

Effect of *Uncaria gambir* and *Uncaria sclerophylla* on Pulmonary-Thromboembolism Mice

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History

- Submission Date: 28-11-2019;
- Review completed: 04-12-2019;
- Accepted Date: 12-12-2019.

DOI : 10.5530/pj.2020.12.29

Article Available online

<http://www.phcogj.com/v12/i1>

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ABSTRACT

Background: Previous studies on virtual screening on P2Y₁₂ receptor of Adenosine Diphosphate (ADP) have showed that Roxburghine B, the compound which is found in *Uncaria* species, can inhibit the receptor function. **Objective:** In this study, we investigated the effect of *Uncaria gambir* and *Uncaria sclerophylla* extract on survival rate and bleeding time as antithrombotic *in vivo*. **Methods:** Animal subjects (ddY strain mice) were divided to two different experimental group (survival rate and bleeding time). *U. gambir* and *U. sclerophylla* were given to the mice orally in three different dose (5 mg, 10 mg, 20 mg/20 g BW and 2.5 mg, 5 mg, 10 mg/20 g BW, respectively) for seven days. **Results:** *U. gambir* and *U. sclerophylla* able to prolong bleeding time from test subjects equivalent to ASA as standard. The results show the increasing number of survived animals in the treated group compared to the negative control group. **Conclusion:** Both of *U. gambir* and *U. sclerophylla* prevent pulmonary thromboembolism on mice subjects represent by the increased of survival rate. Antithrombotic effects that were observed suggested was provide by their antiplatelet activity.

Key words: Antithrombotic, Pulmonary thromboembolism, Bleeding time, Survival rate, *Uncaria gambir*, *Uncaria sclerophylla*.

INTRODUCTION

The third leading cause of death by cardiovascular disease after myocardial infarction (MI) and cerebrovascular accidents (CVA) is venous thromboembolism (VTE).¹ VTE is a clinical manifestation from pathophysiology of acute pulmonary embolism (PE) and deep venous thrombosis (DVT).² The thrombi formation in deep veins itself called DVT, when DVT is released, it will travel to the lungs causing a block in blood supply called pulmonary embolism.³ If this pathology not treated immediately the mortality will increase about 23 -73%.⁴ Data reported the incidence of VTE annually ranges from 104-183 person cases per year, its similar the rate of stroke cases. Whereas the incidence of PE without DVT ranges from 29-78 cases per 100.000 person for each year.⁵ Of course, this condition will impact on global health problem, reducing the quality of life and mortality of patients.

Pathophysiology and clinical manifestations of PE itself are highly depends on the four main factors, including: the size of the embolism and the degree of vascular occlusion, the patient's condition before, the release of thromboxane and serotonin from platelets that attached to the embolus leading the vasoconstriction.⁶ There are a number of PE treatment strategies such as the administration of low molecular weight heparin (LMWH), that followed or combined with antagonist vitamin K. There are also new strategies with administration of thrombin inhibitors or factor Xa.⁴ Another strategy was the treatment using acetylsalicylic acid (ASA) as thromboprophylaxis agent. Five systematic reviews and five non-randomized

primary clinical studies shows that the clinical efficacy of ASA better than LMWH and inhibitors factor Xa.⁷ Low doses oral administration of ASA will provide irreversible inhibition on cyclooxygenase (COX)-1 in order to prevent the thromboxane A₂ formation that responsible to platelet aggregation and vasoconstriction. Doses oral administration of ASA greater than the dose that used for cardiovascular prophylaxis will also inhibit COX-2 and prostaglandin release.⁸ In contrast with the therapeutic effect, administration of ASA like a "double-edged knife". That is because ASA have a number of adverse effects, such as the risk of bleeding that increases at elderly patients. In addition, a number of diseases such as ulcer peptic, cancer, polypharmacy in geriatric will increase the risk of bleeding incident.⁹ Therefore, the investigation and discovery of new safer thromboprophylaxis agents is important to be encouraged.

In the process of thrombus formation, adenosine diphosphate (ADP) is a major component that responsible for the release of activated platelets. ADP acts as an agonist of two purinergic G-protein coupled receptors, namely: P2Y₁ and P2Y₁₂ receptors. Activation of P2Y₁ impact on the shape of platelets changes. Synergistically when P2Y₁₂ is stimulated, the aggregation response occurs will be amplified and stabilized.¹⁰ In order to obtain animal model for anti-platelet activity test, epinephrine is applied because it ability to induce platelet aggregation mediated by α -2 adrenoreceptors. Epinephrine also capable to potentiates aggregation process triggered by a number of platelet agonists such as collagen. Yun-Choi *et al.*, (2000) reported that the combination of epinephrine and collagen result on platelet shape

Cite this article: Sakti AS, Nityasa AR, Saputri FC. Effect of *Uncaria gambir* and *Uncaria sclerophylla* on Pulmonary-Thromboembolism Mice. Pharmacogn J. 2020;12(1): 192-6.

changes without causing an aggregation process.¹¹ That fact makes the collagen-epinephrine (CE) combination promising to induce animal model for determination antiplatelet activity

Uncaria genus rich in polyphenol compounds which have beneficial effects on cardiovascular. Besides polyphenol compounds, alkaloid compounds are also contained in the *uncaria* genus.^{12,13} As reported in previous study, *U. gambir* and *U. sclerophylla* contain a number of alkaloids which have hypotensive activity. Moreover, virtual screening on ADP receptor (P2Y₁₂), revealed that the Roxburghine B as one of the alkaloids contained in the *uncaria* genus can inhibit the receptor function.^{12,14} In this study, the bleeding time and survival rate were performed on a mice model to revealed the pulmonary-thromboembolism effects of *U. gambir* and *U. sclerophylla*.

MATERIALS AND METHODS

Materials

Uncaria gambir and *Uncaria sclerophylla* dried leaf was obtained from Padang, West Sumatra Indonesia. Both of samples were authenticated by Herbarium University of Andalas. Epinephrine was obtained from Sigma-Aldrich (Nucleos, Singapore) and carboxymethylcellulose (CMC) was from Brataco (Jakarta, Indonesia). Acetylsalicylic acid, collagen, and other chemical reagents were purchased from Sigma-Aldrich (Tokyo, Japan).

Preparation of extract

Ethanolic extracts were obtained by extracting dried leaf samples using 70% ethanol (v/v), the extraction process performed by maceration method for 72 hours at room temperature.¹⁵ Liquid extract obtained was then evaporated using a rotary evaporator, until concentrated crude extract yielded. The crude extract was kept at 4°C until use. The characteristics of *U. gambir* Roxb. and *U. sclerophylla* Roxb. leaf have been done and reported in our previous study including phytochemical screening, thin layer chromatography profile and total phenolic content.¹²

Animal experimental design

The Ethics Committee of Faculty of Medicine, Universitas Indonesia (No. 232/UN2.F1/ETIK/2017) was approved this study. The ddY strain of mice (*Mus musculus*) weights ranging from 20 - 30 g were obtained from Faculty of Veterinary, Bogor Agriculture Institute. The acclimatization was carried out for 2 weeks before the experiment, all of mice had free access to a commercial mice pellet and drinking water. The animals were placed in a well-ventilated cage with a constant physical ambient temperature (25 ± 5°C) and with 12 hours light-dark periods. The animals were weighed every day and observed for the activity. Only healthy animals were included in this study.

This study was performed to evaluate antithrombotic effect experimently by bleeding time assay and pulmonary thromboembolism *in vivo*. A total of 30 mice randomly divided into five groups for tail bleeding assay.

Group I: normal control group.

Group II: mice treated with 0.208 mg/20g BW of ASA, orally for 7 d.

Group III: mice treated with the plant extract (*U. gambir*: 5 mg/20 g BW; *U. sclerophylla*: 2.5 mg/20 g BW, orally for 7 d).

Group IV: mice treated with the plant extract (*U. gambir*: 10 mg/20 g BW; *U. sclerophylla*: 5 mg/20 g BW, orally for 7 d).

Group V: mice treated with the plant extract (*U. gambir*: 20 mg/20 g BW; *U. sclerophylla*: 10 mg/20 g BW, orally for 7 d).

Another total of 36 mice randomly divided into six groups for survival rate assay by pulmonary thromboembolism model.

Group I: normal control group.

Group II: CE control group (received 700 µg/mouse of collagen-42 µg/mouse of epinephrine, iv. on 8th day).

Group III: mice treated with 0.208 mg/20g BW of ASA, orally for 7 d, and received 700 µg/mouse of collagen-42 µg/mouse of epinephrine, iv. on 8th day)

Group IV: mice treated with the plant extract (*U. gambir*: 5 mg/20 g BW; *U. sclerophylla*: 2.5 mg/20 g BW, orally for 7 d, and received 700 µg/mouse of collagen-42 µg/mouse of epinephrine, iv. on 8th day).

Group V: mice treated with the plant extract (*U. gambir*: 10 mg/20 g BW; *U. sclerophylla*: 5 mg/20 g BW, orally for 7 d, and received 700 µg/mouse of collagen-42 µg/mouse of epinephrine, iv. on 8th day).

Group VI: mice treated with the plant extract (*U. gambir*: 20 mg/20 g BW; *U. sclerophylla*: 10 mg/20 g BW, orally for 7 d, and received 700 µg/mouse of collagen-42 µg/mouse of epinephrine, iv. on 8th day).

Experimental procedure

An *in vivo* assay similar with the previous study¹⁶ was performed to revealed the effect of both extracts on bleeding time and survival rate. Each of the crude extract with three doses were suspended in 0.5% CMC-Na to obtain the suspension of plant extract. ASA dissolved in water was then used as a positive control. The suspensions of extract and ASA were administered to treated mice orally for 7 d. Both normal control groups in tail bleeding and survival rate assay were given 0.5% CMC-Na orally for 7 d.

Tail bleeding assay

Determination of bleeding time performed by tail bleeding test method. All animal test subjects administrated 10 mg/kg xylazine and 100 mg/kg ketamine as an anesthetic, intermittently 5 hours from the last administration of the plant extract or ASA or 0.5% CMC-Na.¹⁷ The animals were placed in a prone position and the tail dangles down. The tail was cut 10 mm along lateral vein, then immediately immersed in 50 mL falcon tube containing normal saline pre-warmed to 37°C. Observation of bleeding time was performed within 20 min. Re-bleeding was monitored even though the bleeding stopped before 20 min. The sum of bleeding time during 20 min period was used for on/off bleeding cycles. The experiment will be stopped at the end of 20 min to prevent the death of animals.¹⁸

Pulmonary thromboembolism model

An established method from Saputri *et al.* performed to provide a pulmonary thromboembolism (PE) model.¹⁶ After 24 hours from the last administration of the plant extract or ASA or 0.5% CMC-Na, the mixture solution of collagen and epinephrine (CE) was injected through the tail vein to induce PE. While the normal group was given an injection of normal saline. The lethal and paralysis effects are observed for 15 minutes after induction. The survival rate (%) was calculated based on the following formula:

$$\text{Survival rate (\%)} = \left(1 - \frac{\text{amount of dead animals}}{\text{amount of animals in the group}} \right)$$

Stastical analysis

Result of determination bleeding time assay are presented as mean ± SD (Standard Deviation) of five data. Homogeneity of data was determined with Levene test, and the data normality was analyzed with the Shapiro-Wilk test. The differences in mean (Table 1.) was analyzed with ANOVA test followed by Tukey and LSD post hoc analysis using SPSS v.22, values of p < 0.05 were considered to be statistically significant.

RESULTS

This study revealed both of *U. gambir* and *U. sclerophylla* were proven able to prolong bleeding time from test subjects equivalent to ASA as standard. The bleeding time prolongation occurs, was in line with increasing of dose (Figures 1 and 2). Whereas, can observed at Table 1. dose 3 shows the longest bleeding time prolongation. All doses tested compared with control group were significantly different ($p < 0.05$). Pulmonary thromboembolism induction caused 60% of deaths from each group administrated with CE injection alone. In accordance with the bleeding time assay performed, administration of *U. gambir* and *U. sclerophylla* capable to increase the survival rate. The highest survival rate for *U. gambir* was observed at dose 3, that shows 80% of survival rate (Table 2). It means that *U. gambir* was able to increase survival rate 2 times compared to group that administrated CE alone. *U. sclerophylla* provide the highest survival rate at dose 2, however at the higher dose (dose 3) there was observed decreases in survival rate. This phenomenon suggested because at the dose 3 bleeding event or toxic effect was occurs, further investigation needed to clarify this phenomenon.

DISCUSSION

U. gambir and *U. sclerophylla* contain a number of phytochemical compounds such as flavonoids, polyphenols and alkaloids.^{12,14} The major phenolic content from *Uncaria* species is catechins, which are commonly known for their properties as antioxidant.¹⁹ Catechin that can be found more than 70% in *uncaria* extracts, also reported have several antithrombotic effects due its antiplatelet activity.^{20,21} Besides polyphenol, *uncaria* species also reported to contain a number of

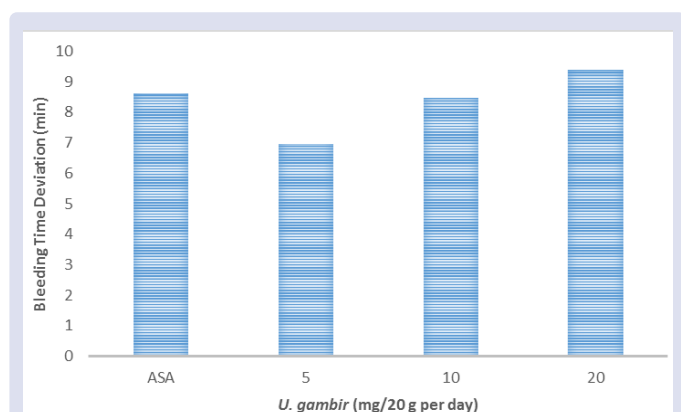


Figure 1: Dose-dependent reduction on bleeding time parameter between *U. gambir* and control. ASA: acetylsalicylic acid.

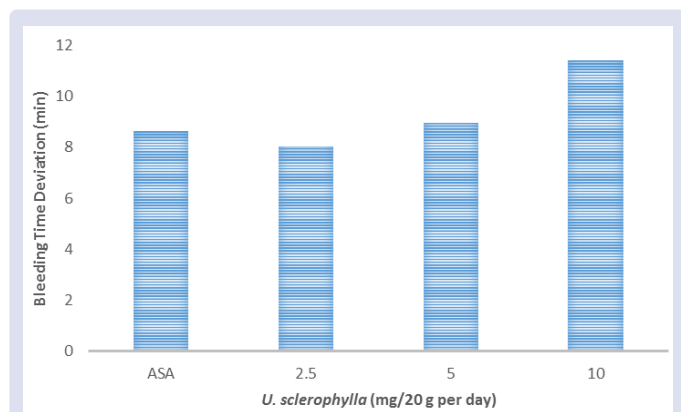


Figure 2: Dose-dependent reduction on bleeding time parameter between *U. sclerophylla* and control. ASA: acetylsalicylic acid.

Table 1: Mean value of bleeding time.

Treatment	Bleeding Time (Mean \pm SD)	
	<i>U. gambir</i> extract	<i>U. sclerophylla</i> extract
Control	5.39 \pm 2.21	5.39 \pm 2.21
ASA	14.02 \pm 4.20*	14.02 \pm 4.20*
Dose 1	12.34 \pm 1.24*	13.42 \pm 3.14*
Dose 2	13.85 \pm 3.50*	14.34 \pm 1.07*
Dose 3	14.78 \pm 3.59*	16.79 \pm 3.39*

Note: Control (0.5% CMC vol: 0.3 ml/20 g BW), ASA (0.208 mg/20g BW), *U. gambir* Dose 1 (5 mg/20 g BW), *U. gambir* Dose 2 (10 mg/20 g BW), *U. gambir* Dose 3 (20 mg/20 g BW), *U. sclerophylla* Dose 1 (2.5 mg/20 g BW), *U. sclerophylla* Dose 2 (5 mg/20 g BW), *U. sclerophylla* Dose 3 (10 mg/20 g BW). * shows $P < 0.05$ as compared to the control group. ASA: acetylsalicylic acid; SD: standard deviation; BW: Body Weight.

Table 2: Survival rate analysis.

Treatment	Survival Rate (%)	
	<i>U. gambir</i> extract	<i>U. sclerophylla</i> extract
Control	-	-
CE	40	40
CE + ASA	60	60
CE + Dose 1	60	80
CE + Dose 2	60	83.33
CE + Dose 3	80	20

Note: Control (0.5% CMC vol: 0.3 ml/20 g BW), CE (0.5% CMC vol: 0.3 ml/20 g BW), ASA (0.208 mg/20g BW), *U. gambir* Dose 1 (5 mg/20 g BW), *U. gambir* Dose 2 (10 mg/20 g BW), *U. gambir* Dose 3 (20 mg/20 g BW), *U. sclerophylla* Dose 1 (2.5 mg/20 g BW), *U. sclerophylla* Dose 2 (5 mg/20 g BW), *U. sclerophylla* Dose 3 (10 mg/20 g BW). CE: collagen-epinephrine; ASA: acetylsalicylic acid; BW: Body Weight.

alkaloid compounds. Various studies reported that indole alkaloids from *uncaria* species are responsible for vasodilation effect. Furthermore, previous *in silico* screening revealed that roxburghine B which is an indole alkaloid from *uncaria*, has an inhibitory activity against P2Y₁₂ receptor.¹⁴ This study confirmed our previous *in silico* findings, *in vivo* data (Tables 1 and 2) show that *U. gambir* and *U. sclerophylla* has antithrombotic effects.

In the mechanism of platelet aggregation, presence of collagen was required for adhesion of platelet also to completes platelet activation itself. Collagen plays an important role, indirectly in regulating formation of thrombin process. Phosphatidylserine as negatively charged phospholipids for the example, it will only form coagulation catalytic site and form a thrombin after interact with collagen.²² Ephineprin is a sympathomimetic catecholamine that works on both of α - and β -adrenergic receptors mediated by a G protein-linked second messenger system. At the small amount, ephineprin has higher affinity for β -adrenergic receptors, whereas at high doses it provides selective activity to α -adrenergic receptors.²³ Related to antithrombotic effect, ephineprin can increases platelet deposition by 55-85%. Mustonen *et al.* reported that platelet aggregation mediated by ephineprin was related to phosphorylation of p38MAPK and cytosolic phospholipase A2 (cPLA2) activity, which responsible to arachidonic acid (AA) production from platelet membrane cell.²⁴

That description above underlies the application of collagen-epinephrin (CE) to induce pulmonary thromboembolism (PE) on animal model. Intravenous CE injection capable to induce platelet activation subsequently implicated lethal incident on animal model immediately.²⁵ Moreover, Konstantinides *et al.* also reported that histological and histochemical examination of CE-induced animal model show the formation of fibrin and fibrinogen rich thrombi.²⁶ We have successfully determined antithrombotic effect from various natural product using this CE-induced mice model at the previous

studies.²⁷⁻³⁰ There are three classes of antithrombotic agents, including: agents that inhibit aggregation of platelet, inhibit fibrin strands formation (known as anticoagulant), and agent that dissolve existing clots (known as fibrinolytic). Both of antiplatelet and anticoagulant work through preventing thrombogenesis, but not affect the clots that already formed.³¹ Administration of both of extracts intervention for 7 days then subsequently CE administrated was reveal whether the intervention able to prevent the clots formation or not. The results show that *U. gambir* and *U. sclerophylla* able to prevent the clots formation and prolong the bleeding time. This result suggested due to antiplatelet activity of *U. gambir* and *U. sclerophylla* through P2Y₁₂ inhibition as reported on in virtual screening before.

CONCLUSION

Uncaria gambir and *Uncaria sclerophylla* able to prolong bleeding time and prevent pulmonary thromboembolism represent by the increased of survival rate compared to control. Antithrombotic effects that were observed suggested was provide by antiplatelet activity from *Uncaria gambir* and *Uncaria sclerophylla*.

ABBREVIATIONS

VTE: Venous thromboembolism; **MI:** Myocardial infarction; **CVA:** Cerebrovascular accidents; **PE:** Pulmonary embolism; **LMWH:** Low molecular weight heparin; **ASA:** Acetylsalicylic acid; **COX:** Cyclooxygenase; **ADP:** Adenosine diphosphate; **CE:** Collagen-epinephrin; **cPLA2:** Cytosolic phospholipase. **AA:** Arachidonic acid (AA).

ACKNOWLEDGMENT

The authors would like to give gratitudes to Universitas Indonesia and Ministry of Research and Higher Education for supporting this research with the grant Penelitian Dasar Unggulan Perguruan Tinggi (PDUPT) No. 1472/UN2.R3.1/HKP.05.00/2019.

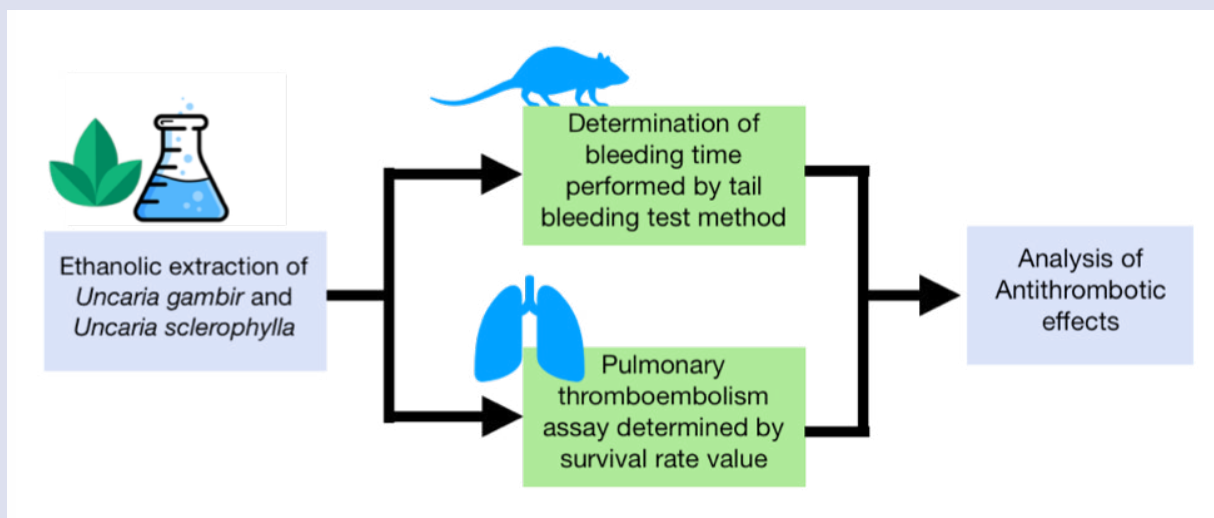
CONFLICTS OF INTEREST

The authors declare there is no conflict of interest.

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GRAPHICAL ABSTRACT



SUMMARY

The present study investigated the effect of *Uncaria gambir* and *Uncaria sclerophylla* extract as antithrombotic by bleeding time assay and pulmonary thromboembolism *in vivo*. Both of extracts able to prolong bleeding time equivalent to ASA as a positive control. The results also showed the increasing number of survived animals in the treated group compared to the negative control group. Both of *U. gambir* and *U. sclerophylla* prevent pulmonary thromboembolism on mice subjects represent by the increased of survival rate. Antithrombotic effects that were observed suggested was provide by their antiplatelet activity.

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Cite this article: Sakti AS, Nityasa AR, Saputri FC. Effect of *Uncaria gambir* and *Uncaria sclerophylla* on Pulmonary-Thromboembolism Mice. Pharmacog J. 2020;12(1):192-6.