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Inhibitory activities of ethanol extracts from *Boesenbergia rotunda*, *Zingiber purpureum*, and *Alpinia galanga* on *in vitro* agonists induced human platelet aggregation

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Abstract
The rhizomes of *Boesenbergia rotunda*, *Zingiber purpureum*, and *Alpinia galangal* are well-known Thai medicinal plants has been used as spice and folk medicine due to its numerous functional and nutritional properties. The present study, the anti-platelet aggregation activities of the ethanol extracts from the rhizomes of rhizomes of *B. rotunda*, *Z. purpureum*, and *A. galangal* were evaluated using 96-well plate platelet aggregation assay. The ethanol extracts from *B. rotunda*, *Z. purpureum*, *A. galanga* (IC$_{50}$ 0.064 ± 0.039, 0.019±0.721, and 0.113±0.031 µg/mL) were more active than the ibuprofen (IC$_{50}$ 0.439 ± 0.064 µg/mL) in ADP-induced platelet aggregation (P<0.05). The inhibitory activity of extract from *Z. purpureum* (IC$_{50}$ 0.024±0.08 µg/mL) was comparably active to the ibuprofen (IC$_{50}$ 0.036±0.006 µg/mL) in the AA induced platelet aggregation; however, the extracts from *B. rotunda* and *A. galangal* (IC$_{50}$ 0.054±0.031 and 0.068±0.026 µg/mL) were less potent than ibuprofen (P<0.05).The ethanol extracts from *B. rotunda* and *Z. purpureum* (IC$_{50}$ 0.025±0.008 and 0.012±0.015 µg/mL) were more potent than ibuprofen (IC$_{50}$ 0.056±0.023 µg/mL) in collagen-induced platelet aggregation (P<0.05). The extracts from *Z. purpureum* and *A. galanga* (IC$_{50}$ 225±0.094 and 190±0.075 µg/mL) possessed inhibitory activities on thrombin-induced platelet aggregation. The ethanol extracts from the rhizomes of rhizomes of *B. rotunda*, *Z. purpureum*, and *A. galangal* were inactive in the platelet aggregation assay using U-46619 and TRAP-6 as agonists.

Keywords: *Boesenbergia rotunda*, *Zingiber purpureum*, *Alpinia galanga*, platelet aggregation inhibitory activity

Introduction
Platelets are the smallest blood elements presented in blood circulation, which are produced from the progenitor cells called megakaryocytes in bone marrow [1]. They play the central roles in the regulation normal vascular and coagulation function [2]. Under physiological condition, platelets play an important role in the hemostatic processes in response to vascular damage. Platelets undergo three crucial steps of physiological and biochemical changes composing of initiation, activation, and perturbation. Multiple pathways of platelet activation contribute platelet activation, which require specific agonists such as adenosine diphosphate (ADP), thrombox-ane A2 (TxA2) and thrombin [3]. As the results of inappropriate and excessive platelet activation in rupture of atherosclerotic plaques, activated platelets stimulate thrombus formation in response to rupture of an atherosclerotic plaque or endothelial cell erosion, promoting atherothrombotic disease. Atherothrombosis is the phenomenon of atherosclerotic lesion disruption with superimposed thrombus formation that is the major cause of acute coronary syndromes and cardiovascular death [4]. Thus, the inhibition of platelet aggregation is important to prevent the progression of platelet-associated cardiovascular diseases. Antiplatelet medications, for example, aspirin, clopidogrel, dipyridamole, ticlopidine, play important roles in the treatment and prevention of thrombotic events. However, antiplatelet therapy is inevitably associated with a risk of hemorrhage, as the same pathways by which anti-platelet drugs antagonize platelet activation or aggregation increase the risk of bleeding [5]. Discovery of newer antiplatelet agents from both edible and medicinal plants with minimal adverse effects raise a challenge. Several plants in the ginger family (Zingiberaceae) are well-known as sources of traditional medicines, spices, and vegetables. *Boesenbergia rotunda* (L.) Mansf., called Kra Chai in Thai, is a well-known ginger as spice in Thai cuisine and as Thai medicinal plant. The rhizomes and roots of this plant were use as carminative medicine, anti-dysentery remedy, as well as spice in Thai cuisine. Several types of flavonoids are the major constituents

from the rhizomes and roots, for example, chalcones, flavanones, and flavones [6]. The biological activities of both crude extracts and pure compounds have been continuously reported from this plant, e.g., anti-inflammatory, anti-HIV-1 protease, anti-bacterial, and anti-cancer activities [7, 11]. *Zingiber purpureum* Roscoe, Plai in Thai, was traditionally used as muscle pain relief, wound healing, and carminative remedies. The major chemical constituents are phenylbutenoids, monoterpenoids, and phenolic compounds [12]. Various biological activities were reported from this plant such as anti-inflammatory, cytotoxic, and anti-bacterial activities [13, 16]. *Alpinia galanga* (L.) Wild, called Kha in Thai, is used as a spice in many Thai cuisines and folk medicines for carminative, antitussive, antiarthritic, and anti-urticaria. Certain terpenoids, lignans, flavonoids, and phenolic compounds are the major chemical constituents from this plant [17]. The important bioactivities were reported from this plant including cytotoxic, anti-cancer, and anti-inflammatory activities [18, 21]. To our best knowledge, there is no report on the anti-platelet aggregation activities by using human platelet model. In the present study, we assess the anti-platelet activities of the ethanol extracts from the rhizomes of *B. rotunda*, *Z. purpureum*, and *A. galanga* on the *in vitro* human platelet aggregation inhibitory using ADP, AA, U-46619, thrombin, and TRAP-6 as aggregating agents.

Materials and Methods

General

The PST-60HL (bioSan, Riga, Latvia) temperature-controlled orbital shaker was used to generate shear stress with the human body-liked constant temperature. The absorbance intensity was measured on the iMark™ Microplate Reader (Bio-Rad Laboratories, Inc., California, USA). ADP, thrombin, TRAP-6 and collagen were purchased from Sigma-Aldrich (Missouri, USA). AA and U-46619 were purchased from Cayman chemical (Michigan, USA). All other chemicals were analytical grade.

Plant materials and preparation

Plant specimens were collected from local farm at Muang District, Phayao Province in November 2016-March 2017. The samples were deposited with voucher specimen numbers at the Herbarium of Walairukavej Botanical Research Institute and identified as *Boesenbergia rotunda* (L.) Mansf. (khumgratok no.01-11), *Zingiber purpureum* Roscoe (khumgratok no.02-11), *Alpinia galanga* (L.) Wild. (khumgratok no.03-12). The rhizomes of three gingers were cut and dried in hot air oven at 60°C for 48 hours. Dried rhizomes (3 kg) were ground and extracted with 95% v/v ethanol by maceration for three days at room temperature. The extraction was repeated for three times of maceration. The ethanol was removed from the extracts under vacuum condition to yield ethanol crude extracts. The extract was kept from light and stored in -20°C refrigerator until use. At the time of experiment, the extract was dissolved in 50% dimethyl sulfoxide (DMSO)-water prior to perform the assay.

Human platelet preparation

The research protocols on human subjects were approved by the University of Phayao Human Ethics Committee (Approval No. 2/240/60). The healthy volunteers are 19-22 year-old subjects without history of medication taken during last 14 days. The human platelet-rich plasma (PRP) was prepared according to the previously described protocols [21]. The 3.8% citrated blood was centrifuged at 110*g*, 22±0.5°C for 5 minutes. Platelet-rich plasma (PRP) was collected, and the remaining packed red cell was further centrifuged at 3500*g*, 22±0.5°C for 5 minutes to obtain platelet-poor plasma (PPP). Washed platelet was additionally prepared for the thrombin-induced platelet aggregation. PRP was washed with calcium-free Tyrode buffer pH 7.35 and centrifuged at 2700*g*, 22±0.5°C for 5 minutes. Platelet was suspended in Tyrode buffer pH 7.35 and kept in a water bath at 37°C prior to assay.

Antiplatelet aggregation assay

Platelet aggregation assay was performed in a 96-well microplate format. The unwashed platelet aggregation, PRP (190 µL) was pre-incubated with 10 mM $CaCl_2$ solution (2 µL) and ethanol extract or referent compounds (4 µL) for 2 minutes at 37°C with orbital agitation of 1000 rpm. At the indicated time, an aliquot (4 µL) of ADP, AA, TRAP-6 or collagen were added into well to yield a final concentration of 0.5 mM, 1 mM, 1.5 mM, 100µg/mL, respectively. After incubation ended (18, 10, 10 minutes for ADP, AA, and TRAP-6/collagen), the absorbance of the suspension was measured on a microplate reader at 595 nm against PPP. Thrombin-induced platelet aggregation was assayed with washed platelet. Aliquot of washed platelet (190 µL) was preincubated with ethanol extract or referent compound (2 µL) in the same condition to PRP. After that, thrombin solution (final concentration of 0.4 IU/mL) was added and further incubated for 10 minutes. The absorbance was read at 595 nm against suspension buffer. The inhibition percentage was calculated according to the following equation.

$$\% \text{ inhibition} = \left[1 - \left(\frac{A_{test}}{A_{blank}}\right)\right] \times 100$$

A solution of 50% DMSO/$H_2O$was used as blank instead of ethanol extract or referent compounds and noted as 0% aggregation. The half inhibition (IC_{50} value) was calculated by the plot between the concentrations of test or referent compounds against inhibition percentage. The duplicate screenings of ethanol extract or referent compounds were assayed at the final concentration of 300 µg/mL. The active compounds were obtained to assay independently with platelets from five volunteers (n=5) and each experiment was duplicate.

Statistical analysis

Data analysis was performed on the IBM SPSS Statistics software version 22 licensed to the University of Phayao. The IC_{50} values were presented as mean±SEM. One-way ANOVA was used to test for overall differences. The significant ANOVA was followed by Duncan multiple comparisons for pair-wise differences between the treatment groups. A P value of less than 0.05 was considered statistically significant.

Results and Discussion

The gold standard platelet function assay is platelet aggregometry that widely applied for the evaluation for an antiplatelet activity of the natural products [23]. However, the standard aggregometry utilizes a large amount of both platelet-rich plasma and test compounds. The microtiter plate-based platelet aggregation assay is a high throughput assay with acceptable reproducibility and accuracy for both clinical study of platelet function and biological investigation of antiplatelet activity [24]. Therefore, we selected this assay format for both screening and determination of the IC_{50} values. The extract showed the inhibition percentage over 50% which indicated the extract were active in the agonists induced platelet aggregation assay. All of the extracts were

screened at the final concentration of 300 µg/mL and the results were summarized in figure 1. The ethanol extract from *B. rotunda*, *Z. purpureum*, and *A. galangal* exhibited the inhibitory effects on the ADP, AA, and collagen induced platelet aggregation. Proteolytic activity of thrombin was inhibited by the ethanol extracts from *Z. purpureum* and *A. galangal* in thrombin induced platelet aggregation. None of the extracts was active in the U-44619 and TRAP-6 induced platelet aggregation. The active extracts were further assayed for the assessment of inhibitory activities (Table 1).

The effects of extracts on the purinergic receptor mediated platelet aggregation were investigated by the ADP, a specific agonist. The extracts from *B. rotunda*, *Z. purpureum*, *A. galanga* (IC_{50} 0.064 ± 0.039, 0.019±0.721, and 0.113±0.031 µg/mL) were more active than the ibuprofen (IC_{50} 0.439 ± 0.064 µg/mL), a reference compound ($P<0.05$). The result indicated that the ethanol extract from the roots of *B. rotunda*, *Z. purpureum*, *A. galanga* had inhibitory effects on the ADP-mediated platelet activation and/or aggregation. The certain platelet G-protein-coupled purinergic receptors such as P2Y1 and P2Y12 are responsible for the secretory ADP to cause platelet shape change and aggregation. The P2X1 receptor is another platelet ligand-gated ion-channel purinergic receptor responsible for ATP, which cause only platelet shape change [25, 26]. These extracts exerted an inhibitory activity on the P2Y1 and P2Y12 receptors mediated platelet aggregation, but the exact mechanisms of inhibition are not clarified by our result. Although P2Y1 and P2Y12 receptor activation causes platelet aggregation, only P2Y1 receptor causes platelet shape change. Our findings obtained from the monitoring of final aggregation which could not differentiate the inhibitory effects of the extract on shape change only or aggregation.

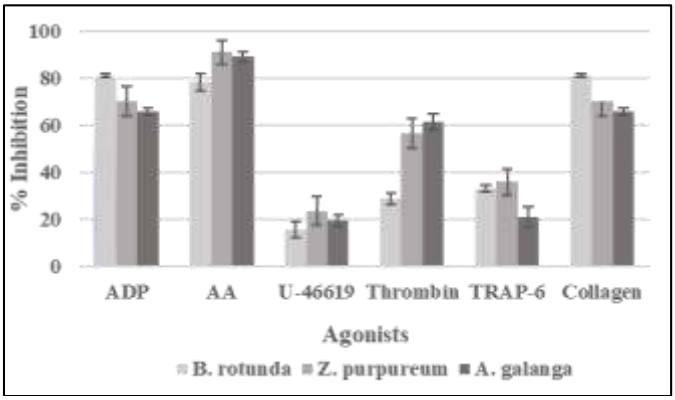

Fig 1: The inhibition percentage of ethanol extract from *B. rotunda*, *Z. purpureum*, and *A. galanga* on the agonist-induced human platelet aggregation (n=5).

The inhibitory effects of the ethanol extract on the cyclooxygenase-thromboxane A2 mediated platelet aggregation was investigated. The inhibitory activity of extract from *Z. purpureum* (IC_{50} 0.024±0.08 µg/mL) was comparably active to the ibuprofen (IC_{50} 0.036±0.006 µg/mL) in the AA induced platelet aggregation. However, the extracts from *B. rotunda* and *A. galangal* were less potent than the referent drug ($P<0.05$) in the same assay. The platelet cyclooxygenase-1 (pCOX-1) is a dimeric heme-iron dioxygenase that responsible for the first step of thromboxane A2 (TxA2) biosynthesis. It could assume that the extracts had inhibitory activities on pCOX-1 and/or TxA2 receptor. According to the screening assay, the result demonstrated that all extracts did not interfere the interaction between the TxA2 analogue, U-46619, and TxA2 receptor. The platelet thromboxane-prostanoid-alpha (TPα) receptor is the predominant in platelet, which is comprised of two G-protein-coupled receptors, Gq and G12/13 [27, 28]. The results clearly demonstrated that the ethanol extracts from *B. rotunda*, *Z. purpureum*, *A. galangal* had the pCOX-1 enzymatic inhibition without TxA2-TPα binding interference.

The inhibitory activities on platelet activation through the induction of collagen is also studied. The results showed that the ethanol extract from *B. rotunda* and *Z. purpureum* (IC_{50} 0.025±0.008 and 0.012±0.015 µg/mL) were more potent than the standard drug (IC_{50} 0.056±0.023 µg/mL) ($P<0.05$). The inhibitory effects of extract from *A. galangal* and ibuprofen were the same degree of inhibition. In our defined assay under shear condition, two surface collagen receptors such as integrin α2β1 and glycoprotein VI (GPVI) receptors response for the collagen-induced platelet activation [29, 31]. The activation of platelets through the collagen receptor requires secondary messengers (ADP, TxA2) which are released by the synthesis/secretion and degranulation from platelets. The inhibitory mechanisms of the ethanol extracts from *B. rotunda* and *Z. purpureum* were complicated. The extract had inhibitory effects on ADP and AA mediated platelet aggregation, therefore, the most possible mechanisms of inhibition could be purinergic intervention, pCOX-1 enzymatic inhibition, and integrin α2β1/GPVI interference.

Thrombin is the proteolytic enzyme that is a strong agonist on the platelet aggregation [32]. The inhibitory mechanisms of the ethanol extract from these gingers on thrombin-induced aggregation was revealed by our findings. The extracts from *Z. purpureum* and *A. galanga* displayed potent inhibitory activities (IC_{50} 225±0.094 and 190±0.075 µg/mL). The extract from *B. rotunda* had no effects on the thrombin-induced platelet aggregation, which clearly demonstrate that the extract did not exert any enzymatic inhibition on the thrombin activity. Moreover, the interfering effect of the extracts from three gingers on the thrombin receptor-activating peptide (TRAP) and thrombin receptor was not observed by our defined assay. this important result allowed us to conclude that the ethanol extract from *B. rotunda*, *Z. purpureum*, and *A. galanga* did not exert inhibitory activities on thrombin mediated platelet aggregation. The results allowed us to conclude that *Z. purpureum* and *A. galangal* inhibited platelet aggregation though the proteolytic cleavage inhibition without the interference on TRAP and TR interaction.

Table 1: Inhibitory activity of ethanol extract from *B. rotunda*, *Z. purpureum*, *A. galanga* on the agonist-induced human platelet aggregation.

Extracts/Standard	IC_{50} (Mean±SEM, n=5)					
	ADP	AA	U-46619	Collagen	Thrombin	TRAP-6
B. rotunda	0.064 ± 0.039*	0.054±0.031*	NA	0.025±0.008*	NA	ND
Z. purpureum	0.019±0.721 *	0.024±0.08	NA	0.012±0.015*	225±0.094	NA
A. galanga	0.113±0.031*	0.068±0.026*	NA	0.051±0.011	190±0.075	NA
Ibuprofen	0.439 ± 0.064	0.036±0.006	NA	0.056±0.023	ND	NA

* $P<0.05$ statistic difference as compared to positive control, Ibuprofen.
NA not active at the concentration of 300 µg/mL
ND not determined

Conclusion

These data suggested us to determine the anti-platelet activity from *B. rotunda*, *Z. purpureum*, and *A. galanga*. Our obtained results clearly demonstrate the significant anti-platelet activities of the ethanol extract from these gingers through the inhibition of ADP, AA and collagen induced platelet aggregation. Moreover, *Z. purpureum* and *A. galanga* exhibited the inhibitory effects on thrombin mediated platelet aggregation. However, the exact mechanisms of inhibition were not clarified by our defined protocol. The extensive studies are needed to reveal the inhibitory effects of the platelet activation pathways of the ethanol extract from these plants. The phytochemical investigation is also required to identify the active constituents from these gingers. According to our preliminary findings, the rhizomes of *B. rotunda*, *Z. purpureum*, and *A. galanga* had pharmacological potential for the preventive and therapeutic application.

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Conflict of interest: Authors declare no conflict of interest.

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