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Triterpenoid-rich fraction of *Centella asiatica* leaves and *in vivo* antihypertensive activity

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Keywords

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Abstract

Antihypertensive herbs in scientification of "jamu" program in Indonesia contained *Centella asiatica* (L.) Urban. The leaf contains several compounds such as triterpenoids, flavonoids, phenolics, tannins, and resins. The total triterpenic fraction of *C. asiatica* could treat venous hypertensive microangiopathy, while ethyl acetate fraction of *C. asiatica* leaf has hypotensive effect in cats. This study aimed to provide triterpenoid-rich fraction of *C. asiatica* leaf, to analyze asiaticoside contents, and to examine the *in vivo* antihypertensive effect on phenylephrine-induced hypertensive rats by non-invasive tail-cuff method. The results showed that triterpenoid contents in chloroform fraction of *C. asiatica* (CFCA) were more dominant than the flavonoid/phenolic contents. TLC-densitometric data showed that asiaticoside contents of CFCA were 0.402 ± 0.02%. The CFCA showed antihypertensive effect on phenylephrine-induced hypertensive rats. The ED_{50} values, a parameter of drug potency, of these effects on systolic blood pressure, diastolic blood pressure, and mean arterial pressure were 10.40 ± 0.98, 9.05 ± 1.95, and 9.37 ± 1.69 mg/kg, respectively.

Introduction

The top rank of global risks for mortality in the world was high blood pressure (WHO, 2009). The basic health research 2007 conducted in Indonesia showed that hypertension prevalence based on measurement and disease history was 32.2%. However 75.8% of cases had not been diagnosed and reached yet by the health care system. Seven of 10 patients were not getting good treatment resulting in complications with renal failure, stroke, and coronary heart disease (Rahajeng and Tuminah, 2009). This failure was estimated to be 4.5% case of global disease (WHO and ISH, 2003).

Empirically, hypertension could be treated by traditional medicines (Koffi *et al*., 2009). Antihypertensive herbs in scientification of "jamu" program in Indonesia contained *Centella asiatica* (L.) Urban leaf. This plant contains several compounds such as triterpenoids (asiaticoside, madecassoside, asiatic acid, madecassic acid), glycosides, flavonoids, alkaloids, steroids, volatile and fatty oils (Subban *et al*., 2008; James and Dubery, 2011). Traditionally, people used *C. asiatica* in the treatment of venous disorders, diuretic and blood cleanser (Sudarsono *et al*., 2002). Clinical trials of *C. asiatica* extract had been done to venous insufficiency (WHO, 1999), whereas the total triterpenic fraction of *C. asiatica* was effective in chronic venous hypertensive and in protecting the venous endothelium (Incandela *et al*., 2001). The chloroform fraction of ethanol extract of *C. asiatica* had been reported as antibacterial agent (Rachmawati *et al*., 2011) and its triterpenoid could improve cognitive function in mice (Herlina and Hutasoit, 2011).

Reportedly, ethyl acetate fraction of *C. asiatica* leaf had hypotensive effect in cats (Khuzaimah, 1997), while its extract dose of 500 mg/kg had diuretic activity (Roopesh *et al.*, 2011). Further more, the diuretic effect might cause hypotensive effect. However, the active compounds of *C. asiatica* which have antihypertensive effect are unknown yet. Based on the facts, chloroform fraction of *C. asiatica* leaf (CFCA) was investigated for its in vivo antihypertensive effects and its asiaticoside contents. In phytochemistry, separation method by fractionation of *C. asiatica* ethanolic extract with chloroform was performed to provide triterpenoid-rich fraction. Based on Indonesian herbal pharmacopoeia, determination of asiaticoside content was conducted

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by TLC densitometry as standardization of quality (National Agency for Drug and Food Control, 2003; Department of Health Republic of Indonesia, 2008). The combination of TLC and densitometry was a new method has been developed to determine asiaticoside in crude extracts and commercial products (Chaisawadi and De-Eknamkul, 2012), also to analyze concentration of the four major triterpenoids in fresh material (James and Dubery, 2011). Phenylephrine was used to increase blood pressure acutely (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

The materials that were used as follows: ethanol 70%, chloroform, phenylephrine-HCl, and captopril from Sigma Chemical Co. (St. Louis, MO, USA), asiaticoside (98.5% purity of HPLC, Fluka, Switzerland), Titrated extract of *Centella asiatica* (TECA) from Syntex Laboratories (France), Liebermann-Burchard (LB), anisaldehide-H_2SO_4 and citroboric reagent.

Animals

Male Wistar rats weighing 150 - 300 g were obtained from Laboratory of Pharmacology and Toxicology, Faculty of Pharmacy, Universitas Gadjah Mada, Indonesia. The animal handling protocols of this study were in accordance with the guidelines for laboratory animal care. Ethical clearance for the animal study was obtained from Research Ethics Committee, Integrated Research and Testing Laboratory Universitas Gadjah Mada, Indonesia (No. 120/KEC-LPPT/X/2013).

Extraction and fractionation

Centella asiatica leaves were collected from Medicinal Plant and Traditional Medicine Research and Development Centre Tawangmangu, Solo, Indonesia and had been identified by a botanist. These leaves were then dried and powdered. One kg of powder was macerated by ethanol 70% for 24 hours. Subsequently, the residue was remacerated four times by the same solvent, and the extract was mixed into the previous ones. The collected extract was then evaporated under reduced pressure to give viscous ethanolic extract (EECA), then fractionated for yielding soluble (CFCA) and insoluble fractions of chloroform (CIF), then were concentrated by rotary vacuum evaporator.

Identification of Triterpenoid in CFCA

All samples (CFCA, CIF, EECA, TECA, and asiaticoside) were spotted on silica gel 60 F254 plate and developed in chloroform: methanol: water (65:25:4 v/v), then the samples were sprayed with Liebermann-Bourchard and heated at 110^{o}C for 10 minutes or until the coloured bands appeared. These spot were observed under Visible and UV366 light, then its hRf (100 x Rf) values were determined. Based on the TLC profile, CFCA would be proved as triterpenoid-rich fraction and was ensured to be separated from CIF whose flavonoid-rich.

TLC-Densitometric analysis

A CAMAG TLC system (Linomat, Switzerland), equipped with an automatic TLC sampler, a TLC scanner and a CATS software, was used. Both CFCA (50 mg) and asiaticoside standard solutions (0,1; 0,4; and 0,8 µg) were spotted on silica gel 60 F254 plate and developed in chloroform: methanol: water (65:25:4 v/v), then sprayed with anisaldehyde-sulfuric acid (AS) reagent and heated at 110^{o}C for 10 minutes. After that, the TLC plate was scanned using the wavelength of 506 nm according to Indonesian herbal pharmacopoeia (Department of Health Republic of Indonesia, 2008).

In vivo antihypertensive study

As many as 40 male Wistar rats were grouped into 8 treatment groups, each group consisted of 5 rats. Group 1, was normal control (0.5% per oral CMC-Na.), group 2 was negative control (phenylephrine 0.9 mg/kg subcutaneous), and group 3 was positive control (captopril 2.5 mg/kg per oral). Groups 4 to 8 were given per oral CFCA with respective doses 5, 10, 15, 20 mg/kg, and EECA dose of 400 mg/kg. Groups 2 to 8 also were given subcutaneous injection of phenylephrine dose of 0.9 mg/kg at 30 minutes after the administration of a single dose per oral treatment. The rats blood pressure were measured and recorded by non-invasive tail-cuff method. Systolic blood pressure (SBP) before being induced by phenylephrine was stated as a basal blood pressure (BP_0). If $SBP_0 \leq 130$ mmHg or normotensive, the rats were given treatment immediately, then 30 minutes later were induced by phenylephrine. These blood pressure was measured again after achieving the onset (BP1) and the phenylephrine effect duration (BP2).

Data analysis and statistical

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

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

Explanation:

P_{phe}: blood pressure difference in negative control groups

P_{tre}: blood pressure difference in treatment groups

P_{nor}: blood pressure difference in normal control groups

Next, the ED_{50} value was determined by non-linear regression analysis from logaritmic of doses-response curve with this formula (Kenakin, 1997):

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

Explanation:

X_1 : Logaritmic of dose with response exactly under 50%

X_2 : Logaritmic of dose with response exactly upper 50%

Y_1 : % response exactly under 50%

Y_2 : % response exactly upper 50%

Cardiovascular parameter datas 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

Triterpenoid separation from other compounds

The results of extraction produced 206.56 g ethanolic extract from 1 kg *C. asiatica* leaves dry powder with 20.66% rendement. Results of fractionation produced chloroformic fraction whose viscous, dark green color, and total 7.14 g or 8% rendement calculated from ethanolic extract. The rendement of CFCA was less than the chloroform insoluble fractions (87.81%) because non polar compounds in *C. asiatica* leaves were smaller than polar compounds (Harwoko *et al*., 2012).

Column packed with HPD100 resin revealed a good ability to separate asiaticoside, madecassoside, and other triterpene saponins from herbal raw materials (Jia and Lu, 2008). But the present study separated triterpene from other components on a silica gel plate by a normal TLC (Chivapat *et al*., 2011).

The TLC profile showed that CFCA spots have the hRf value as 24 (purple), 70 (blue-purple), and 80 (blue-purple) which indicating the triterpenoid compounds. These results are similar to literature by Wagner and Bladt (1996) with asiaticoside Rf is 0.2 to 0.35 (brown-violet), while its aglycone seen blue at Rf 0.85. James and Dubery (2011) also reported Rf

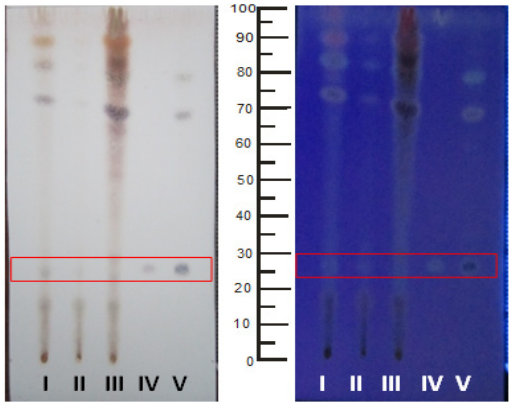

Figure 1. The TLC profile of EECA, CIF, CFCA, Asiaticoside, and TECA (I–V) on Visible (left) and UV_{366} (right).

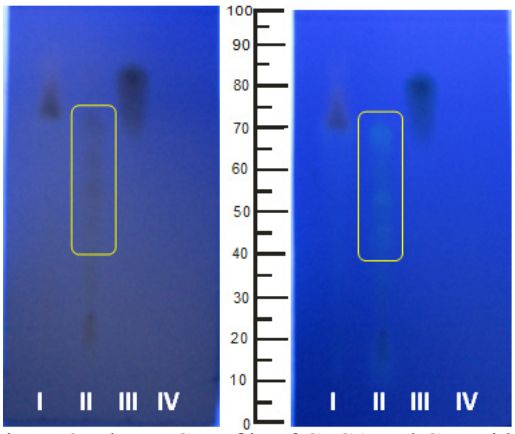

Figure 2. The TLC profile of CFCA and CIF with quersetin and TECA standard (I–IV) on UV366 before (left) and after (right) sprayed by citroboric reagent

0.45 was asiaticoside and 0.55 was madecassoside, while the spots at Rf 0.94 and 0.97 were asiatic acid and madecassic acid, respectively. Sathiyanarayanan *et al.* (2010) also reported that R_F value of 0.26 was obtained for asiaticoside. Thus, CFCA spots at hRf 24, 70, and 80 were identified as asiaticoside, asiatic acid, and madecassic acid, while brown spot at hRf 16 was suspected as madecassoside (Figure 1).

Based on the TLC profiles, the chloroform insoluble fraction contained flavonoids on hRf 40-70 whose yellow fluorescence and higher intensity after being sprayed with citroboric become brownish yellow (Figure 2). However, at the range of this hRf, CFCA spots did not seem, but only one spot on the hRf 71 which is likely impurities. Thus, CFCA did not contain many flavonoids as reported by Rachmawati *et al*. (2011) that the chloroform fraction of ethanol extract of *C. asiatica* contained terpenoids and phenol compounds, but did not contain flavonoids.

Asiaticoside quantification

Standard curve of plotting asiaticoside concentration versus area under the curve (AUC) was Y = 0.8233 + 0.965X with correlation coefficient

Table 1. Quantitative determination of asiaticoside in CFCA with TLC densitometry

Concentration (mg/mL)	AUC/1000	% Asiaticoside content (w/w)
100	1.5920	0.398
100	1.6645	0.436
100	1.5438	0.373
Mean ± SEM		0.402 ± 0.02

AUC = Area Under the Curve of the samples spot

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
CFCA-5	30.83 ± 12.72*	33.33 ± 33.82	32.30 ± 21.88*
CFCA-10	60.9 ± 11.91	49.33 ± 17.31	54.84 ± 14.27
CFCA-15	70.68 ± 5.10	78.67 ± 27.86	81.72 ± 15.89
CFCA-20	87.22 ± 10.05	100 ± 22.25	96.77 ± 15.07
EECA-400	83.46 ± 8.36	141.33 ± 16.60	116.13 ± 11.20
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.

(r) 0.99597. The AUC values were used to calculated the asiaticoside content in CFCA. It can be seen that CFCA contained 0.402 ± 0.02% of asiaticoside (Table 1). This result is greater than asiaticoside in *C. asiatica* extract from Karanganyar only 0.21% (Pramono and Ajiastuti, 2004), but smaller than that determined by Bermawie *et al.* (2008) with HPLC was 0.71%. TLC densitometry method has been developed to determine triterpenoid contents because of their sensitivity and selectivity, also good precision and accuracy (James and Dubery, 2011; Chaisawadi and De-Eknamkul, 2012).

Phenylephrine induced hypertensive rats

The phenylephrine profile in increasing rat blood pressure from preliminary experiment indicated that the onset of phenylephrine was 15 - 30 minutes and duration of effect was 1 hour. These results are consonant with the pharmacokinetic data of phenylephrine hydrochloride, an α1-adrenergic receptor agonist, which has 10 - 15 minutes onset and 1 hour duration in subcutaneous injection (Nugroho *et al*., 2008; Lacy *et al.*, 2013).

The negative control group that phenylephrine-induced was stated as a hypertension rat model with an average increase in SBP (25 mmHg), DBP (15-20 mmHg), and MAP (18 - 22 mmHg). This increase as same as in two kidney one clip hypertensive rats (20 mmHg) or L-NAME induced rats model (5 - 25 mmHg) (Monassier *et al*., 2006). Normal control group which given 0.5% CMC-Na 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 presented in figure 3 showed that both treatment and normal control groups differ significantly in comparison to that of negative control. However, in treatment of CFCA doses of 5 and 10 mg/kg, the DBP change was not significant

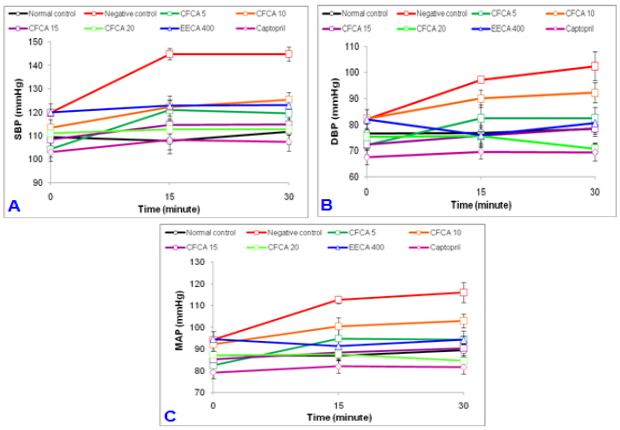

Figure 3. The changes in systolic blood pressure (A), diastolic blood pressure (B), and mean arterial pressure (C) during the measuring time in all groups

different with negative control ($p > 0.05$). Thus, low-dose CFCA could not inhibit DBP increasing that caused by phenylephrine-induced. However, all CFCA dose regiments exhibited hypotensive effect on SBP and MAP. Phenylephrine mildly decrease the heart rate (6%), while CFCA and EECA can increased the heart rate (5 - 15%). However high doses of CFCA mildly decrease the heart rate as well as captopril (12 - 19%).

The percentage of decrease in blood pressure indicated the response or the effect for each treatment group (Table 2). This percentage for CFCA on SBP and MAP at dose of 5 mg/kg was significantly different with captopril 2.5 mg/kg. But it was not significantly different for CFCA at dose of 10, 15, 20 mg/kg, and EEDP dose of 400 mg/kg for all blood pressure parameters. Although at 30th minutes after injecting of phenylephrine, CFCA dose of 20 mg/kg has hypotensive effect on DBP higher and significantly different with captopril ($p < 0.05$).

The results of this study may proved that the triterpenoids in CFCA whose containing 0.4% of asiaticoside had potency and efficacy as antihypertensive. The hypotensive effect of CFCA was started at dose of 5 to 20 mg/kg with gradual response. The ED50 value of CFCA hypotensive effect on SBP (10.40 ± 0.98 mg/kg) as similiar as its effect on DBP (9.05 ± 1.95 mg/kg) and MAP (9.37 ± 1.69 mg/kg). This doses were equivalent to 85 - 100 mg in 60 kg of human (Laurence and Bacharach, 1964) or 1/100 times of lethal dose in mice (Chivapat *et al*., 2011).

Several actions of the total triterpenic fraction of *C. asiatica* in vascular diseases makes the use of this compound very interesting in venous and arterial problems (Incandela *et al.*, 2001). Khuzaimah (1997) reported that the ethyl acetate fraction of *C. asiatica* leaf was suspected to contain triterpene/ saponins could lower systemic blood pressure in

cats. Reportedly, the triterpenoid-rich extract from bamboo shavings could reduce SBP in spontaneously hypertensive rats (Jiao *et al.*, 2007). In addition, the total triterpenic fraction of *C. asiatica* could treat venous hypertension microangiopathy (Incandela *et al.*, 2001), improve microcirculation and capillary permeability (Belcaro *et al*., 1990). *Centella asiatica* extract also showed potent diuretic activity (Jamil *et al*., 2007; Roopesh *et al.*, 2011) and *in vivo* antioxidant activity (Hussin *et al.*, 2007).

Indonesia is a second largest biodiversity country that provides many traditional medicines for various diseases. However, the scientific data are still limited so the Indonesia government declared a program entitled "Evidence based Jamu development" or "Scientification of Jamu". This program have developed antihypertensive formula that contains *C. asiatica* leaf. In Indonesia, this plant is known as herba pegagan or kaki kuda and is used for food, vegetable or traditional medicine.

Centella asiatica (L.) Urban. (Apiaceae) mostly used in herbal medicines industries as compound of herbs or an extract raw material. Phytopharmaca, one criteria of Indonesian herbal medicines, has a hypotensive effect in the cats, either with normal or hypertension by epinephrine-induced (Djatmiko *et al*., 2001). The purified extract of fraction is potential to develope as an antihypertensive agent (Nugroho *et al*., 2013). This study reported that triterpenoid-rich fraction of *C. asiatica* had *in vivo* antihypertensive effect on phenylephrine-induced hypertensive rats. In addition, the detailed mechanisms and the long term impact of antihypertensive effects of this plant needs to be studied.

Conclusion

Triterpenoid-rich fraction (CFCA) could be separated from flavonoid fraction by fractionation with chloroform. The TLC-densitometric data showed that asiaticoside contents of CFCA were 0.402 ± 0.02%. The triterpenoid-rich fraction showed antihypertensive effect on phenylephrine-induced hypertensive rats. The ED_{50} values, a parameter of drug potency, of these effects on SBP, DBP, and MAP were 10.40 ± 0.98, 9.05 ± 1.95, and 9.37 ± 1.69 mg/kg, respectively.

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