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Original Article

Antithrombotic Effect of *Kaempferia galanga* L. and *Curcuma xanthorrhiza* Roxb. on Collagen-epinephrine Induced Thromboembolism in Mice

Fadlina Chany Saputri[1*], Chavella Avatara[2]

ABSTRACT

Objective: *Kaempferia galanga* L. and *Curcuma xanthorrhiza* Roxb. have been proven to possess antiplatelet activity *in vitro*. The aim of this study is to investigate the antithrombotic effect of the rhizome extracts of *Kaempferia galanga* L. and *Curcuma xanthorrhiza* Roxb in a mouse thrombotic model. **Methods:** The ethanol extracts of *K. galanga* and *C. xanthorrhiza* were orally administered with three different doses (7, 14 and 28 mg/20 g BW) in two experimental mouse models. Bleeding time prolongation was observed on mice tail that had been cut and the survival rate of mice was observed after thromboembolism induction by collagen-epinephrine. These two experiments were observed after 7 days extracts pre-treatment and compared to the positive control, aspirin. **Results:** A potent effect of *K. galanga* and *C. xanthorrhiza* extracts were demonstrated through significant bleeding time prolongation compared to control group. *C. xanthorrhiza* extract exhibited better activity than *K. galanga* extract. Moreover, both *K. galanga* and *C. xanthorrhiza* extracts significantly protected mice from thromboembolic death, where the protective effect of *C. xanthorrhiza* extract was stronger than *K. galanga* extract in a dose-dependent manner. **Conclusion:** *K. galanga* and *C. xanthorrhiza* extracts have a potential to be developed as antithrombotic agents against platelet thromboembolism.

Key words: Antithrombotic, *Kaempferia galanga* L., *Curcuma xanthorrhiza* Roxb., Bleeding time, Survival rate.

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INTRODUCTION

Thrombosis is the formation of blood clots both in arteries or veins, which can be caused by platelet aggregation. It is the risk factor of cardiovascular disease. Thrombosis may cause venous thromboembolism (VTE).[1,2] The most serious complication of VTE is pulmonary embolism (PE), which occurs in more than a one-third of VTE patients and contributing to 12% of patient deaths.[3-5] Aspirin is one of the drugs which can inhibit platelet aggregation. This drug inhibits cyclooxygenase (COX) enzyme in cyclooxygenase pathway thus preventing thrombus formation.[6]

Kaempferia galanga L. and *Curcuma xanthorriza* Roxb. (family: Zingiberaceae) have been reported to exhibit antithrombotic activity *in vitro*.[7,8] Cinnamaldehyde and curcumin have been identified as the major bioactive compounds in *K. galanga* and *C. xanthorrhiza*, respectively. These compounds have been reported to be able to inhibit platelet aggregation induced by collagen, arachidonic acid, and adenosine diphosphate (ADP) which reduced the formation of thromboxane A_2 (TxA_2) in cyclooxygenase pathway.[9-10] Cinnamaldehyde had shown to inhibit collagen- and thrombin-induced aggregation of rat platelets *in vitro* in a concentration-dependent manner.[9] A research also showed that curcumin inhibited thromboxane B_2 (TXB_2) production from exogenous [^{14}C] arachidonate in washed platelets.[10]

In this study, we examined the *in vivo* antithrombotic effect of the ethanol extracts of *K. galanga* and *C. xanthorrhiza* rhizomes by evaluating the bleeding time and survival rate in an *in vivo* thrombosis model.

MATERIALS AND METHODS

Materials

The ethanol extracts of *Kaempferia galanga* and *Curcuma xanthorriza* were prepared and obtained from the Indonesian Spice and Medicinal Crops Research Institute, Bogor, Indonesia. Voucher specimen was deposited at Center for Plant Conservation Botanic Gardens (No. B-1693/IPH.3./KS/VI/2017). Carboxymethylcellulose (CMC) was procured from Brataco, Indonesia. Normal saline was supplied by Euro-Med, Indonesia. Aspirin was obtained from Medifarma, Indonesia.

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Total Flavonoid and Phenolic Content Assay

Total flavonoid content was measured using aluminum chloride colorimetry method. Working solutions of extracts prepared by mixing 200 mg of the ethanol extracts with one ml of hexamethylentetramine (HMT) 0.5% w/v solution. Twenty ml acetone was then added and followed by two ml of 25% chloride acid solution. The mixture obtained was refluxed for 30 min, then the obtained residue filtered, and the filtrate was collected. Acetone followed by ethyl acetate was added to the filtrate. Ten ml working solutions from the bottom layer of the obtained mixture was added by one milliliter of 2% aluminum chloride in glacial acetic acid solution to obtain the final solution. The absorbance was measured at λ = 510 nm.[11] The total phenolic compound was measured using the folin-ciocalteau method and the absorbance was measured at λ = 725 nm.[12]

Phytochemical screening

The phytochemical screening indicated a notable presence of flavonoids in all extracts. Other metabolites and biologically active components were recognized such as saponin and terpenoids. Glycosides and alkaloid were noticed in *C. xanthorrhiza* extract, but not in *K. galanga* extract, while tannin could only be noticed in *K. galanga* extract (Table 1).

Animals

Male mice (*Mus musculus*) Deutschland Denken Yoken (DDY) strain weighing between 20-30 g were obtained from the Faculty of Veterinary, Bogor Agriculture Institute. This study was approved by The Ethics Committee of Faculty of Medicine, Universitas Indonesia (Number. 232/UN2.F1/ETIK/2017).

Antithrombotic Activity Test

Bleeding Time Test

Bleeding time was observed in the treated mice according to the protocols with minor modification.[13] The mice were divided into five groups as shown in Table 2. After acclimatization for a week, different doses of the plant samples or aspirin were administered to the mice by oral route for 7 days. The dose of aspirin was 80 mg/day.[13-15] Antithrombotic activity was evaluated by measuring the bleeding time, 5 h after sample administration on day-7. The animals were anesthetized with ether by inhalation and placed in a horizontal position. About 10 mm segment of the tail was amputated with a scalpel. The ends of severed tails were immediately immersed in a 15 mL falcon tubes containing isotonic saline. The tail was vertically positioned with the tip horizontally placed about 2 cm below the body. The bleeding observation was conducted for 20 min.[13] The increase in bleeding time percentage (%) of extract groups was known by comparing the effect of sample to the vehicle group.

Survival Rate Assay

Treatment of mice for determination of the survival rate was similar to the bleeding time treatment as shown in Table 3. Antithrombotic activity was tested by calculating the survival rate 24 h after day-7. A mixture of collagen (700 µg) and epinephrine (42 µg) was injected into the tail vein of each treated mouse to induce pulmonary thrombosis.[13,16] Whereas, normal control mice were given the isotonic saline solution intravenously. After 15 min, the number of dead or paralyzed mice was recorded, and the percentage of protection was calculated as follows:

$$[1- (\text{number of death or paralysis}) / \text{total number of mice}] \times 100$$

The increase in survival rate percentage (%) of mice was obtained by comparing the effect of the samples to the vehicle group.[13]

Table 1: Phytochemical compounds identified in different extracts.

Metabolite	Reagent	*K. galanga*	*C. xanthorrhiza*
Alkaloids	Bourchardat	-	+
	Dragendorff	-	+
	Mayer	-	+
Tannins	$FeCl_3$ 3%	+	-
	NaCl-Gelatin	+	-
Saponins	HCl 2N	+	+
Flavonoids	Mg+HCl	+	+
Terpenoids	Lieberman Bourchard	+	+
Glycosides	Molisch	-	+

Table 2: Design experiment of bleeding time assay in mice.

Group	Number of mice	Bleeding Time Treatment	
		Day-1 to 7	Day-7 (5 h after last administration)
Normal	5	CMC 0.5%	Tail Bleeding Assay
ASA	5	Aspirin	
Dose 1	5	Each of the Extract	
Dose 2	5		
Dose 3	5		

Note: Normal (CMC 0.5%), ASA (aspirin 0.208 mg/20 g BW), dose I (each extract 7 mg/20 g BW), dose II (each extract 14 mg/20 g BW), dose III (each extract 28 mg/20 g BW)

Table 3: Design experiment of survival rate assay in mice.

Group	Number of mice	Survival Rate Treatment	
		Day-1 to 7	Day-8
Normal	5	CMC 0.5%	Saline Injection
Model	5	CMC 0.5%	Thrombotic Induction
ASA	5	Aspirin	
Dose 1	5	Each of the Extract	
Dose 2	5		
Dose 3	5		

Note: Normal (CMC 0.5%), Model (CMC 0.5%), ASA (aspirin 0.208 mg/20 g BW), dose I (each extract 7 mg/20 g BW), dose II (each extract 14 mg/20 g BW), dose III (each extract 28 mg/20 g BW).

Statistical Analysis

The data obtained were further statistically processed using SPSS (version 18). Homogeneity of data was tested using Levene method, and the normality was confirmed using Shapiro-Wilk method. Normally distributed and homogeneous data were analyzed by one-way ANOVA test to assess the overall differences among the groups. LSD was performed to assess the differences between groups.

RESULTS

Total Flavonoid and Phenolic Contents

Total flavonoid of the extract was measured by using aluminum chloride assay. While the total phenolic content was measured by using the Folin-Ciocalteu assay. The extract of *C. xanthorrhiza* had higher total flavonoid and phenolic contents than *K. galanga* with value of 0.146% and 20.01%, respectively. While *K. galanga* extract give 0.004% and 0.930% for total phenolic and flavonoid content, respectively.

Table 4: The effect of extracts on bleeding time in mice.

Group	Bleeding Time (Mean ± SD)	
	***K. galanga* extract**	***C. xanthorrhiza* extract**
Normal	7.29 ± 1.31	5.91 ± 1.23
ASA	14.78 ± 2.85*	16.88 ± 3.53*
Dose 1	17.71 ± 1.96*	16.82 ± 4.38*
Dose 2	17.07 ± 2.80*	17.50 ± 1.51*
Dose 3	18.69 ± 1.58*	17.66 ± 1.24*

Note: *: $p \leq 0.05$ Comparison with the normal group

Bleeding Time

Table 4 shows that single oral doses of *K. galanga* and *C. xanthorrhiza* extracts significantly increased bleeding time in mice ($p \leq 0.05$) compared to the normal group. *K. galanga* extract at a dose of 7 mg/20 g BW showed an increase in bleeding time by 142.94%, at a dose of 14 mg/20 g BW by 134.16%, and at a dose of 28 mg/20 g BW by 156.38% compared to the normal group (Figure 1). While the extract of *C. xanthorrhiza* at a dose 7 mg/20 g BW resulted in an increase of bleeding time by 184.6%, at a dose of 14 mg/20 g BW by 196.11%, and at a dose of 28 mg/20 g BW by 198.82% (Figure 2). These results were qualitatively similar to the effect of ASA. ASA in the *K. galanga* extract group showed an increase in bleeding time by 102.74% compared to the normal group, while *C. xanthorrhiza* extract group by 185.62%. The increasing bleeding time indicated antithrombotic effects of ASA, *K. galanga* extract, and *C. xanthorrhiza* extract. For *C. xanthorrhiza* extract, there was a tendency to increase bleeding time with increasing dose, even though this did not reach statistical significance.

Survival Rate

Table 5 shows that *K. galanga* and *C. xanthorrhiza* extracts increased the survival rate in mice. *K. galanga* extract inhibited collagen-epinephrine induced thromboembolism by 80%, but there was a tendency to increase survival rates as dose increased. *C. xanthorrhiza* extract at dose 3 had the same result with ASA in inhibiting thromboembolism by 100%.

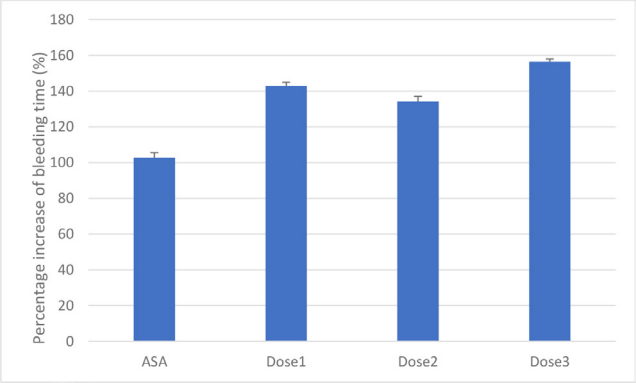

Figure 1: Bleeding time percentage increase of *Kaempferia galanga* extract and Aspirin group.

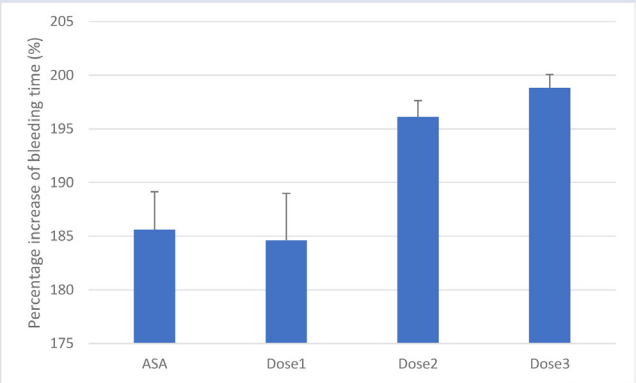

Figure 2: Bleeding time percentage increase of *Curcuma xanthorrhiza* extract and Aspirin group.

DISCUSSION

The antithrombotic effect of *K. galanga* and *C. xanthorrhiza* extracts was evaluated by comparing the bleeding time of samples to the normal group. While the bleeding time of the two samples did not differ significantly with ASA, which meant the effect was similar for both groups as antithrombotic. There was a tendency to increase the bleeding time of *C. xanthorrhiza* extract. A similar result was also found in the previous study, which showed that the bleeding time of *Ramulus mori* extract at dose 2 was lower than the dose 1.[18] According to the results of this experiment, the highest bleeding time prolongation of *K. galanga* and *C. xanthorrhiza* extracts was achieved at dose 3 (28 mg/20 g BW).

Antithrombotic activity of *K. galanga* and *C. xanthorrhiza* extracts had similarities with aspirin. Aspirin inhibits collagen-induced platelet aggregation optimally at doses of 81-162 mg/day.[19] The mechanism of action of aspirin is by inhibition of COX and non-COX. Inhibition of COX-1 will inhibit TxA_2 formation and stimulate platelet aggregation. In non-COX, aspirin alters GP IIb/IIIa receptor function and affects the permeability of clots. In addition, aspirin also inhibited acetylation of prothrombin, antithrombin, fibrinogen, and factor XIII.[19]

The thrombosis induction method used was a combination of collagen and epinephrine. Collagen is a major activator of granular secretions (serotonin and ADP) that works by binding to glycoprotein receptors on platelet surfaces and helping von willebrand factor (vWF) to increase cytosolic calcium concentration.[20] When injured, collagen activates platelets to attach to the subendothelium and form aggregates. Meanwhile, epinephrine can cause platelet aggregation induced by ADP.[13] Epinephrine is a β_2-adrenergic agonist which blocks the activation of adenylyl cyclase thereby increasing intracellular calcium and plays a role in vasoconstriction.[21] Epinephrine causes a disruption of the potassium ions exchange with sodium and calcium. Therefore, the number of potassium ions in extracellular is less than intracellular or commonly called hypokalemic. Hypokalemic causes stimulation of the muscle membrane is disrupted leading to paralysis or paralysis.[22-23] The deadly effects of collagen and epinephrine in mice were due to microcirculation by thromboembolism or vasoconstriction caused by an increase in TxA2 and prostaglandins from platelets.[9]

The active compound of *K. galanga* and *C. xanthorrhiza* extracts, cinnamaldehyde and curcumene, can increase bleeding time and survival rate significantly due to inhibition of platelet activation and aggregation. These compounds inhibited platelet aggregation induced by collagen, arachidonic acid, thrombin, and ADP.[10,24] They reduced the formation of TxA_2 on the cyclooxygenase pathway by suppressing the release of arachidonic acid from membrane phospholipids of platelets and inhibiting the conversion of arachidonic acid.[9-10] TxA_2 is a potent agonist of the platelets activation and thrombus formation. TxA_2 causes

Table 5: The effect of extracts on survival rate in mice.

Group	Survival Rate (%)	
	***K. galanga* extract**	***C. xanthorrhiza* extract**
Normal	-	-
Model	0	0
ASA	100	100
Dose 1	80	80
Dose 2	60	80
Dose 3	80	100

irreversible platelet aggregation, vasoconstriction, and proliferation of smooth muscle cells.[9]

In addition to inhibiting the cyclooxygenase pathway, cinnamaldehyde also served to inhibit the effect of vasoconstriction caused by vascular damage (vasodilator) and thromboxane A_2. This will disturb the calcium vasodilator influx and release of calcium so that the vascular smooth muscle becomes dilated.[25] The curcumene compound may also play a role in the lipooxygenase (LOX) pathway. In lipooxygenase pathway, curcumene increased the production of 12-hydroxyeicosatetraenoic acid (12-HETE) which can reduce the formation of TxA_2 through its precursor.[10] If the formation of thrombus was inhibited, the blood flow will be smooth and bleeding time will increase. The deadly effects of collagen-epinephrine induced thromboembolism or vasoconstriction in mice can be prevented by *K. galanga* and *C. xanthorrhiza* extracts.

CONCLUSION

The extract of *Kaempferia galanga* L. and *Curcuma xanthorriza* Roxb. had antithrombotic potency as its highest dose exhibited an increase in bleeding time and survival rate. The greatest antithrombotic potency was discovered at dose of 28 mg/20 g BW on both extracts. The most effective antithrombotic activity in both bleeding time and survival rate parameters was indicated by *C. xanthorrhiza* extract.

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CONFLICT OF INTEREST

No conflict of interest associated with this work.

ABBREVIATIONS

VTE: venous thromboembolism; PE: pulmonary embolism; COX: cyclooxygenase; ADP: adenosine diphosphate; TxA_2: thromboxane A_2; TXB_2:thromboxane B_2; CMC: Carboxymethylcellulose; HMT: hexamethylentetramine; DDY: Deutschland Denken Yoken; ASA: acetyl salicylic acid; LOX: lipoxygenase; vWF: von willebrand factor; 12 HETE: 12-hydroxyeicosatetraenoic acid.

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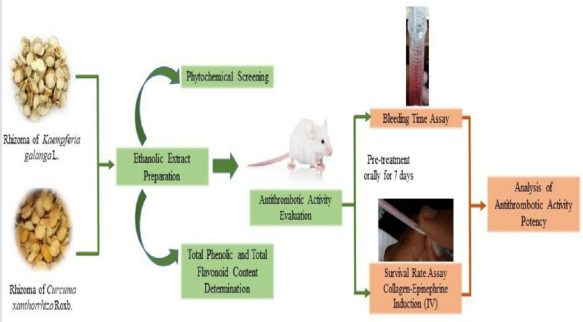
GRAPHICAL ABSTRACT



SUMMARY

- The present study provides the evidence that pre-treatment of *Kaempferia galanga* L. and *Curcuma xanthorriza* Roxb. affect bleeding time prolongation and protect mice from thromboembolic death induced by collagen-epinephrine in mice.

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