014). The viscous ethanolic

extract of *C. asiatica* (EECA) was fractionated for yielding chloroform fraction (CFCA) and chloroform insoluble fraction (CIFCA), then was concentrated by a rotary vacuum evaporator.

### Identification of flavonoid in CIFCA

All samples (CFCA, CIFCA, and quercetin) were spotted on silica gel 60 F254 plate and developed in n-butanol: glacial acetic acid: water (BAW 4:1:5 v/v, upper phase). Subsequently, the samples were sprayed with citroboric reagent. These spots were observed under UV<sub>366</sub> light, then its *R<sub>f</sub>* values were determined. Based on the TLC profile, CIFCA was proved as a flavonoid -rich fraction and was ensured to be separated from CFCA whose triterpenoid-rich.

### Determination of total flavonoid

Total flavonoid content was determined based on modified colorimetric method of Chang *et al.* (2002) and had been validated by Mujahid (2011) using quercetin as a reference standard. Quercetin (10 mg) were dissolved in 10 mL methanol, then diluted to provide concentrations series (6; 7; 8; 9 and 10 mg/100 mL). Either 0.5 mL CIFCA or quercetin was added with 1.5 mL of methanol, 0.1 mL of 10%  $\text{AlCl}_3$ , 0.1 mL potassium acetate 1M and 2.8 mL distilled water, and then incubated for 30 minutes. The absorbance was measured in 415 nm wavelength, and the blank that used was distilled water with 10%  $\text{AlCl}_3$  and 1M potassium acetate. Total flavonoid content was expressed in mg quercetin equivalent (QE) of each 100 mg of sample dry weight.

### In vivo hypotensive assay

As many as 35 male Wistar rats were grouped into 7 treatment groups, each group consisted of 5 rats. Group 1-3, were normal control (0.5% per oral CMC-Na.), negative control (phenylephrine 0.9 mg/kg subcutaneous), and positive control (2.5 mg/kg per oral captopril), respectively. Groups 4 to 7 were orally administered with CIFCA at doses 25, 50, 100 mg/kg, and EECA at dose of 400 mg/kg, respectively. Groups 2 to 7 were also given subcutaneous injection of phenylephrine at dose of 0.9 mg/kg at 30 minutes after oral administration of a single dose of drug treatment. The blood pressure were measured in conscious rats and recorded by non-invasive tail-cuff method. Systolic blood pressure (SBP) before being induced by phenylephrine was stated as a basal blood pressure ( $\text{BP}_0$ ). If  $\text{SBP}_0$  was  $\leq 130$  mmHg or normotensive, the rats were treated with the drug or vehicel immediately, then 30 minutes later were induced by phenylephrine. The blood pressure was measured again after achieving the onset ( $\text{BP}_1$ ) and

the duration of phenylephrine effect (BP<sub>2</sub>).

*Data analysis*

The data were presented as mean ± the standard error of mean (SEM). In the *in vivo* study, the response was stated as antihypertensive capacity percentage (AHCP) which formulated as below:

$$\% \text{ AHCP} = \frac{(P_{\text{phe}} - P_{\text{tre}})}{(P_{\text{phe}} - T_{\text{nor}})} \times 100\% \dots\dots\dots \text{Eq. (1)}$$

Explanation:

- P<sub>phe</sub>: blood pressure difference in phenylephrine groups
- P<sub>tre</sub>: blood pressure difference in treatment groups
- P<sub>nor</sub>: blood pressure difference in normal control groups

Then the ED<sub>50</sub> value was determined by non-linear regression analysis from logarithmic of doses-response curve with following formula (Kenakin, 1997):

$$\text{Log ED}_{50} = \left[ \frac{50 - Y_1}{Y_2 - Y_1} \times (X_2 - X_1) \right] + X_1 \dots\dots\dots \text{Eq. (2)}$$

Explanation:

- X<sub>1</sub>: A logarithmic value of dose that produce the response of upper 50%
- X<sub>2</sub>: A logarithmic value of dose that produce the response of under 50%
- Y<sub>1</sub>: % response of upper 50%
- Y<sub>2</sub>: % response of under 50%

Cardiovascular parameter data were analyzed statistically using one-way analysis of variance (ANOVA) followed by least significant difference (LSD) test. The P-values less than 0.05 were considered significant.

**Results and Discussion**

*Extraction and fractionation*

In the present study, extraction of one kg *C. asiatica* dry leaves with 70% ethanol yielded 206.56 g of a viscous ethanolic extract (20.66%) which is accordance with requirements of Indonesian Herbal Pharmacopoeia (Department of Health Republic of Indonesia, 2008). The fractionation process yielded chloroform insoluble fraction with characteristics of viscous and yellowish brown color. The process provided 14.05 g or 87.81% rendement calculated from ethanolic extract. The rendement of CIFCA was more than that of chloroform fraction (CFCA) due to the polar compounds in *C. asiatica* leaves higher than non polar ones.

*Identification and quantification of flavonoid content*

Based on the chromatogram, the chloroform

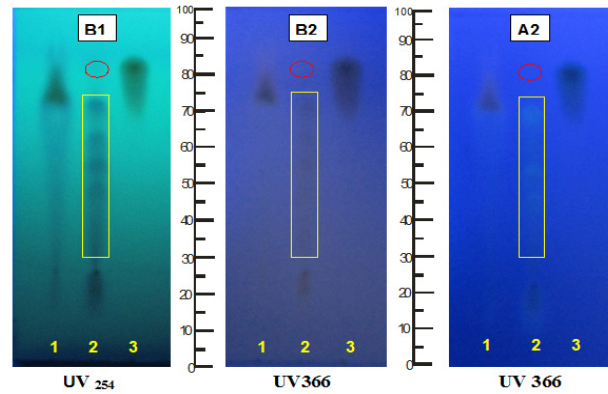


Figure 1. The chromatogram profiles of CFCA (1), CIFCA (2), and quercetin (3) on UV<sub>254</sub>-UV<sub>366</sub> before (B1, B2) and after (A2) sprayed by citroboric reagent. The spots of CIFCA (2) indicated the presence of quercetin (red circle) and another flavonoids content (yellow square)

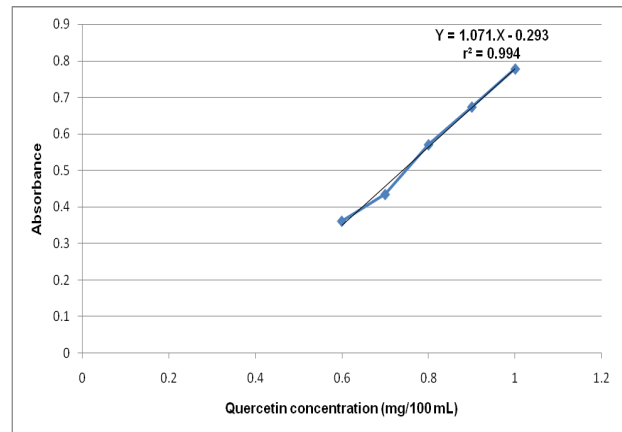


Figure 2. Linear curve of quercetin

insoluble fraction (CIFCA) exhibited a clear separation when developed using a mobile phase of BAW (4:1:5 v/v). The TLC profile exhibited some spots that have values of hRf 35; 41; 50; 58; 69 with brownish yellow fluorescens under UV<sub>366</sub> after sprayed by citroboric reagent. While quercetin standard spot showed a yellowish brown fluorescens (hRf 80) under UV<sub>366</sub> after sprayed by this reagent. In TLC profile of CIFCA, hRf 40-70 whose yellow fluorescence showed higher intensity of flavonoid (Figure 1). However, at this range CFCA did not exhibit any yellow fluorescens spot, and one red spot at hRf of 71 is likely impurity.

In the previous study, CFCA did not contain many flavonoid as reported by Rachmawati *et al.* (2011). The chloroform fraction of ethanol extract of *C. asiatica* contained terpenoids and phenol compounds, however did not contain flavonoid. This result was similar to our preliminary study that chloroform fraction consisted of high level of triterpenoid and chloroform insoluble fraction consisted of high level of flavonoid (Harwoko *et al.*, 2012). Flavonoid in the *C. asiatica* are flavons and flavonons group that do



Table 1. Percentage of total flavonoid content in the CIFCA

Concentration (mg/100 mL)	Absorbance (n) ( $\lambda$ 415 nm)	Total flavonoid content (QE % b/b)
100	0.478	1.20
	0.471	1.19
	0.473	1.19
Mean $\pm$ SEM		1.19 $\pm$ 0.01

QE = Quercetin Equivalent

not contain free 5-OH, and the others are flavonols group i.e quercetin and rutin without free 5-OH but substituted at the 3-OH that caused less in polarity (Fatmawati, 2005).

#### Determination of the total flavonoid content

Flavonoid are the most compounds of plant phenolics. Reportedly, *C. asiatica* leaf consisted of high concentration of flavonoid including quercetin, rutin, naringin, kaempferol, myricetin, apigenin, luteolin, and catechin (Zainol *et al.*, 2009; Andarwulan *et al.*, 2010). In this study, total flavonoid content was determined by Chang *et al.* (2002) validated by Mujahid (2011) using quercetin as a reference standard. Principally, the procedure is related to the formation of complex between flavonoid and  $AlCl_3$  that produces a yellow coloured solution. The absorbance is measured spectrophotometrically at maximum wavelength of 415 nm. The total flavonoid content was equivalent to quercetin in milligram per gram dry material of the fraction. The absorbances of a series concentrations of quercetin were plotted to their concentration to yield a linear calibration curve of quercetin ( $y = 1.071x - 0.293$ ) with coefficient of correlation ( $r^2$ ) value of 0.9941 (Figure 2).

In the present study, total flavonoid content of CIFCA was  $1.19 \pm 0.01\%$ . It means that each 100 g dry weight of CIFCA contained total flavonoid equivalent to quercetin of  $1.19 \pm 0.01$  g (Table 1). This total flavonoid content was higher than this of ethanolic extract without fractionation. In previous study, the leaves were extracted without fractionation yielding total flavonoid content of  $3.5 \pm 0.1$  mg/100 g extract. The sample (CIFCA) showed a small quantity of quercetin because *C. asiatica* contains some type of flavonoids (Fig. 1). The highest flavonoids content had been reported in *C. asiatica* leaf was naringin, followed by quercetin, catechin, and rutin. The others, include luteolin, apigenin, and kaempferol were inconsistently present in some parts of *C. asiatica* (Zainol *et al.*, 2009).

The major constituents in *C. asiatica* are triterpenoids such as asiatic acid, madecassic acid, asiaticoside and madecassoside that have effects in wound healing, memory enhancement, and

antidepressant (WHO, 1999; Chen *et al.*, 2005; Hashim *et al.*, 2011). To date, flavonoids regarded as the main contributors to the antioxidant activity exhibited by the plants (Mustafa *et al.* 2010). *Centella asiatica* extract is one that showed free radical scavenging activity (Hashim *et al.*, 2011). Each gram of *C. asiatica* ethanolic extract has antioxidant activity equal to  $43.198 \pm 2.048$  mg of quercetin (Salamah and Farahana, 2014). In addition, Pittella *et al.* (2009) reported that the aqueous extract of *C. asiatica* contained flavonoid (0.361 g/100 g) which showed the radical scavenging activity by the  $IC_{50}$  value is 31.25  $\mu$ g/mL.

#### Profile of blood pressure on phenylephrine-induced hypertensive rats

Phenylephrine is an agonist of alpha-1 adrenergic receptor in vascular smooth muscle. Activation of this receptor could increase intracellular calcium that caused increasing in vascular smooth muscle contraction. Furthermore, vasoconstriction causes rising in peripheral resistance so that blood pressure would increase. Based on the preliminary study, profile of phenylephrine in increasing rat blood pressure indicated that the onset of phenylephrine was 15-30 minutes and duration of effect was 1 hour. These results are inline with the pharmacokinetic data of phenylephrine hydrochloride which has onset of 10-15 minutes and duration of 1 hour after subcutaneous injection (Lacy *et al.*, 2013).

In the study, the control group that only receive phenylephrine induction exhibited an increase in SBP (25 mmHg), DBP (15-20 mmHg), and MAP (18-22 mmHg). This increase was as same as in L-NAME induced rats model (5-25 mmHg) or two kidney one clip hypertensive rats (20 mmHg) (Monassier *et al.*, 2006). Normal control group administered with aquadest showed that the average change in SBP (-1,6 to 2,4 mmHg), DBP (0.2 to 1.8 mmHg), and MAP (-0,2 to 2,2 mmHg) that is stated as the normotensive group.

#### Effect of treatment on cardiovascular parameters

Blood pressure changes were presented in Figure 3. The profiles of blood pressure changes of both treatment and normal control groups were significant different in comparison to that of negative control. When compared to negative control, the CIFCA treatment at doses of 50 and 100 mg/kg was significantly different ( $p < 0,05$ ), but at dose of 25 mg/kg was not significantly different ( $p > 0,05$ ). The phenylephrine mildly decrease the heart rate (6%), while lower dose of CIFCA can increase the heart rate until 50%. However high doses of CIFCA and

Table 2. The percentage of decrease in blood pressure in treatment groups after phenylephrine-induced

Treatment Groups	% of decrease in blood pressure (Mean±SEM)		
	SBP	DBP	MAP
CIFCA-25	29.22±12.20	45.98±28.00	52.00±17.25
CIFCA-50	62.50±9.26	72.41±16.92	70.00±10.79
CIFCA-100	95.31±16.69	109.20±24.12	52.00±17.25
EECA-400	83.46±8.36	141.33±16.60	16.13±12.13
Captopril-2.5	74.44±6.66	86.67±22.45	83.87±12.88

\* $(p < 0,05)$  : The difference is significant compared to captopril group by LSD test

EECA at dose 400 mg/kg strongly decrease the heart rate as well as captopril (0,5-2%).

The percentage of decrease in blood pressure indicated the effect of each treatment group (Table 2). The CIFCA treatment at dose of 25 and 50 mg/kg showed blood pressure lowering effect that much different with captopril treatment. However the CIFCA at dose of 50 mg/kg has higher hypotensive effect on MAP and was not significantly different with captopril ( $p > 0,05$ ). The higher dose of CIFCA (100 mg/kg) and EECA at dose of 400 mg/kg were also not significantly different with captopril at dose of 2.5 mg/kg ( $p > 0,05$ ).

The results of this study may prove that CIFCA whose containing 1.2% of total flavonoid had potency and efficacy as antihypertensive agent. The hypotensive effect of CIFCA was started at dose of 25 to 100 mg/kg with gradual response. Thus, low-dose CIFCA could not inhibit the increase of blood pressure caused by phenylephrine. The CIFCA that enriched with flavonoid at dose of 50 mg/kg showed a fairly potent *in vivo* hypotensive effect by lowering blood pressure up to 150% on phenylephrine-induced rats. The ED<sub>50</sub> value of CIFCA hypotensive effect on SBP (27.7±1.52 mg/kg) as similar as its effect on DBP (29.50±1.61 mg/kg) and MAP (27.76±1.08 mg/kg). The doses were equivalent to 270-300 mg in 60 kg of human (Laurence and Bacharach, 1964) that can be used until 3 times a day under the lethal doses (Chivapat *et al.*, 2011). Most of the flavonoid showed *in vitro* hypotensive effect as Angiotensin-Converting Enzyme (ACE) inhibitors (Balasuriya and Rupasinghe, 2012) and exhibited antihypertension effects in rats isolated-aorta (Nugroho *et al.*, 2013). Quercetin supplementation (150 mg/d for 6 weeks) decreased SBP by 2-9 mmHg in the subgroup of hypertensive subjects and by 3-7 mmHg in the subgroup of younger adults aged 25–50 years (Egert *et al.*, 2009).

Most of the herbal physician in Java and Bali, Indonesia prescribed Indonesian herbal medicine in this decade. A total of 61.8% of physicians use

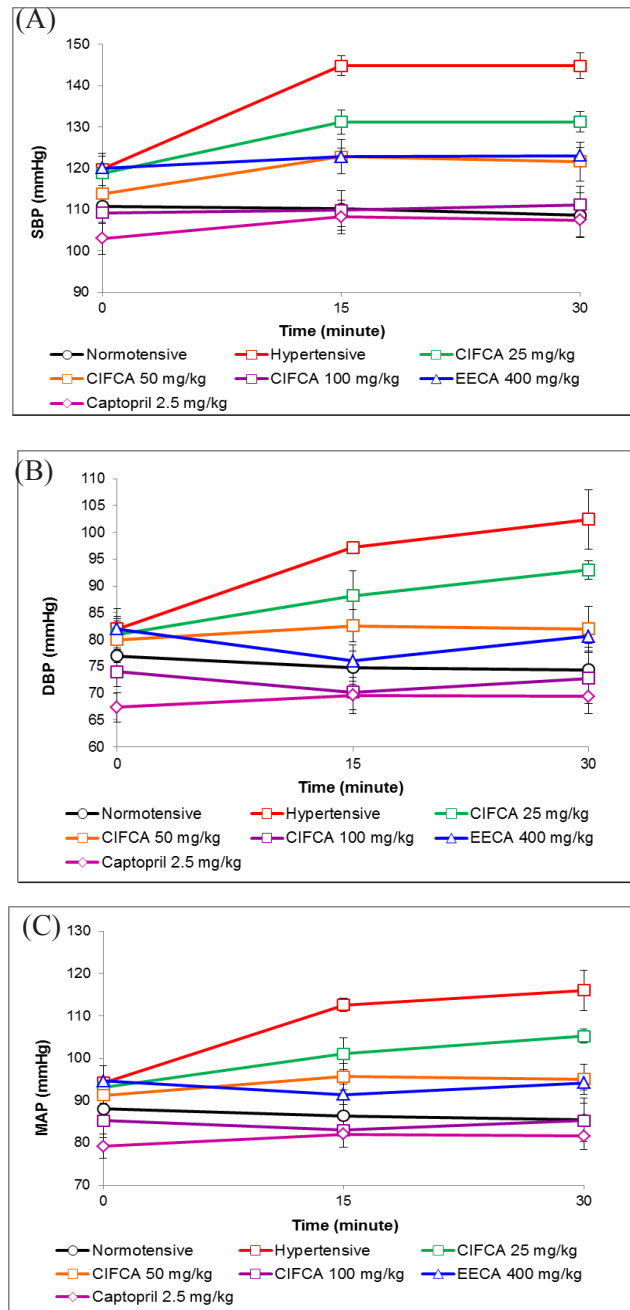


Figure 3. The changes in systolic blood pressure (A), diastolic blood pressure (B), and mean arterial pressure (C) during the measuring time in control groups (normotensive and hypertensive) and treatment groups (CIFCA 25; 50; 100 mg/kg, EECA 400 mg/kg, and captopril 2.5 mg/kg)

herbal medicine as complementary treatment of hypertension (Delima *et al.*, 2012). One of the prime plant that widely used in antihypertensive formula is *Centella asiatica* (L.) Urban, Apiaceae family, that well known as pegagan and has used for food, vegetable or traditional medicine. In addition, it is mostly used in herbal medicines industries as an extract raw material or traditional herb composition.

The present study reported that the flavonoid-rich fraction of *C. asiatica* (CIFCA) had *in vivo* hypotensive effect on phenylephrine-induced

hypertension rat model. Few mechanisms which support that *C. asiatica* as an antihypertensive including diuretic activity (Roopesh *et al.*, 2011), potential inhibitory against ACE (Loh and Hadira, 2011), and vasodilatation effects on rats isolated-aorta (Nugroho *et al.*, 2013). Flavonoid and phenolic compounds in *C. asiatica* have the major contribute to the antioxidant activity (Zainol *et al.*, 2003) and potential use in the management of cardiovascular diseases (Pang *et al.*, 2008). Biomarkers of ROS excess are increased in patients with hypertension and oxidative damage is important in the molecular mechanisms associated with cardiovascular and renal injury in hypertension (Montezano and Touyz, 2012). However, the detailed mechanisms and the long term outcome of hypotensive effect of this plant needs to be studied.

This study showed that *C. asiatica* leaf extract is potential to develop as a high blood pressure-lowering agent for hypertension patients. The present findings can also add information on the pharmacological activities of plants originated from southeast asia especially Indonesia. Southeast asia countries have the biggest biodiversity including medicinal plants that some of them are being conducted intensive studies (Nugroho *et al.*, 2011a; Nugroho *et al.*, 2011b; Nugroho *et al.*, 2012).

## Conclusion

Chloroform insoluble fraction of *C. asiatica* leaf (CIFCA) which contains higher content of flavonoid compounds exhibited hypotensive effect on phenylephrine-induced hypertensive rats. The ED<sub>50</sub> values, a parameter of drug potency, of these effects on systolic, diastolic, and mean arterial blood pressure were 27.7±1.52; 29.50±1.61; and 27.76±1.08 mg/kg, respectively.

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