Available online at www.ijpcr.com
International Journal of Pharmaceutical and Clinical Research 2016; 8(4): 280-283

ISSN- 0975 1556

Research Article

In Vitro Antiplatelet Aggregation Activity of *Centella asiatica* (L.) Urban Ethanolic Extract

Agatha Budi Susiana Lestari[1,2], Achmad Fudholi[3*], Akhmad Kharis Nugroho[3], Erna Prawita Setyowati[4]

[1]*Postgraduate program of Pharmaceutical Sciences, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia*
[2]*Department of Pharmaceutical Technology, Faculty of Pharmacy, Sanata Dharma University, Yogyakarta, Indonesia*
[3]*Department of Pharmaceutics, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia*
[4]*Department of Pharmaceutical Biology, Faculty of Pharmacy, Gadjah Mada University, Yogyakarta, Indonesia*

Available Online:01st April, 2016

ABSTRACT

The death on cardiovascular disease can be caused by thrombosis. Thrombosis is triggered by irreversible activity of thrombocyte aggregation. However, almost all prescribed drugs exhibiting antiplatelet activity are associated with a large number of side effects. Then, exploration of medicinal plants which have cardioprotective activity especially as antiplatelet aggregation agent are needed. One of the medicinal plant, *Centella asiatica* (L.) Urban, has been known to keep people's health. This study was undertaken to investigate anti-platelet aggregation activity of *Centella asiatica* ethanolic extract (CEE). There were two groups of CEE, the first group was n-hexane purified CEE (pCEE) while the second group was non-purified CEE (npCEE). In vitro study of antiplatelet aggregation was evaluated by Born method using a Model 490 Chrono Log aggregometer. The blood plasma of male Sprague dawley rats were collected and 10 µM adenosine diphosphat (ADP) was used as agonist. The results showed that both of pCEE and npCEE had anti-platelet aggregation activity on the range concentration of 200, 400, and 800 µg/ml. However, antiplatelet aggregation activity of pCEE and npCEE were not different statistically. Purification process using n-hexane on *Centella asiatica* herbs powder does not influence its antiplatelet aggregation activity.

Keywords: antiplatelet aggregation, *Centella asiatica*, n-hexane purification

INTRODUCTION

Platelet aggregation has many benefits for organism such as on homeostasis, xenobiotic phagocytoses, interaction with virus, bacteria or antigen-antibody complexes. However, it also has dangerous effects. Hyperactivity of thrombocyte function leads to the increase of thrombocyte aggregation which can cause arterial thrombosis and embolism. Thrombosis and embolism can increase the risk factor of cardiovascular disease[1]. Thrombocyte aggregation assay was done to detect the thrombocyte abnormality function and it can be analysed by macroscopic, microscopic, or using other agonist like ADP, collagen, epinephrine, or ristosetin. Commonly used of antiplatelet agents such as clopidogrel and aspirin are widely used clinically[2], but they have various side effects. For this reason, studies have been conducted to search for naturally derived antiplatelet aggregation with fewer side effects.

From in vitro study, *Centella asiatica* (CA) has been known to have some pharmacodynamic activities such as wounds healing[3], anti peptic ulcer[4], mental disorders, antiviral, fungicidal, antibacterial and many others[5]. CA herbs are usually used for some therapy like gastrointestinal disease, dermatitis, infection disease, etc[5]. The main compound of CA and also as a marker constituent on quality control is asiaticoside, a triterpene compound[6]. CA has been shown to decrease the malonaldehyde (MDA) level, in addition, catalase activity was found to be significantly higher[7]. These effects may be attributed to the antioxidant compound present in CA, and in particular the flavonoids, quercetin, cathecin and rutin, that are known to be potent antioxidant[7]. The antioxidant activity of CA could be developed as the prevention agent for degeneration diseases such as antiplatelet aggregation which could lead to stroke or heart disease.

Extraction was used to obtain higher number of active compounds from CA herbs. To obtain high quality extract, it could be done by purification or without purification. In previous study, n-hexane purification process affects some quality parameters of CEE such as the number of asiaticoside content, IC_{50}, and total phenolic content[8].

The aim of this research is to determine the influence of n-hexane purification process on CA herbs powder towards its ethanolic extract anti-platelet aggregation activity. Platelet aggregation tests were done by Born method using a 490 model Chrono-Log.

**Author for Correspondence*

Table 1: The effect of pCEE and npCEE on % platelet aggregation

Concentration (µg/ml)	% aggregation					
	1	2	3	4	5	(mean±SEM)
100	29	19	18	17	20	20.6 ± 2.15
200	9	18	25	13	9	14.8 ± 3.03
400	17	12	35	14	10	17.6 ± 4.50
800	19	23	23	16	17	19.6 ± 1.46
1600	19	16	29	15	27	21.2 ± 2.87

level of significance p<0.05.

Table 2: The effect of non purified CEE on % platelet aggregation

Concentration (µg/ml)	% aggregation					
	1	2	3	4	5	(mean±SEM)
100	20	34	24	18	28	24.8 ± 2.87
200	17	16	13	24	10	16.0 ± 2.34
400	19	28	19	16	12	18.8 ± 2.63
800	35	14	18	12	18	19.4 ± 4.06
1600	28	27	14	20	14	20.4 ± 2.92

level of significance p<0.05

Table 3: The effect of DMSO on % platelet aggregation

% aggregation					
1	2	3	4	5	(mean±SEM)
24	25	25	39	28	28.2 ± 2.78

MATERIALS AND METHODS

Materials

CA herbs were obtained from Balai Besar Penelitian dan Pengembangan Tanaman Obat dan Obat Tradisional (B2P2TOOT/Research and Development Centre of Medical Plants and Traditional Medicine) Tawangmangu, Central Java, Indonesia, acetosal (PT Konimex), ginkgo biloba dried extract (Javaplant).

Reagents

Ethanol (Brataco), n-hexane (Brataco), DMSO (p.a., Merck), ADP (Chrono-Par. cat 384), saline (NaCl 0.9%, 308 mOsm/L, Otsuka).

Animals

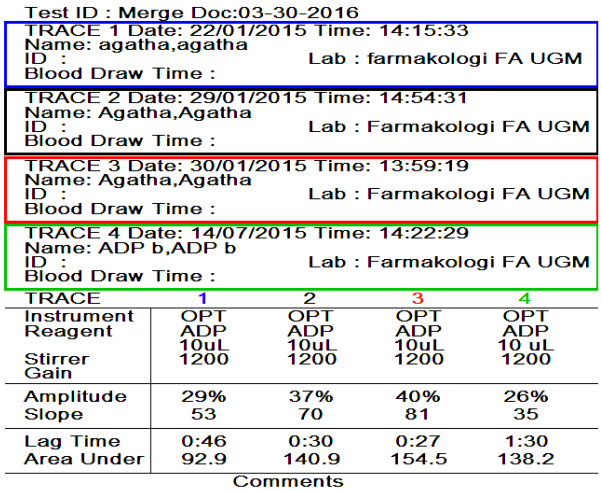
Two months old male Sprague dawley rats (180-200 grams) were purchased from Imono Laboratory, Faculty of Pharmacy, Sanata Dharma University, Yogyakarta. Each 4-5 rats were placed on the box at 24-25^{o}C in a room with an automatically controlled 12hours light-dark cycle. Upon arrival, rats were fed by BR2 once a day and acclimated to the facility for 7 days before the experiment. Rats had free access to de-ionized water and the assigned diet for along the study. Ethical clearance was acquired from ethical commission for animal study of Laboratorium Penelitian dan Pengujian Terpadu (LPPT/Integrated Research and Testing Laboratory) Gadjah Mada University.

Extraction of Centella asiatica herbs

Dried herbs were sorted and grinded into powder with the size of 12/40. There were two kinds of powders which would be extracted, n-hexane purified CA herbs powders (group I) and non-purified CA herbs powders (group II). In group I, CA powder which had been purified using n-hexane was evaporated until all solvent gone away. After that, both of them (group I and II) were extracted by maceration using ethanol and water (3:1). Each dried powder and solvent (1:10) were mixed in erlenmeyer and macerator was turned on at 177 rpm for 24 hours. The supernatant was separated and re-maceration was started again. All of the supernatant were collected and the solvent was evaporated to obtain viscous *Centella asiatica* ethanolic extract (CEE).

Test sample preparation

To obtain 2 mg/ml stock solution, CEE was dissolved in 50 ml DMSO. After that, further dilutions from stock solution were done to make range concentration of 100, 200, 400, 800, and 1600 µg/ml. Ginkgo biloba extract was used as a reference with the range concentration of 50, 100, 200, 400, and 800 µl/ml and 1 mM acetosal.


Test ID : Merge Doc:03-30-2016
TRACE 1 Date: 22/01/2015 Time: 14:15:33
Name: agatha,agatha
ID :
Blood Draw Time :
Lab : farmakologi FA UGM
TRACE 2 Date: 29/01/2015 Time: 14:54:31
Name: Agatha,Agatha
ID :
Blood Draw Time :
Lab : Farmakologi FA UGM
TRACE 3 Date: 30/01/2015 Time: 13:59:19
Name: Agatha,Agatha
ID :
Blood Draw Time :
Lab : Farmakologi FA UGM
TRACE 4 Date: 14/07/2015 Time: 14:22:29
Name: ADP b,ADP b
ID :
Blood Draw Time :
Lab : Farmakologi FA UGM

TRACE	1	2	3	4
Instrument	OPT	OPT	OPT	OPT
Reagent	ADP 10uL	ADP 10uL	ADP 10uL	ADP 10 uL
Stirrer	1200	1200	1200	1200
Gain				
Amplitude	29%	37%	40%	26%
Slope	53	70	81	35
Lag Time	0:46	0:30	0:27	1:30
Area Under	92.9	140.9	154.5	138.2

Comments

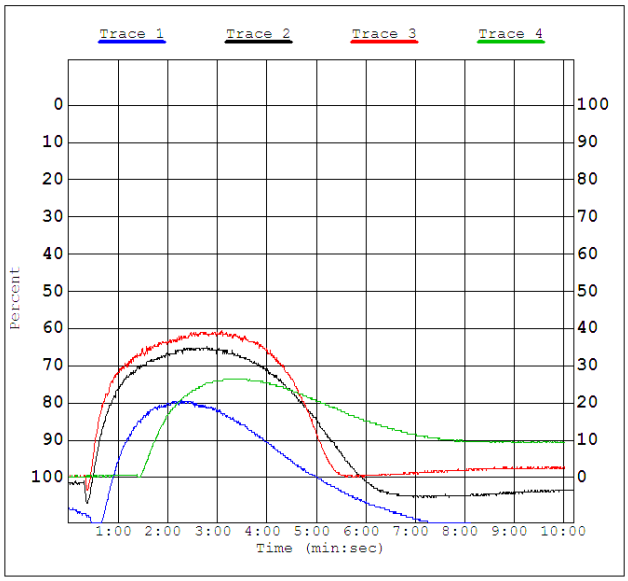


Figure 1. Baseline curve of 10µM ADP

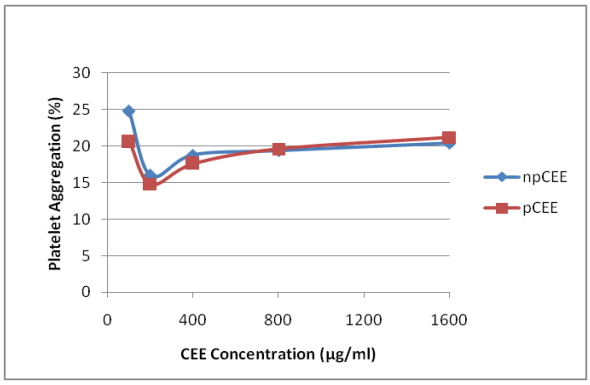


Figure 2: Curve of CEE (pCEE and npCEE) activity on platelet aggregation (%).

Preparation of platelet-rich plasma (PRP) and platelet-poor plasma (PPP)

Rats (Sprague dawley) arterial blood were collected and anti-coagulated with 0.11 M trisodium citrate (3% citrate-whole blood, 1:9, v/v)[9]. The citrated whole blood was centrifuged at 1000 rpm for 15 minutes and the PRP aspirated. PPP was obtained by further centrifugation (3500 rpm, 15 minutes) of the residual blood. After that, PPP was moved to other tube.

ADP Preparation

ADP was dissolved in a bottle which contained 5 ml saline solution. Each 40 µl of ADP solution was moved to microtube and stored in -70ºC (stable for one year). The ADP solution was melted on temperature room and it will stable for 8 hours in 2-8ºC.

Platelet Aggregation

CEE (pCEE and npCEE) ability on anti-platelet aggregation was determined using Born method with some modifications. This method based on turbidimetric principal using a Model 490 Chronolog aggregometer. In aggregometer cuvette, PRP and PPP were added for about 500 µl each cuvette. After that, all of them were incubated for 5 minutes in incubation wells. For about 5 µl of test compound (CEE) was added to pre-treated PRP aliquots and after one min aggregation was initiated by addition of 10 µM ADP and the responses were recorded for ten minutes. A magnetic bar was added in each cuvette and then the cuvette were inserted in optical chamber. The chambers were stirred for 3 minutes. For about 5 µl CEE was added in PRP cuvette and stirred for 1 minute. For about 5 µl of 10 µM ADP agonist was added in PRP cuvette and let the reaction flow for 10 minutes.

RESULTS AND DISCUSSION

Platelet aggregation was performed using the turbidimetric method based on the change of light transmissions. When the light is passed by plasma which contained PRP, a little light transmission will be resulted. Thrombocyte aggregation will appear after agonist addition. Therefore, the plasma becomes clear and it causes bigger light transmission. Platelet aggregation activity was determined by the difference of light transmission[10].

ADP concentration as the agonist should be looked carefully. Lower concentration of ADP can cause thrombocyte aggregation which is followed by deaggregation. Meanwhile, higher concentration of ADP causes monophasic curve which cannot differentiate the

Table 4: The effect of positive control on % platelet aggregation

No	Treatment	Concentration	% aggregation					
			1	2	3	4	5	(mean±SEM)
1	Ginkgo Biloba Extract (GBE)	50 µg/ml	16	13	15	12	28	16.8 ± 2.88
		100 µg/ml	22	15	16	20	5	15.6 ± 2.94
		200 µg/ml	15	8	13	20	18	14.8 ± 2.08
		400 µg/ml	19	17	11	15	7	13.8 ± 2.15
		800 µg/ml	20	13	7	16	9	13.0 ± 2.34
2	Acetosal	1 mM	11	12	18	21	18	16.0 ± 1.92

level of significance p<0.05

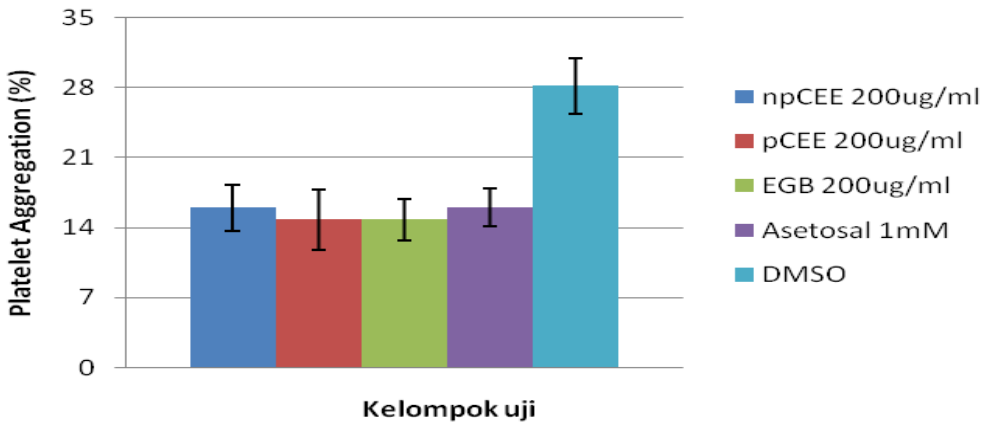


Figure 3: Comparative effect of pCEE, npCEE, GBE, Acetosal, and DMSO on platelet aggregation (%)

normal thrombocyte and the hyperactive thrombocyte[10]. In this study, 10µM ADP was used, and the baseline curve was shown at figure 1. ADP as agonist performed high platelet aggregation activity, which is 34.2 ± 2.81 % (taken from 5 replications, mean±SEM). When CEE was added on PRP, after 10 minutes, the percentage of platelet aggregation has smaller than ADP baseline. It means that CEE can decreased the platelet aggregation activity. Figure 2 showed that the ability of pCEE and npCEE to induce platelet aggregation were dose independent manner. Both of them also had similar profile, having the lowest % platelet aggregation on the concentration of 200 µg/ml. When the bigger concentrations (>200 µg/ml) were given, the percentage of platelet aggregation tended to increase although still lower than the smallest concentration (100 µg/ml). Both of pCEE and npCEE at concentration 200, 400, and 800 µg/ml performed antiplatelet aggregation activity significantly compared to negative control DMSO. The lower number of % platelet aggregation indicated stronger antiplatelet aggregation activity. At the concentration of 200 µg/ml, % platelet aggregation of pCEE (15.4 ± 3.24) and npCEE (16.0 ± 2.34) were not different statistically. It showed that n-hexane purification process on CA herbs powder did not affect the antiplatelet aggregation activity. In this study, two kinds of positive control were used, ginkgo biloba extract (GBE) and acetosal. GBE was used as medicinal plant representative which had been proven to has antioxidant and antiplatelet activities at the concentration of 80 µg/ml11. We used serial concentration of GBE: 50, 100, 200, 400, and 800 µg/ml. At the concentration used, GBE also performed platelet aggregation activity significantly compared to negative control DMSO. Acetosal was used as chemical drugs representative which had been known to has antiplatelet aggregation activity. Based on the Table 3, 1 mM acetosal induced platelet aggregation as much as npCEE (200 µg/ml), for about 16.0%. The comparative effect of Comparative effect of pCEE, npCEE, GBE at same concentration, acetosal, and DMSO on platelet aggregation (%) was shown in figure 3.

CONCLUSIONS

In vitro study showed that *Centella asiatica* herbs ethanolic extract (CEE) had antiplatelet aggregation activity. In this study, antiplatelet aggregation activity of CEE was performed at the concentration of 200, 400, and 800 µg/ml. n-hexane purification on the *Centella asiatica* herbs powder does not affect CEE antiplatelet aggregation activity significantly.

ACKNOWLEDGEMENTS

The authors would like to say thanks to:

1. Indonesia Higher Education Ministry (DIKTI) who had given the dissertation research funding through BPPS 2012 and Doctoral dissertation research grant 2015.
2. Institution of Sanata Dharma who had given the opportunity to take doctoral postgraduate degree and the scholarship for dissertation research.
3. drh. Sitarina Widyarini, M.P., Ph.D., Faculty of Veterinary Medicine, Gadjah Mada University, who had given the guidance about platelet aggregation study.
4. PT. Konimex Surakarta, who had given the drug sample, acetosal.

REFERENCES

1. Freedman JE, Loscalzo J, Arterial and Venous Thrombosis, in Longo D, Fauci A, Kasper D, Hauser S, Jameson J, Loscalzo J. *Harrison's Principles of Internal Medicine*. 18th Edition, Vol. 1, McGraw Hill Professional, 2011, 983-988.
2. Gaglia Jr MA, Manoukian SV, Waksman R, Novel antiplatelet therapy. American Heart Journal 2010; 160: 595-604
3. Shukla A, Rasik AM, Jain GK, Shankar R, Kulshrestha DK, Dhawan BN. In vitro and in vivo wound healing activity of asiaticoside isolated from *Centella asiatica*. Journal of Ethnopharmacology 1999; 65(1): 1–11
4. Cheng CL, Guo JS, Luk J, Koo MWL. The healing effects of Centella extract and asiaticoside on acetic acid induced gastric ulcers in rats. *Life Sci.*, 2004; 74(18): 2237–2249
5. Brinkhaus B, Lindner M, Schuppan D, Hahn EG. Chemical, pharmacological and clinical profile of the East Asian medical plant *Centella asiatica*. *Phytomedicine* 2000; 7(5): 427–448.
6. Zheng C, Qin L. Chemical components of Centella asiatica and their bioactivities. *Journal of Chinese Integrative Medicine* 2007; 5(3): 348–351
7. Hussin M, Abdul-Hamid A, Mohamad S, Saari N, Ismail M, Bejo MH. Protective effect of Centella asiatica extract and powder on oxidative stress in rats. Food Chemistry 100 (2007); 535-541
8. Lestari ABS, Fudholi A, Nugroho AK, and Setyowati EP. The effect of simplex powder n-hexane purification on asiaticoside content, free radical scavenging and total phenolic content of gotu kola (Centella asiatica (L.) Urban) ethanolic extract. *Jurnal Ilmu Kefarmasian Indonesia* 2015; 13 (1): 10-16.
9. Seo EJ, Lee DU, Kwak JH, Lee SM, Kim YS, Jung YS. Antiplatelet effects of Cyperus rotundus and its component (+)-nootkatone. Journal of Ethnopharmacology 2011; 135: 48–54.
10. Gawaz M. Blood Platelets: Physiology, Pathophysiology, Membrane Receptors, Antiplatelet Drugs, Coronary Heart Disease, Stroke, Peripheral Arterial Disease; 47 Tables. 2001; 42-46.
11. Ryu KH, Han HY, Lee SY, Jeon SD, Im GJ, Lee BY, Kim K, Lim KM, Chung JH.Ginkgo biloba extract enhances antiplatelet and antithrombotic effects of cilostazol without prolongation of bledding time. *Thrombosis Research* 2009; 124: 328-334.