Natural Product Sciences
26(4) : 295-302 (2020)
https://doi.org/10.20307/nps.2020.26.4.295

Antiplatelet Effect of Cudraxanthone L Isolated from *Cudrania tricuspidata* via Inhibition of Phosphoproteins

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Abstract – *Cudrania tricuspidata* (*C. tricuspidata*) is a deciduous tree found in Japan, China and Korea. The root, stems, bark and fruit of *C. tricuspidata* has been used as traditional herbal remedies such as eczema, mumps, acute arthritis and tuberculosis. In this study, we investigated the potential efficacies of this natural compound by focusing on the inhibitory effect of cudraxanthone L (CXL) isolated from the roots of *C. tricuspidata* on human platelet aggregation. Our study focused on the action of CXL on collagen-stimulated human platelet aggregation, inhibition of platelet signaling molecules such as fibrinogen binding, intracellular calcium mobilization, fibronectin adhesion, dense granule secretion, and thromboxane A_2 secretion. In addition, we investigated the inhibitory effect of CXL on thrombin-induced clot retraction. Our results showed that CXL inhibited collagen-induced human platelet aggregation, intracellular calcium mobilization, fibrinogen binding, fibronectin adhesion and clot retraction without cytotoxicity. Therefore, we confirmed that CXL has inhibitory effects on human platelet activities and has potential value as a natural substance for preventing thrombosis.
Keywords – Cudraxanthone L, Ca^{2+} mobilization, αIIb/β3 affinity, clot retraction, cyclic adenosine monophosphate

Introduction

Platelet aggregation is essential for the maintenance of hemostasis, but it can also trigger thrombosis. The thrombosis is a critical factor for patients who have risks such as hypertension, atherosclerosis, and cardiovascular disease Despite numerous investigations into the discovery and development of more effective antiplatelet and antithrombotic drugs, the mortality rate is still weak. Therefore, inhibition of platelet function is a promising approach in preventing platelet-mediated risks.[1] Collagen, a platelet agonist, is known to stimulate platelet aggregation by binding to the integrin α2β1 and glycoprotein VI on platelet surface. This binding hydrolyzes membrane phosphatidylinositol 4, 5-bisphosphate (PIP_2) to inositol 1, 4, 5-trisphosphate (IP_3) and diacylglycerol. IP_3 mobilizes Ca^{2+} from endoplasmic reticulum and activates granule secretion. Another way to increase $[Ca^{2+}]_i$ level is influx from extracellular spaces. Depletion of the $[Ca^{2+}]_i$ level is known to connect the influx of extracellular Ca^{2+}, which is stimulated by extracellular signal-regulated kinases (ERK).[2,3] These signaling cascades facilitate to the activation of glycoprotein IIb/IIIa (αIIb/β_3), an abundant integrin at the platelet surface. αIIb/β_3 can bind to adhesive proteins (i.e. fibrinogen, fibronectin, vitronectin, and thrombospondin) and induce Ca^{2+} mobilization, granule secretion, and formation of thrombus.[4]

Cudrania tricuspidata (*C. tricuspidata*) is widespread throughout Asia and used in traditional medicine.[5] The beneficial effects of *C. tricuspidata* have been traditionally associated with anti-inflammatory activity,[6] anti-tumor effect,[7] a-glucosidase inhibition,[8] and antioxidant and cytotoxic activities.[9] It has been also found to exert an anti-platelet effects on collagen-induced rat platelet aggregation,[10] and antihypertensive effect through increase of nitric oxide in vascular tissues.[11] Aforementioned various effects are achieved by xanthones and flavonoids, major constituents in *C. tricuspidata*. Our previous study showed that the inhibitory effects of steppotenin and isoderone on collagen-induced human platelet aggregation.[12,13] Therefore, we searched for a new substance from various xanthones and flavonoids in *C. tricuspidata*. We investigated 8 single compounds such as alboctalol, cudraxanthone D,

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cudraflavanon B, isolupalbigenin, xanthone V1a, cudraflavone B, shuterin, and Cudraxanthone L (CXL) and we found CXL was an anti-platelet substance. Therefore, we studied to find the anti-platelet mechanism of CXL and compared the inhibitory activity with isoderrone and steppogenin previously studied. We expect that the discovery of new physiological activities can affect the development of drugs for the prevention and treatment of cardiovascular diseases.

Experimental

Chemicals and reagents sources – ChemFaces (Wuhan, China) supplied CXL. Chrono-Log corporation (Havertown, PA, USA) supplied platelet collagen. Cayman chemical (Ann Arbor, MI, USA) supplied cAMP EIA kit, thromboxane B_2 assay kit. Cell signaling (Beverly, MA, USA) supplied antibodies against phospho-VASP (Ser^{157}), phospho-inositol-3-phosphate receptor type I (Ser^{1756}), phospho-cPLA$_2$ (Ser^{505}), phospho-Akt (Ser^{473}), phospho-ERK (1/2), β-actin, and anti-rabbit secondary antibody. Invitrogen (Eugene, OR, USA) provided fura 2-AM (2-acetoxymethyl) and alexa fluor 488 conjugated fibrinogen. Fibronectin-coated cell adhesion kit as procured from Cell Biolabs (San Diego, CA, USA). Serotonin ELISA kit was purchased from Labor Diagnostika Nord GmbH & CO. (Nordhorn, Germany).

Preparation of human platelets suspension – The human platelet-rich plasma was procured from Korean Red Cross Blood Center (Suwon, Korea), and study protocols were approved by the Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Republic of Korea) (PIRB-P01-201812-31-007). The platelet-rich plasma was centrifuged for 10 min at 1,300*g*, and pellet was washed twice using washing buffer (pH 6.5) and re-suspended them with suspension buffer (pH 6.9).[14] All procedures were performed at room temperature. The suspension of platelets was adjusted to 5×10^8/mL concentration.[15]

Platelet aggregation – For platelet aggregation, human platelets suspension (10^8/mL) was pre-incubated for 3 min in presence or absence of CXL along with 2 mM $CaCl_2$ at 37 ºC, then agonists were added for stimulation. Collagen (2.5 μg/mL) triggers full platelet aggregation and we used these agonists for aggregation. The aggregation assay was conducted for 5 minutes under continuous stirring condition. An increase in light transmission converted into the platelet aggregation rate (%). 0.1% dimethyl sulfoxide solution was used to dissolve the CXL.

Cytotoxicity assay – CXL was examined for any cytotoxic effects *via* lactate dehydrogenase (LDH) leakage from cytosol of platelets. Human platelets suspension (10^8/mL) was incubated with different concentrations of CXL for 2 hours and centrifuged for 2 min at 12,000 g. The supernatant was used to detect the cytotoxic effects using ELISA reader (TECAN, Salzburg, Austria).

Intracellular calcium concentration – The Fura 2-AM (5 μM) and PRP mixture was pre-incubated with at 37 ºC for 60 min and then human platelets suspension (10^8/mL) was washed with washing buffer. After washing step, platelets were suspended using suspending buffer and pre-incubated with or without CXL for 3 min at 37 ºC. The platelets were stimulated with collagen (2.5 μg/mL) in the presence of 2 mM $CaCl_2$. A spectrofluorometer (Hitachi F-2700, Tokyo, Japan) was used to measure Fura 2-AM fluorescence according to the Grynkiewicz method.[16]

Measurement of Thromboxane B_2 – Thromboxane A_2 (TXA_2) synthesized by platelets is unstable and quickly transforms into thromboxane B_2 (TXB_2), therefore, TXA_2 generation was measured by detecting TXB_2 production. After platelet activation, the reaction was stopped by adding indomethacin (0.2 mM) with EDTA (5 mM). The amounts of TXB_2 was measured using TXB_2 ELISA kit with ELISA reader (TECAN, Salzburg, Austria).

Measurement of serotonin – Human platelets suspension (10^8/mL) was pre-incubated for 3 min at 37 ºC with CXL, then stimulated with collagen (2.5 μg/mL) in the presence of 2 mM $CaCl_2$ to terminate serotonin release, followed by centrifugation. The supernatant was used for detection of serotonin release. Measurement of serotonin release was conducted using serotonin ELISA kit with ELISA reader (TECAN, Salzburg, Austria).

Immunoblotting – Platelet aggregation performed for 5 min and stopped by addition of lysis buffer and lysates of platelet were calculated using a bicinchoninic acid protein assay kit (Pierce Biotechnology, IL, USA). For Western blotting, proteins (15 μg) from platelet lysates were divided by SDS-PAGE (8%) and transferred onto PVDF membranes which were then probed with the primary (1:1,000) and secondary antibodies (1:10,000). The bands were analyzed by Quantity One, Ver. 4.5 (BioRad, Hercules, CA, USA).

Measurement of Fibrinogen binding to αIIb/β3 – Human platelets suspension (10^8/mL) was incubated with CXL were treated with fibrinogen (30 μg/mL, Alexa Flour 488-conjugated) at 37 ºC for 5 mins. 0.5% paraformaldehyde in cold PBS was added to fix the interaction between platelet integrin and Alexa Flour 488-conjugated human

fibrinogen. All procedures were conducted in the absence of light. The fibrinogen binding to integrin αIIb/β3 was conducted by the fluorescence of fibrinogen using flow cytometry (BD Biosciences, San Jose, CA, USA), and data were analyzed by the CellQuest software (BD Biosciences).

Fibronectin adhesion – Human platelets suspension (10^8/mL) was pre-incubated with CXL and $CaCl_2$ (2 mM) for 1h at 37 ºC in the presence of collagen (2.5 μg/mL) and washed five times with PBS followed by addition of cell stain solution and was placed for 10 min. Extraction solution was added after a washing step to detach the adhesive platelet plaque from fibronectin coated well. Bovine serum albumin coated well is used for negative control. Each sample was examined by detecting absorbance using ELISA reader (TECAN, Salzburg, Austria).

Platelet-mediated fibrin clot retraction – Human platelet-rich plasma (300 μL) was poured into a polyethylene tube and samples were pre-incubated in presence or absence of various concentration of CXL for 15 min and 30 min at 37 ºC, and clot retraction was triggered by adding thrombin (0.05 U/mL). Pictures of fibrin clot were taken using a digital camera at 30 min interval. Image J Software was used to calculate the clot area (v1.46, National Institutes of Health, USA).

Measurement of cAMP – Washed human platelets (10^8/mL) were preincubated for 3 min at 37 ºC with or without CXL in the presence of 2 mM $CaCl_2$, then stimulated with collagen (2.5 μg/mL) for 5 min for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. cAMP was measured using cAMP EIA kit with ELISA reader (TECAN, Salzburg, Austria).

Statistical analyses – Experimental data have been presented as the mean ± standard deviation included with the various number of observations. To determine major differences among groups, Analysis of variance was performed followed by Tukey-Kramer method. SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA) was employed for statistical analysis and $p<0.05$ values were considered as statistically significant.

Result and Discussion

Collagen at 2.5 μg/mL was used for optimum aggregation of human platelets to determine anti-platelet effects by CXL (MW 396.4) (Fig. 1). However, collagen induced platelet aggregation was decreased by CXL (75 to 150 μM) dose-dependently (Fig. 2A) and its half maximal inhibitory concentration (IC_{50}) was 100.7 μM (Fig. 2B).

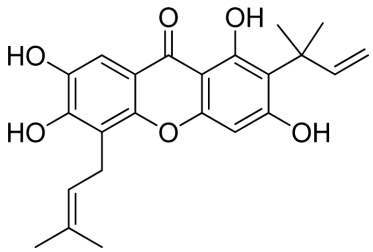

Fig. 1. Chemical structure of cudraxanthone L (CXL).

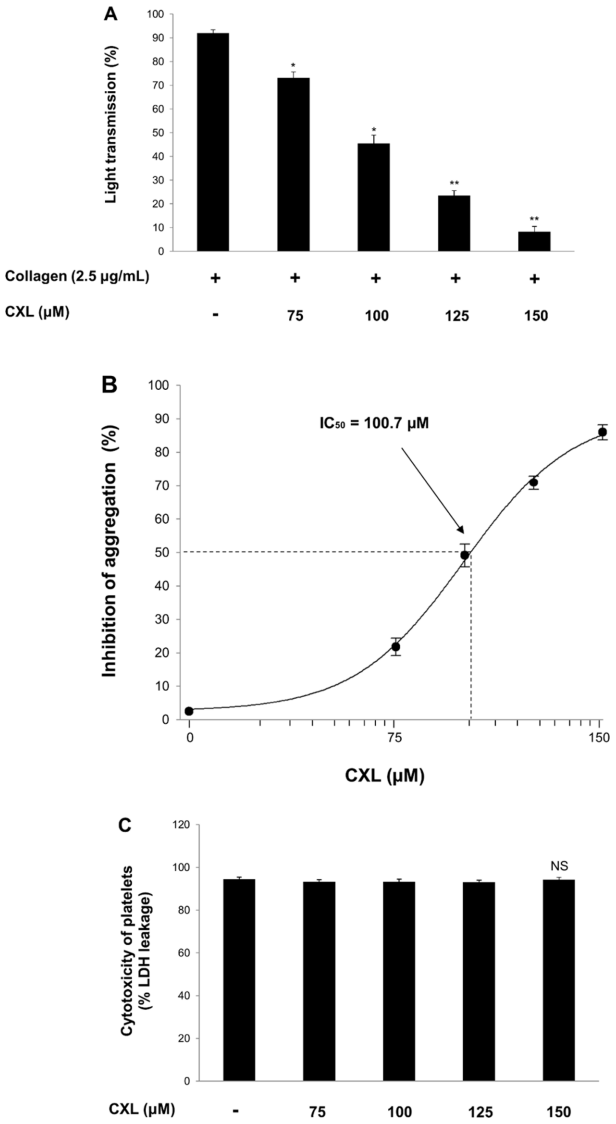

Fig. 2. Effects of CXL on platelet aggregation, half maximal inhibitory concentration, and cytotoxicity (A) Effect of CXL on collagen-induced human platelet aggregation. (B) Half maximal inhibitory concentration (IC_{50}) value of CXL in collagen-induced human platelet aggregation. (C) Effect of CXL on cytotoxicity. Platelet aggregation and cytotoxicity were carried out as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). $^{*}p<0.05$, $^{**}p<0.01$ versus each agonist-stimulated human platelets. NS, not significant.

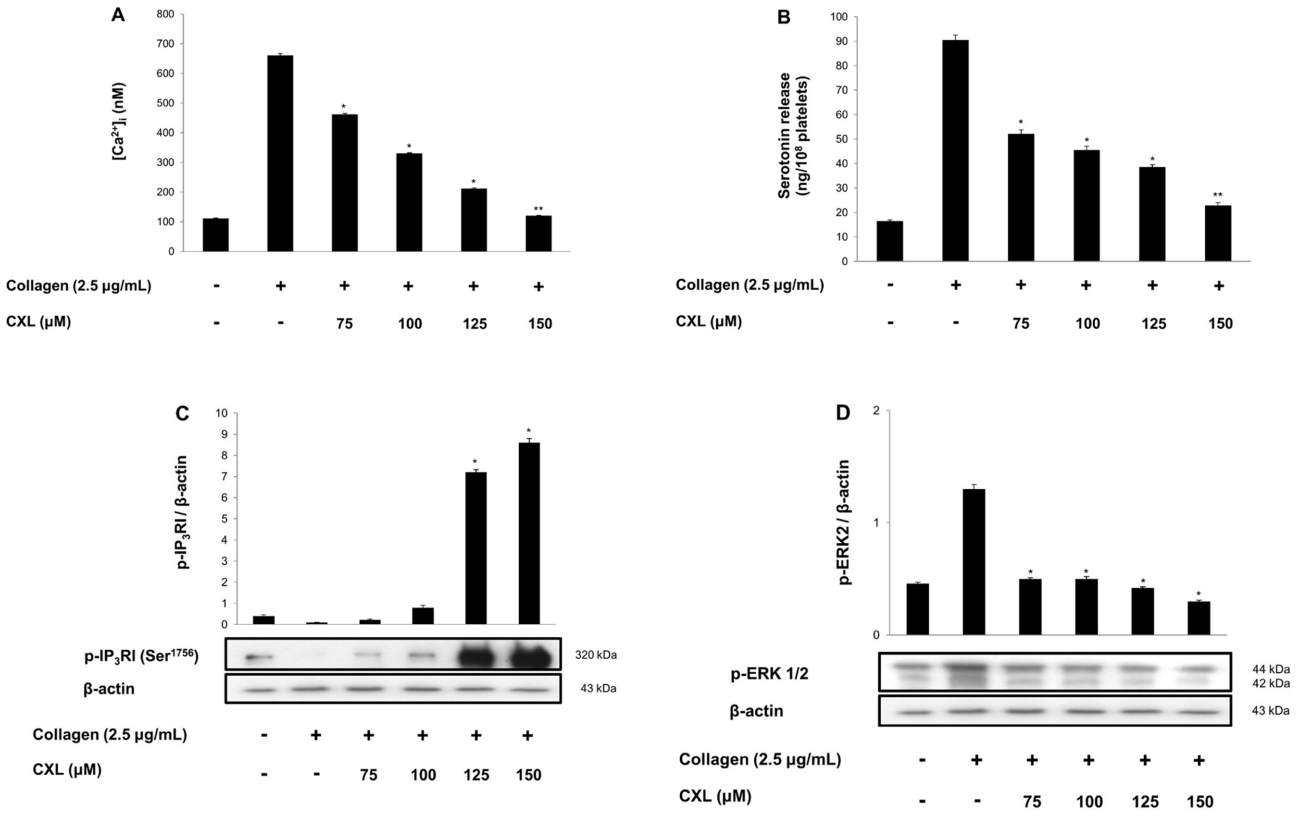

Fig. 3. Effects of CXL on $[Ca^{2+}]_i$ mobilization, Serotonin release, and IP_3RI, ERK (1/2) phosphorylation (A) Effect of CXL on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) Effect of CXL on collagen-induced serotonin release. (C) Effect of CXL on collagen-induced IP_3RI phosphorylation. (D) Effect of CXL on collagen-induced ERK (1/2) phosphorylation. Measurement of $[Ca^{2+}]_i$ mobilization, serotonin release Western blot was performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). $^{*}p<0.05$, $^{**}p<0.01$ versus the collagen-stimulated human platelets.

In addition, CXL (75 to 150 μM) did not affect the release of LDH as compared with intact platelets (Fig. 2C).

We focused the effect of artocarpesin on $[Ca^{2+}]_i$ mobilization. As shown in Fig. 3A, collagen (2.5 μg/mL) induced $[Ca^{2+}]_i$ levels were elevated from 111.5 ± 0.7 nM to 660.5 ± 6.5 nM, but CXL dose (75 to 150 μM)-dependently reduced the collagen-increased $[Ca^{2+}]_i$ levels. It is well known that increased Ca^{2+} activates granule secretion, thus, we investigated whether CXL involves in dense granule release of platelets through detecting serotonin release. As shown in Fig. 3B, CXL (75 to 150 μM) dose-dependently inhibited collagen-stimulated serotonin secretion. Next, we investigated $[Ca^{2+}]_i$ mobilization associated signaling molecule, inositol 1, 4, 5-triphosphate receptor type I (IP_3RI). As shown in Fig. 3C, CXL (125 and 150 μM) increased IP_3RI phosphorylation. This result suggests that the decrease of $[Ca^{2+}]_i$ level by CXL is due to IP_3RI phosphorylation. It is known that Ca^{2+} mobilized from ER is involved in ERK phosphorylation to influx extracellular Ca^{2+}, thus, we investigated the effect of CXL on dephosphorylation of ERK (1/2). As shown in Fig. 3D, ERK2 (42 kDa) was potently phosphorylated by collagen (2.5 μg/mL) as compared with unstimulated platelets. However, CXL inhibited collagen-elevated ERK2 phosphorylation.

Next, we investigated fibrinogen binding to αIIb/β_3, which is an important reaction in binding integrin and adhesive proteins. Collagen elevated the binding of fibrinogen to αIIb/β3 from 4.4 ± 1.2% to 90.3 ± 2.1%, but CXL dose (75 to 150 μM)-dependently decreased the collagen-increased $[Ca^{2+}]_i$ fibrinogen binding to αIIb/β3. Activated αIIb/β3 also serves as a binding receptor of fibronectin. Thus, we investigated whether CXL inhibits fibronectin adhesion. As shown in Fig. 4C, collagen induced fibronectin adhesion was suppressed by CXL dose-dependently.

Vasodilator-stimulated phosphoprotein (VASP) phosphorylation has been known as a negative signaling in αIIb/β_3 activation and Akt phosphorylation has been known as a positive signaling in αIIb/β_3 activation. Thus,

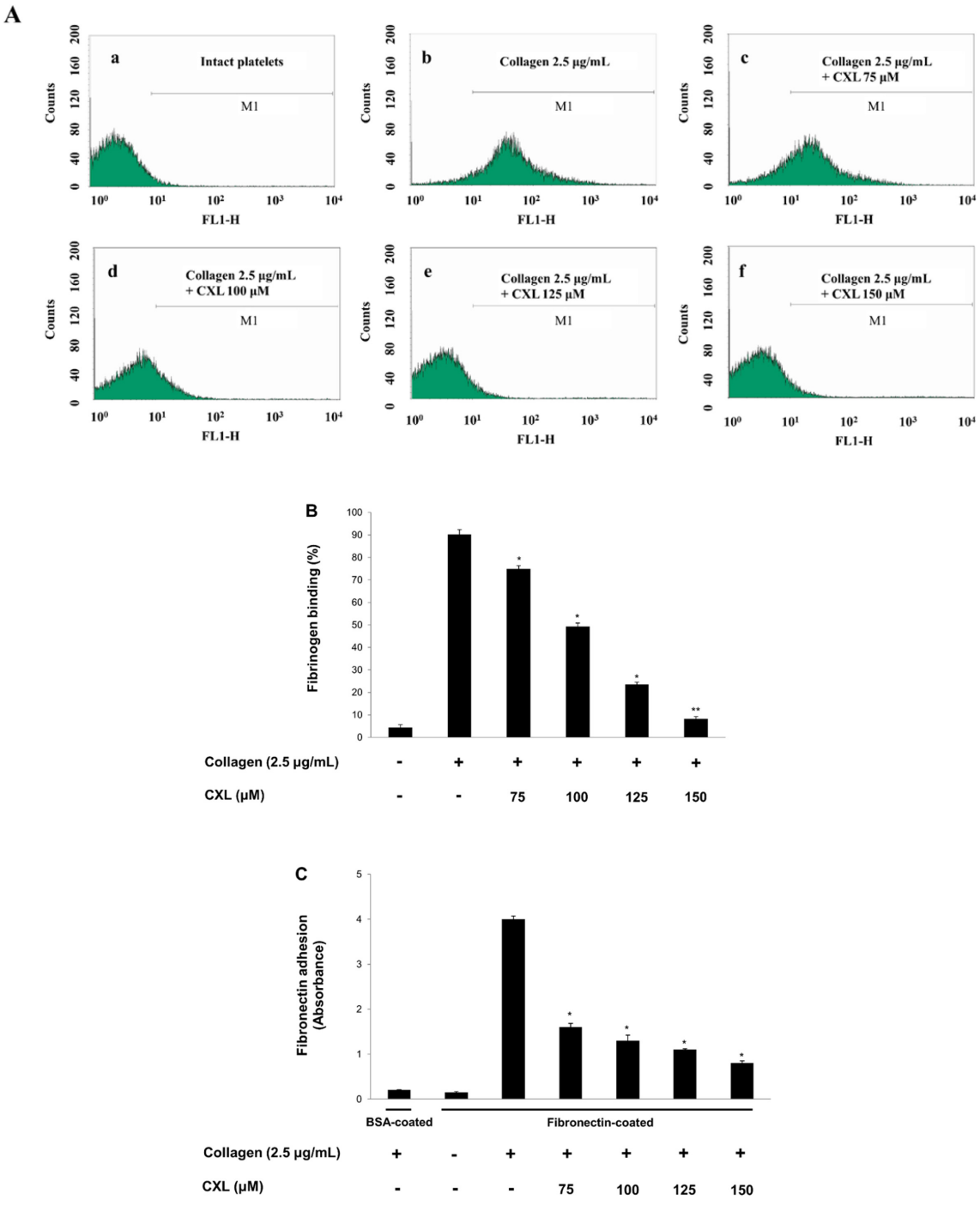

Fig. 4. Effects of CXL on fibrinogen binding to αIIb/β3 and fibronectin adhesion (A) The flow cytometry histograms on fibrinogen binding. (B) Effects of CXL on collagen-induced fibrinogen binding (%). (C) Effects of CXL on collagen-induced fibronectin adhesion. Measurement of fibrinogen binding and fibronectin adhesion was carried out as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). $^{*}p<0.05$, $^{**}p<0.01$ versus the collagen-stimulated human platelets.

we examined whether CXL affects the VASP and Akt phosphorylation. Our results showed that CXL increased VASP (Ser^{157}, Ser^{239}) phosphorylation dose-dependently (Fig. 5A) and CXL decreased Akt (Ser^{473}) phosphorylation dose-dependently. These results suggest that the inhibition of fibrinogen binding and fibronectin adhesion by CXL is due to VASP (Ser^{157}, Ser^{239}) phosphorylation and Akt (Ser^{473}) dephosphorylation.

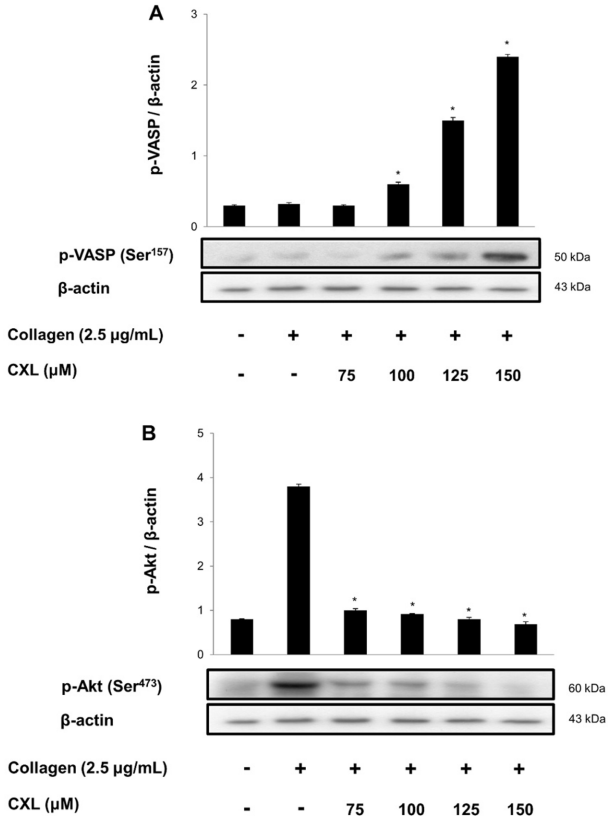

Fig. 5. Effects of CXL on VASP and Akt phosphorylation (A) Effect of CXL on collagen-induced VASP (Ser^{157}) phosphorylation. (B) Effect of CXL on collagen-induced Akt (Ser^{473}) phosphorylation. Measurement of Western blot was carried out as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). $^{*}p<0.05$ versus the collagen-stimulated human platelets.

We investigated TXA_2 production (determined as TXB_2) and associated signaling molecule. Collagen (2.5 μg/mL) stimulated human platelet TXA_2, but CXL inhibited TXA_2 production dose-dependently (Fig. 6A). Cytosolic phospholipase A_2 ($cPLA_2$) is a major regulator for arachidonic acid release. As shown in Fig. 6B, the $cPLA_2$ is phosphorylated by collagen, but CXL inhibited $cPLA_2$ phosphorylation dose-dependently. Next, we investigated the effect of CXL on the production of cAMP and cGMP in platelets. As shown in Fig. 6C, collagen elevated cAMP production was suppressed by CXL dose-dependently. However, CXL did not affect cGMP (data not shown).

Activated platelets trigger various actions such as platelet spreading, adhesion, granule secretion and fibrin clot retraction. Therefore, we examined the inhibitory effects of CXL on clot retraction. As shown in Fig. 7A, human platelet rich plasma is retracted by thrombin (0.05 U/mL). The inhibition rate compare with unstimulated

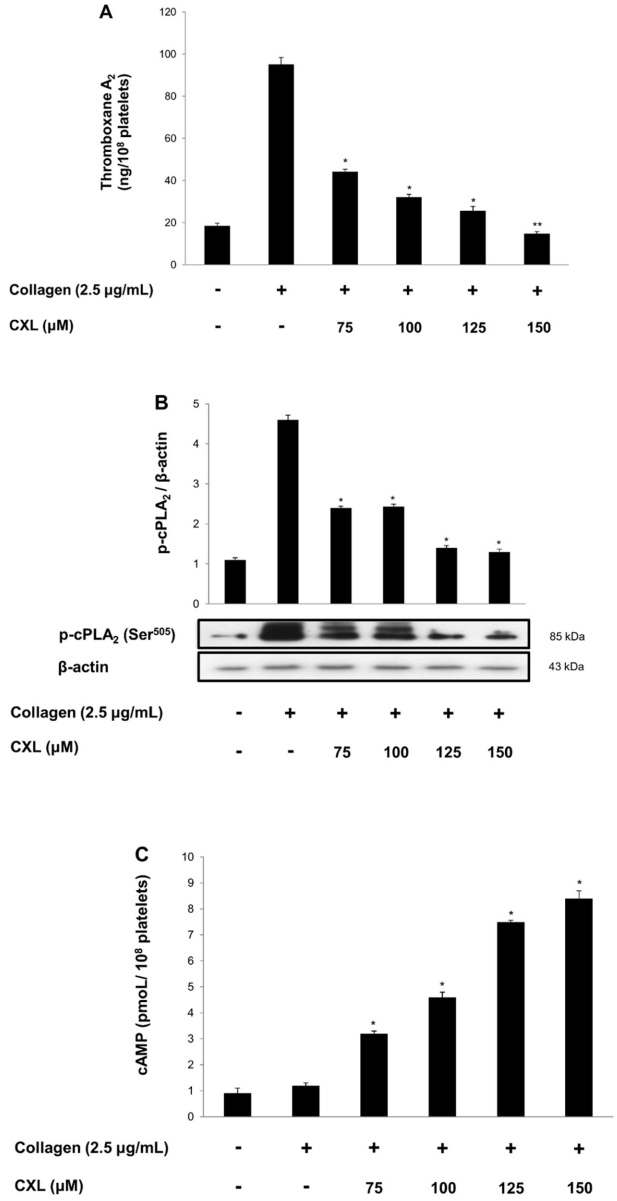

Fig. 6. Effects of CXL on TXA_2 production, $cPLA_2$ phosphorylation, and cAMP level (A) Effect of CXL on collagen-induced TXA_2 production. (B) Effect of CXL on collagen-induced $cPLA_2$ (Ser^{505}) phosphorylation. (C) Effect of CXL on collagen-induced cAMP production. Measurement of Western blot and cAMP level was performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). $^{*}p<0.05$, $^{**}p<0.01$ versus the collagen-stimulated human platelets.

platelet rich plasma was 90.1% at 30 min. However, the retraction was suppressed by CXL (75 to 150 μM) dose-dependently, compared with unstimulated platelet rich plasma (Fig. 7B). Y27632 (5 μM) was used as a positive control.

Prostacyclin and nitric oxide synthesize cAMP and cGMP within platelets, which inhibit platelet activities

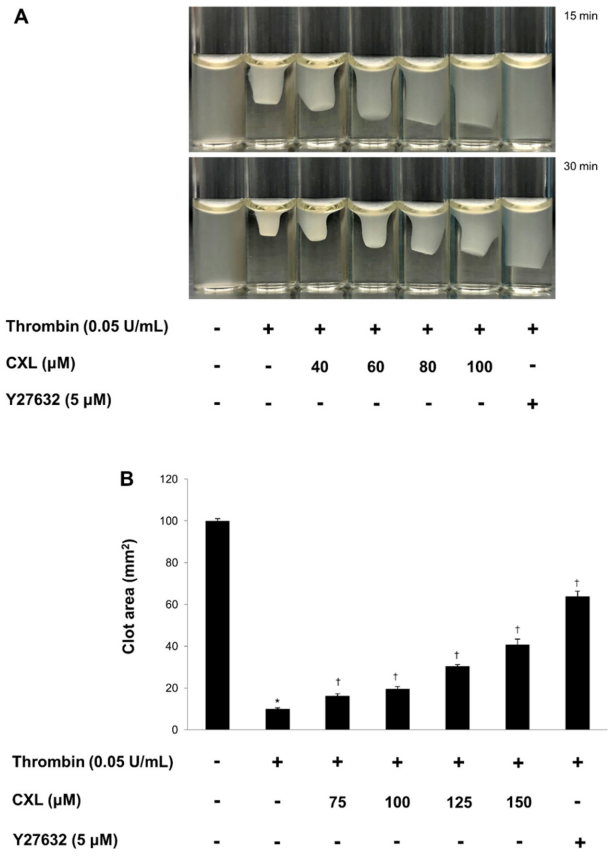

Fig. 7. Effects of CXL on fibrin clot retraction.
(a) Photographs of fibrin clot (b) Effects of CXL on thrombin-retracted fibrin clot (%). Quantification of fibrin clot retraction was performed as describe in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n=4). $^{*}p<0.05$ versus the unstimulated human PRP, $^{\dagger}p<0.05$ versus the thrombin-stimulated human PRP

and make them resting status, which regulates thrombosis and hemostasis. Increased cAMP and cGMP down-regulates platelets through, protein kinase A (PKA) and protein kinase G (PKG).[17] The PKA and PKG have two major substrates in platelets; inositol 1, 4, 5-trisphosphate receptor type I (IP_3RI) and vasodilator-stimulated phosphoprotein. The IP_3RI is located on endoplasmic reticulum surface, which induces Ca^{2+} mobilization ($[Ca^{2+}]_i$) by binding with inositol 1, 4, 5-trisphosphate.[18] The Increased $[Ca^{2+}]_i$ causes both the phosphorylation of myosin light chain (20 kDa) and pleckstrin (47 kDa) to trigger granule secretion.[19,20] However, the IP_3RI (Ser^{1756}) phosphorylation inhibits its activity.[18] CXL suppressed $[Ca^{2+}]_i$ level and serotonin release (Fig. 3A, 3B) and affected IP_3RI (Ser^{1756}) phosphorylation and ERK dephosphorylation (Fig. 3C, 3D). Therefore, we confirmed Ca^{2+}-antagonistic effect of CXL on collagen stimulated human platelets.

Another substrate of PKA and PKG is vasodilator-stimulated phosphoprotein (VASP). VASP is a major protein for shape change of platelets, such as spreading, adhesion and filopodia formation. This change is achieved by actin filament dynamics and VASP supports actin filament elongation.[21] VASP influences on integrin glycoprotein IIb/IIIa (αIIb/β3) on platelet surface and triggers structural change of αIIb/β3. Activated αIIb/β3 allows for interaction with its plasma ligands (i.e. fibrinogen, fibronectin, and vitronectin), causing activation of thrombus formation. Therefore, αIIb/β3 activation is crucial factor for platelet aggregation and hemostasis. In our study showed that CXL suppressed fibrinogen binding to αIIb/β3 and fibronectin adhesion to αIIb/β3 (Fig. 4A, 4C). About these inhibitory effects of CXL, we investigated associated signaling molecules, VASP and Akt and discovered CXL increased VASP phosphorylation at Ser^{157} and decreased Akt phosphorylation at Ser^{473} (Fig. 5A, 5B).[22,23]

CXL also suppressed TXA_2 generation through dephosphorylation of $cPLA_2$ dose-dependently (Fig. 6A, 6B) and produced cAMP (Fig. 6C). Intracellular cAMP and cGMP are strong negative molecules and regulated by the balance between cyclic nucleotide-producing enzymes, adenylate/guanylate cyclase, and hydrolyzing enzymes, phosphodiesterases. These cyclic nucleotides can inhibit αIIb/β3 activity and $[Ca^{2+}]_i$ mobilization. In our study, CXL increased cAMP (Fig. 5a, 5b), which can elevate the phosphorylation of VASP (Ser^{157}) and IP_3RI (Ser^{1756}). Moreover, we confirm that anti-platelet effect of CXL on thrombin-induced clot retraction (Fig. 7A, 7B).

We compare the effects of isoderrone and steppogenin with CXL, isoderrone and steppogenin increased both cAMP and cGMP level,[12,13] while CXL increased only cAMP level in collagen-induced human platelet aggregation. Regarding calcium influx, ERK is a major signaling regulator, and CXL and steppogenin affect ERK phosphorylation.[12] Moreover, CXL showed strong inhibitory effect on αIIb/β3 affinity (Fig. 4A, 4C). Our previous studies of isoderrone and steppogenin, these molecules showed weak inhibition of fibrinogen binding to αIIb/β3 and we thought that the difference is due to the down regulation of Akt phosphorylation (Fig. 5B). The three substances have different structures and have different effects in human platelets. The difference between these results was markedly different in the clot retraction test (Fig. 7A, 7B), the final thrombotic inhibition experiment, and CXL showed a clear inhibitory effect on clot retraction compared to the previous two substances. Therefore, CXL is the most potent thrombus inhibitor among the components of *C. tricuspidata*.

In conclusion, we confirmed that CXL decreases inside-out signaling pathway through the IP_3RI phosphorylation and ERK, $cPLA_2$ dephosphorylation. In addition, CXL suppressed outside-in signaling pathway through VASP phosphorylation and Akt dephosphorylation. These two inhibition lead downregulation of $[Ca^{2+}]_i$ mobilization and αIIb/β3 affinity. Therefore, we suggest that CXL isolated from *C. tricuspidata* would be a useful compound for prevention of platelet mediated circulatory diseases.

Acknowledgements

This study was supported by a grant (NRF-2018R1C1B5083580) from the Basic Science Research Program via the National Research Foundation of Korea (NRF), funded by the Ministry of Education, Science and Technology, Korea

Conflict of interest

The authors declare no conflict of interest.

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Received September 4, 2020
Revised November 3, 2020
Accepted November 17, 2020