Alkaloids from *Nauclea orientalis* Inhibited *in vitro* ADP and Thrombin Induced Human Platelet Aggregation

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ABSTRACT

A series of alkaloids isolated from the roots of *N. orientalis* is investigated for the inhibitory activities on *in vitro* agonists induced human platelet aggregation. Human platelet samples were obtained to investigate the anti-platelet activity by high throughput 98-well microtiter plate format. Adenosine diphosphate (ADP), arachidonic acid (AA), thrombin and thrombin receptor activating peptide-6 (TRAP-6) were used as agonists for *in vitro* human platelet aggregation. All alkaloids were inactive in the AA induced platelet aggregation. Compound 2 was the only alkaloid to inhibit ADP induced platelet aggregation with the IC_{50} value of 27.01±7.67 μM and was more potent than the standard drug, ibuprofen ($P < 0.05$). The compounds 1, 3, 4, 5 and 7 were more potent than the standard drug to inhibit thrombin induced platelet aggregation with the IC_{50} values of 3.05±0.22, 4.41±0.47, 7.50±0.22, 45.69±1.74 and 4.89±0.13 μM ($P <0.05$), respectively. None of the potent alkaloids in thrombin-mediated platelet aggregation exhibited the inhibitory effect in TRAP-6 induced platelet aggregation. Compound 2 could inhibit platelet aggregation through the interference of platelet purinergic receptors (P_2Y_1 and P_2Y_{12} receptors). Moreover, compounds 1, 3, 4, 5 and 7 could have inhibitory effects on thrombin-induced platelet aggregation through the proteolytic inhibition without the interferences of ligand-receptor interaction.

Keywords: *Nauclea orientalis*; anti-platelet activity; alkaloids

Introduction

Platelets, the smallest blood element in the circulation, play the important roles in normally hemostatic processes including adhesive and cohesive functions in the thrombus formation and the activation function of the circulatory coagulation factors. The morphological and biochemical changes of platelets are initiated by the exposure to several specific agonists (ADP, AA, thrombin, collagen) and lead to the platelet activation, adhesion and eventually

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aggregation [1]. Under pathological conditions, excessive and unappropriated platelet activation are associated with the morbidity and mortality of the atherosclerosis-related diseases such as cardiovascular and cerebrovascular disorders [2]. Therapeutic drugs with anti-platelet properties are utilized for the clinical prevention and treatment of these diseases such as aspirin, clopidogrel, ticlopidine and dipyridamole. Although these anti-platelet drugs are clinically used, they have reportedly serious adverse effects such as risks of gastrointestinal, retroperitoneal, and intra-cerebral hemorrhage [3]. Thus, anti-platelet substances with minimal side effects from natural origins are primary targets for drug discovery. Several classes of natural products have been investigated for the anti-platelet activity, including flavonoids, stilbenoids, coumarins, and indole alkaloids [4-5]. The plant species *Nauclea orientalis*, Thai name Kanluang, is a medium-sized tree belonging to the family Rubiaceae. Several parts of this plant are used as Thai traditional medicine. The roots and leaves of this plant are used as pain relief and antipyretics. Recently, two new indole alkaloids along with several known alkaloids were isolated from the roots of *N. orientalis* with cytotoxic activities [6-7]. To our best knowledge, there is no report on anti-platelet activity from this medicinal plant. In the present study, we obtained the isolated alkaloids from this plant to evaluate the *in vitro* human platelet aggregation inhibitory using ADP, AA, thrombin and TRAP-6 as aggregating agents.

Materials and Methods

General materials

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

Test compounds

The alkaloids were obtained from the roots of *N. orientalis*, namely, naucleaoral A (1), naucleaoral B (2), naucleactonin A (3), naucleficine (4), 19-*epi*-naucleidinal (5), naucleidinal (6), pumiloside (7) and strictosamide (8) (Figure 1). The isolation and structural elucidation were performed on chromatographic and spectroscopic basis as previously described [6-7].

Human platelet preparation

The research protocols on human subjects were approved by the Human Ethic Committee of University of Phayao (Project No. 2/018 and 048-9/58). The healthy subjects were suggested and not allowed to take any medication from 2 weeks prior to the study to the end of the study. Blood samples were collected by venipuncture from the median cubital vein. Platelet rich plasma (PRP) was prepared by differential centrifugation of 3.8% citrated blood with the anticoagulant-whole blood ratio of 9:1. The citrated blood was then 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 diluted with calcium-free Tyrode buffer pH 7.35 (136 mM NaCl, 1.27 mM KCl, 12 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 1 mM $MgCl_2$ and 5.5 mM glucose) with the ratio of 1:10 and centrifuged at 2700*g*, 22±0.5°C for 5

minutes. Supernatant was discarded then packed platelets were washed again in the same condition. Platelets were suspended in Tyrode buffer pH 7.35 (136 mM NaCl, 1.27 mM KCl, 12 mM $NaHCO_3$, 0.34 mM NaH_2PO_4, 2 mM $CaCl_2$, 1 mM $MgCl_2$, 0.1% bovine serum albumin and 5.5 mM glucose) and kept in water bath at 37°C prior to assay. Both PRP and platelet suspension were counted and adjusted to 350,000-450,000 platelets/mL with autologous PPP or Tyrode buffer pH 7.35.

Anti-platelet aggregation assay

Platelet aggregation assay was performed in a 96-well microplate format [8]. The unwashed platelet aggregation, PRP (190 µL) was preincubated with 10 mM $CaCl_2$ solution (2 µL) and test or referent compounds (4 µL) for 2 minutes at 37°C with orbital agitation of 1000 rpm. At the indicated time, aliquots (4 µL) of ADP, AA and TRAP-6 were added to yield final concentrations of 0.5, 1 and 1.5 mM, respectively. After incubation ended (18, 10, 10 minutes for ADP, AA and TRAP-6), the absorbance of suspension was measured on the microplate reader at 595 nm against PPP. Thrombin induced platelet aggregation was assayed with washed platelets. Aliquot of washed platelet (190 µL) was preincubated with test or referent compound (2 µL) in the same manner as PRP. After that, thrombin solution (final concentration of 0.4 IU/mL) was added and further incubated for 10 minutes. The absorbance (A) was read at 595 nm against suspension buffer. The inhibition percentage was calculated according the following equation;

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

Normal saline solution was used as blank instead of test 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. The test or referent compounds were assayed independently with platelets from five volunteers (n=5) and each experiment was duplicated. The platelet plug formation in a microtiter plate was transferred to a glass slide and observed under a light microscope.

Statistical analysis

Data were presented as mean±SEM. One-way ANOVA was used to test for overall differences. The significant ANOVA was followed by Tukey HSD test for pair-wised differences between the treatment groups. A P value of less than 0.05 was considered statistically significant.

Results and Discussion

Platelet aggregation is a hemostatic process to provide appropriate blood coagulation under both normal and pathological conditions. Platelets play more crucial roles in certain vascular disorders; e. g. , atherosclerosis, cardiovascular disorders, and cerebrovascular diseases. Inappropriate activation and platelet hyperreactivity are associated with severe life-threatening conditions such as ischemic thrombosis, myocardial ischemia, myocardial infarction, ischemic stroke, etc. [9]. Therapeutic and preventive medications, anticoagulants, and anti-platelet agents, are utilized to reduce those of clinical outcomes. Although several types of anti-platelet drugs are used for treatment of thrombosis-related disorders, some seriously adverse effects of these drugs are noticed as increased risk of bleeding. Therefore, searching for new anti-platelet agents with minimal side effects from natural origins has been extensively studied. However, the investigation of anti-platelet activity is primarily based on Born's platelet aggregometry, the gold standard method for platelet function assay [10]. The required smaller amounts of platelets usage, reproducible, practical, and reference-equivalent methods are preferable. The microtiter plate platelet aggregation is a high throughput assay with acceptable

reproducibility and accuracy for both clinical study of platelet function and biological investigation of antiplatelet activity [8]. Therefore, we selected this method for the study both screening and determination of the IC_{50} values.

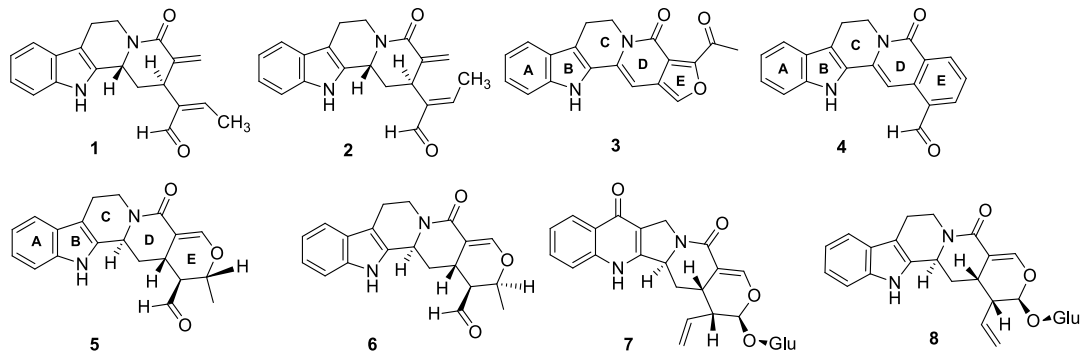

Fig. 1. The chemical structures of alkaloids isolated from *N. orientalis*

Table 1. Anti-platelet activity of the alkaloids from *N. orientalis*.

Compounds	Half Inhibition (μM±SEM; $n = 5$)			
	ADP	AA	Thrombin	TRAP-6
1	>50	>50	3.05±0.22*	>50
2	27.01±7.67*	>50	>50	>50
3	>50	>50	4.41±0.47*	>50
4	>50	>50	7.50±0.22*	>50
5	>50	>50	45.69±1.74*	>50
6	>50	>50	>50	>50
7	>50	>50	4.89±0.13*	>50
8	>50	>50	>50	>50
Indomethacin	ND	4.72±0.45	ND	ND
Ibuprofen	168.11±3.26	ND	1,770 ± 128	ND

*$P<0.05$ as compared to positive control.
ND Not determined

As for the results, 2 was the only potent alkaloid to inhibit the formation of platelet aggregates induced by ADP at the screening concentration of 50 μM (Figure 2C) . As compared to 2, compound 1 was completely inactive under the assayed condition (Table 1). It was indicated that the presence of *trans* configuration in four-member ring indole alkaloid 2 could enhance inhibitory activities as compared to the *cis* configuration alkaloid 1. The compound 2 probably possessed the inhibitory mechanisms through the interferential effects between ADP and platelet P_2Y_1/ P_2Y_{12} receptors mediated platelet aggregation. However, the results could not differentiate the exactly inhibitory activity of 2 on a G_q-coupled P_2Y_1 or the G_i-coupled P_2Y_{12} receptors [11]. Thromboxane A_2, eicosanoid, is synthesized by platelet cyclooxygenase-1/ thromboxane synthase complex using AA as substrate and capable

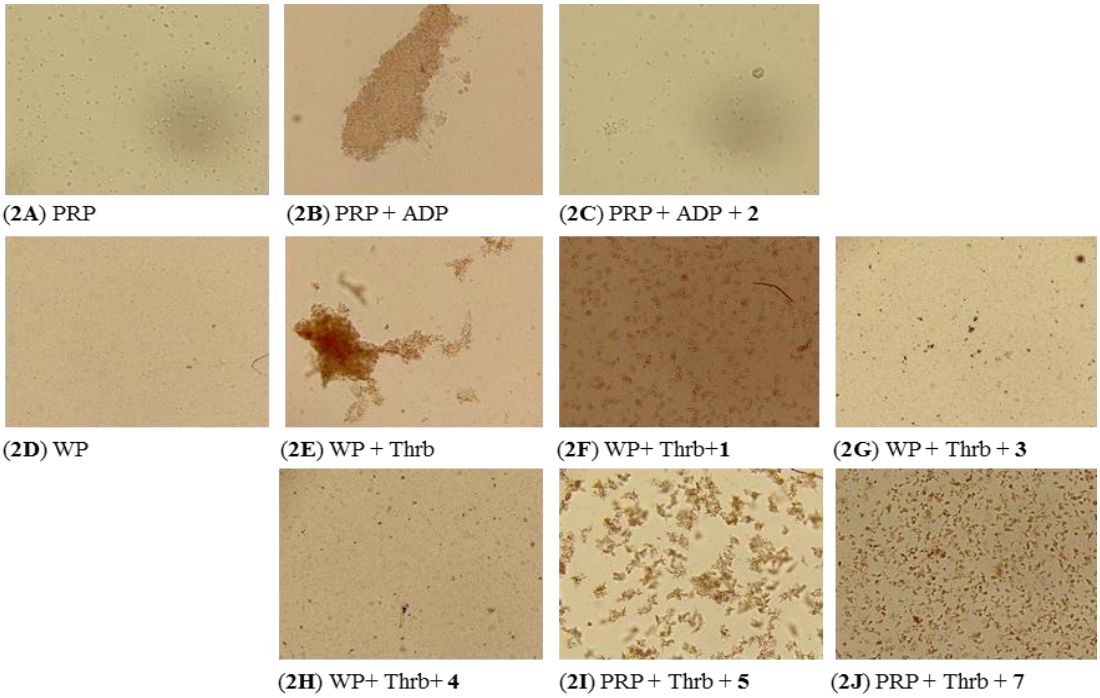

Fig. 2. Human platelet aggregation was induced by ADP (2B) and thrombin (Thrb) (2E) to form platelet aggregate as compared to PRP or washed platelets (WP). Compound 2 (2C) could inhibit the formation of platelet aggregate as compared to ADP-induced PRP (2B). Compounds 1 (2F), 3 (2G), 4 (2H), 5 (2I) and 7 (2J) could reduce platelet aggregate size as compared to the absence of alkaloids (2D). The pictures were taken under light microscope with 400X (2A-2C) and 100X (2D-2J) magnification.

of activating platelets through the interaction with thromboxane-prostanoid (TP) receptors [12]. The results showed that all the alkaloids had no inhibitory effects on the AA induced platelet aggregation at the screening concentration. It could be concluded that the selected alkaloids were unable to inhibit either platelet cyclooxygenase-dependent platelet activation or the interaction between of thromboxane A_2 and thromboxane-prostanoid (TP) receptors signaling pathway. The results showed that all the alkaloids had no inhibitory effects on the AA induced platelet aggregation at the screening concentration. The inhibitory activity of the alkaloids on protease activating receptors (PARs) mediated platelet aggregation was evaluated by using human thrombin as aggregating agent. Compounds 1, 3, 4, 5 and 7 were the active alkaloids to inhibit platelet plug formation which is induced by human thrombin (Figure 2F-2J). The compounds 2, 6 and 8 were inactive for thrombin induced platelet aggregation. The *cis* configuration of 1 had more influential effects on the inhibitory activity than the *trans* configuration of 2 ($P<0.05$) which caused complete inactivity under the defined assay condition. It was indicated that the geometrical isomerism affected the inhibitory activity. The differences in stereoisomerism also affected the inhibitory activity as observed in 5 and 6, of which the later compound was inactive ($P<0.05$). The differences in the types of ring E could affect the inhibitory activities, which heightened the potency of compound 3 more potent than 4, 5 and 6 ($P<0.05$). The presence of 2-acetylfuranyl moiety in 3

might increase the inhibitory activity as compared to the 1-formylphenyl moiety in 4, and the 2-formylpyranyl moiety in 5 and 6, respectively. Compounds 3 and 7 exhibited the same degree of inhibitory activity. Thrombin, the endolytic serine protease, cleaves between the amino acid residue Arg-41 and Ser-42 of the N-terminal-extracellular domain of protease activating receptors (PARs) located on the surface of platelet to unmask a new active Ser-Phe-Leu-Leu-Arg-Asn-containing peptide (SFLLRN), a so-called thrombin receptor activating peptide (TRAP). This peptide residue further interacts with PARs and initiates platelet activation [13]. To investigate the mechanism of action, the synthetic SFLLRN peptide (TRAP-6) was utilized to identify the mechanism of inhibition of the active alkaloids through the thrombin signaling pathway. It was found that those of the active alkaloids were inactive in TRAP-6 induced platelet aggregation. This critical result allowed us to presume that 1, 3, 4, 5 and 7 inhibited platelet aggregation through proteolytic inhibition of thrombin without the interference of TRAP-6 and PARs interaction.

In conclusion, the indole alkaloids 1, 3, 4 and 5, and alkaloid glycoside 7 inhibited platelet aggregation by inhibition of thrombin activity without interfering with the purinergic receptor, platelet cyclooxygenase-1, or TRAP signaling pathways. Our results clearly demonstrated that the most likely extracellularly inhibitory mechanism of these alkaloids was the enzymatic inhibition of thrombin. The enzyme kinetic studies are needed to clarify the mechanism of enzyme inhibition of the indole alkaloids 1, 3, 4 and 5. Additional investigations are also needed to determine the plausible effects of these active alkaloids on the G-protein-coupled receptor intracellular signaling pathways such as Rho-guanine-nucleotide exchange factors (RhoGEFs) dependent platelet shape change and phospholipase Cβ mediated calcium mobilization, mitogen-activated protein (MAP) kinase and protein kinase C activation [14]. Furthermore, the studies are also needed to differentiate the exact mechanism of 2 on ADP induced platelet activation through the platelet purinergic receptor P_2Y_1 or P_2Y_{12} receptors [15]. The obtained results clearly reveal the significance and possibility of these plant constituents in searching for new drugs to build the next platform of new antiplatelet agents.

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Conflict of Interest

The authors have declared that there is no conflict of interest.

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