Antiplatelet effect of cudraxanthone B is related to inhibition of calcium mobilization, αIIbβ3 activation, and clot retraction

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Abstract
Cudrania tricuspidata (C. tricuspidata) is widespread throughout Asia and has known to have various physiological activities such as, inflammation, diabetes, obesity and tumor. Cudrania tricuspidata, a rich source of xanthones and flavonoids, have been investigated phytochemically and biologically. However, research of these compounds on platelets is limited. Therefore, we searched for a new substance from various xanthones and flavonoids in C. tricuspidata. We confirmed the results that steppogenin and isoderrone suppress human platelets among the various components isolated from C. tricuspidata, and as a result of analyzing the antiplatelet effect using additional new samples, we found that cudraxanthone B (CXB) has the effect of suppressing human platelets. Therefore, we studied the potential efficacies of CXB on human platelet aggregation and its inhibitory mechanism. Inhibitory effects of CXB on platelet aggregation were assessed using washed platelets, followed by measurement of [Ca²⁺], mobilization and dense granule release, fibrinogen binding, fibronectin adhesion assay, and clot retraction. Our data showed that CXB suppressed collagen-induced human platelet aggregation, [Ca²⁺], mobilization, fibrinogen binding, fibronectin adhesion and clot retraction without cytotoxicity. Thus, our results show that inhibitory effects of CXB on human platelet activation and thrombus formation, suggesting its potential use as a natural substance for preventing platelet-induced thrombosis.

Keywords: Cudraxanthone B, Ca²⁺ mobilization, αIIbβ3 activation, Granule secretion, Clot retraction

Introduction
An area of damaged vascular wall exposures collagen and circulatory platelets can bind to the collagen through receptors of α2β1 and glycoprotein VI on platelet surface. After platelet activation, phospholipase Cγ 2 hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP₂) into inositol 1,4,5-trisphosphate (IP₃) and diacylglycerol [1, 2]. IP₃ mediated calcium mobilization activates calcium/calmodulin-dependent Myosin light-chain (MLC) kinase and phosphorylates MLC affecting granule release. These signaling cascades are called "inside-out signaling" and facilitates interaction with plasma adhesive molecules (i.e. fibrinogen, fibronectin, vitronectin) and glycoprotein IIb/IIIa (also called αIIb/β3). After interaction between adhesive molecules with αIIbβ3, “outside-in signaling” is subsequently processed to promote platelet aggregation affecting thrombus formation [3]. Platelets are essential for the maintenance of hemostasis, but it can also cause thrombosis. The production of thrombosis is a fatal risk for patients who have thrombus mediated cardiovascular disease. Therefore, more various anti-platelet drugs and functional food are necessary without serious complications [4, 5]. Therefore, further research is needed for the development of more effective and safer drugs to ensure better treatment and prevention of cardiovascular disease.

In normal circulatory system, vascular endothelial cells to produce nitric oxide and prostaglandin I₂ which elevates cyclic AMP (cAMP) and cyclic GMP (cGMP).
concentration within circulatory platelets. Synthesized cAMP and cGMP down-regulates platelet activities and make them resting form. These two cyclic nucleotides work through cAMP/cGMP-dependent kinases, protein kinase A (PKA) and protein kinase G (PKG) [6]. Vasodilator-stimulated phosphoprotein (VASP) is a major substrate of PKA and PKG in platelets and VASP contributes to αIIb/β3 activation, but its phosphorylation at Ser157 and at Ser239 leads to the inhibition of αIIb/β3 activation [7, 8]. In addition, the cAMP/cGMP-dependent kinases phosphorylate substrate protein, inositol 1, 4, 5-triphosphate receptor type I (IP3RI) phosphorylation [9]. The action of IP3RI is inhibited by its phosphorylation at Ser1756. IP3RI phosphorylation involves in inhibition of [Ca2+]i mobilization [10, 11].

It has been reported that C. tricuspidata extracts have various physiological activities. Regarding the effect of improving blood circulation of C. tricuspidata extracts, it has been reported that root extract of C. tricuspidata has anti-platelet effects on collagen-induced rat platelet aggregation [12]. Therefore, we examined the potential efficacies of CXB from root of C. tricuspidata on human platelet aggregation.

Materials and methods

Chemicals and reagents sources

ChemFaces (Wuhan, China) supplied cudraxanthone B (CXB). Chrono-Log corporation (Havertown, PA, USA) supplied chrono-log agonists (collagen and thrombin). Cayman chemical (Ann Arbor, MI, USA) supplied U46619, cAMP EIA kit and cGMP enzyme immunoassay (EIA) kit, thromboxane B2 assay kit. Cell signaling (Beverly, MA, USA) supplied the lysis buffer and antibodies against phospho-VASP (Ser157), phospho-VASP (Ser239), phospho-inositol-3-phosphate receptor type I (Ser1756), phospho-cPLA2 (Ser505), phospho-p38 MAPK, phospho-Akt (Ser473), β-actin, and anti-rabbit secondary antibody. Invitrogen (Eugene, OR, USA) provided fura 2-AM (5 μM) and Alexa fluor 488 conjugated antibody. Invitrogen (Eugene, OR, USA) supplied fura 2-AM (5 μM) 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 (PRP) 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-P01-201,812-31-007). The PRP was centrifuged for 10 min at 1300 g, and pellet was washed twice using washing buffer (pH 6.5) and re-suspended them with suspension buffer (pH 6.9) according to the previous research [13]. All procedures were performed at room temperature. The suspension of platelets was adjusted to 5 × 10^8/mL concentration [14].

Platelet aggregation

For platelet aggregation, human platelets suspension (10^8/mL) was pre-incubated for 3 min in presence or absence of CXB along with 2 mM CaCl2 at 37 °C, then agonists were added for stimulation. Collagen (2.5 μg/mL), U46619 (200 nM), and thrombin (0.05 U/mL) trigger full platelet aggregation and we used these agonists for aggregation. The aggregation assay was conducted for 5 min 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 CXB.

Cytotoxicity assay

CXB 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 CXB for 2 h 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 °C 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 CXB for 3 min at 37 °C. The platelets were stimulated with collagen (2.5 μg/mL) in the presence of 2 mM CaCl2. A spectro-fluorometer (Hitachi F-2700, Tokyo, Japan) was used to measure Fura 2-AM fluorescence according to the Grynkiewicz method [15] for calculate the [Ca2+]i values.

Measurement of thromboxane B2

Since thromboxane A2 (TXA2) is unstable and transforms into thromboxane B2 (TXB2) quickly, therefore, TXA2 generation was measured by detecting TXB2 production. After platelet activation, the reaction was stopped by adding indomethacin (0.2 mM) with EDTA (5 mM). The amounts of TXB2 was measured with ELISA reader (TECAN, Salzburg, Austria) using TXB2 ELISA kit.

Measurement of serotonin

Human platelets suspension (10^8/mL) was pre-incubated for 3 min at 37 °C with CXB, then stimulated with collagen (2.5 μg/mL) in the presence of 2 mM CaCl2 to
terminate serotonin release, followed by centrifugation. The supernatant was used for detection of serotonin release. Measurement of serotonin release was conducted with ELISA reader (TECAN, Salzburg, Austria) using serotonin ELISA kit.

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). Result bands were analyzed by using the 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 CXB and CaCl₂ (2 mM) for 1 h at 37 °C 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. Each sample was examined by detecting absorbance using ELISA reader (TECAN, Salzburg, Austria). Bovine serum albumin coated well is used for negative control.

Fibronectin adhesion
Human platelets suspension (10^8/mL) was pre-incubated with CXB and CaCl₂ (2 mM) for 1 h at 37 °C 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. Each sample was examined by detecting absorbance using ELISA reader (TECAN, Salzburg, Austria).

Measurement of cAMP and cGMP
Washed human platelets (10^8/mL) were preincubated for 3 min at 37 °C with or without CXB in the presence of 2 mM CaCl₂, 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 and cGMP were measured using EIA kit with ELISA reader (TECAN, Salzburg, Austria).

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

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.

Results
Effects of CXB on human platelets aggregation and cytotoxicity
To determine anti-platelet effects by CXB (MW 394.4) (Fig. 1a), we used three agonists, collagen, thrombin and U46619 (TXA2 analogue). Collagen at 2.5 μg/mL, thrombin at 0.05 U/mL, and U46619 at 200 nM were used for optimum aggregation of human platelets (Fig. 1b–d). However, collagen induced platelets treated with CXB (10, 20, 30, and 40 μM) were most significantly reduced (23.0, 54.3, 86.8, and 97.8%, respectively) (Fig. 1b) and its half maximal inhibitory concentration (IC50) was 27.8 μM (Fig. 1e). DMSO 0.1% seemed to have no affect for agonist-induced platelet aggregation [16]. To investigate the cytotoxicity of CXB, we used various concentrations (10 to 40 μM) of CXB. As shown in Fig. 1f, CXB (10 to 40 μM) did not affect the release of LDH as compared with intact platelets.

Inhibitory effects of CXB on [Ca^{2+}] mobilization, IP₃RI phosphorylation and serotonin secretion
Intracellular ion concentration ([Ca^{2+}]) plays essential factor for platelet activation, thus we focused the effect of CXB on antagonistic activity of Ca^{2+}. As shown in Fig. 2a, [Ca^{2+}], levels were elevated from 101.5 ± 0.5 nM to 670.8 ± 10.2 nM by collagen (2.5 μg/mL). However, CXB dose (10 to 40 μM)-dependently reduced the collagen-increased [Ca^{2+}], levels (Fig. 2a). Next, we investigated calcium mobilization associated signaling molecule, inositol 1, 4, 5-triophosphate receptor type 1 (IP₃RI) phosphorylation. As shown in Fig. 2b,
CXB (30 to 40 μM) increased IP₃RI phosphorylation at Ser¹⁷⁵⁶ in collagen-stimulated human platelet aggregation. This result means that the decrease of [Ca²⁺]ᵢ level by CXB is due to IP₃RI phosphorylation. In addition, we explored whether CXB involves in inhibition of granule secretion. The serotonin is stored in dense granules in platelets. We examined dense granules release and as shown in Fig. 2c, CXB (10 to 40 μM) dose-dependently inhibited collagen-stimulated serotonin secretion.

Measurement of TXB₂ and cPLA₂ and p38MAPK dephosphorylation
Collagen induced human platelet suspension increased TXA₂ (determined as TXB₂) levels to 45.2 ± 2.1 ng/10⁸ platelets. However, CXB inhibited TXA₂ production dose-dependently (Fig. 2d). For identification of inhibitory effect of CXB on TXA₂ production, Next, we investigated TXA₂ production associated signaling molecule. The cPLA₂ has been reported to play key role in arachidonic acid release in human platelets. As shown
in Fig. 2e the cPLA₂ was phosphorylated at Ser⁵⁰⁵ by collagen, but CXB significantly inhibited cPLA₂ phosphorylation dose-dependently. It is well known that the cPLA₂ activity is achieved by mitogen-activated protein kinases p38 (p38MAPK) and the p38MAPK also being activated through phosphorylation. As shown in Fig. 2f, collagen increased p38MAPK phosphorylation, but CXB inhibited collagen-elevated p38MAPK phosphorylation dose-dependently.

Inhibitory effects of CXB on fibrinogen binding to integrin αIIb/β₃ and fibronectin adhesion
Next, we investigated fibrinogen binding to αIIb/β₃, which is an important reaction in outside-in signaling. Collagen elevated the binding of fibrinogen to αIIb/β₃ (Fig. 3A-b, B), with a rate of 80.6 ± 4.2%. However, CXB significantly attenuated the fibrinogen interaction with αIIb/β₃ dose-dependently (Fig. 3A-c-f, B). Moreover, αIIb/β₃ also serves as a binding molecule of fibronectin
which is crucial for platelet adhesion to vascular endothelium. Thus, we examined whether CXB affect fibronectin adhesion. As shown in Fig. 3C, CXB suppressed collagen-stimulated fibronectin adhesion.

**Effects of CXB on regulation of VASP, Akt phosphorylation, cyclic nucleotides levels and clot retraction**

Phosphorylated vasodilator-stimulated phosphoprotein (VASP) inhibits actin dynamics which activates αIIb/β3 [7, 8]. As CXB showed the inhibitory action on collagen-induced αIIb/β3 activation (Fig. 3A, C), we investigated the effect of CXB on VASP Ser157 phosphorylation in collagen-stimulated platelets. CXB upregulated VASP Ser157 and VASP Ser239 phosphorylation significantly (Fig. 4a, b). Akt phosphorylation has been known as a positive signaling in αIIb/β3 activation. Thus, we examined whether CXB inhibits the phosphorylation of Akt. Collagen-induced Akt phosphorylation was inhibited by CXB dose-dependently (Fig. 4c). Next, we investigated the effect of CXB on the production of cAMP and cGMP in collagen-induced human platelet aggregation. As shown in Fig. 4d and e CXB elevated cAMP and cGMP levels. Activated integrin αIIb/β3 transduces signals into the cell which triggers various actions in platelets such as platelet spreading, adhesion and contraction, ultimately lead to stable thrombus formation and clot retraction. Thus, we finally examined the inhibitory effects of CXB on thrombin-stimulated fibrin clot retraction. Figure 4f shows thrombin-induced fibrin clot build up and contraction with an inhibition rate of 67.1% compared with
unstimulated PRP. However, the retraction was effectively suppressed by CXB (10 to 40 μM) dose-dependently, with inhibitory degrees of 64.4, 57.2, 49.2 and 43.9%, respectively, compared with unstimulated PRP (Fig. 4g). Y27632 (5 μM) was used as a positive control and its inhibitory degree was 16.3% compare with unstimulated PRP.

Discussion

Cudrania tricuspidata is a perennial plant of the family Moraceae and its roots, leaves, barks, stems and fruits contain diverse phytochemicals. Among various phytochemicals, xanthones and flavonoids are the major constituents in Cudrania tricuspidata which have effects on anti-inflammatory, obesity, diabetes, and anti-tumor [17]. In addition, it has been reported that steppogenin, isoderrone and cudratricusxanthone A have anti-platelets effect [18–20]. Thus, we searched for a new substance and investigated that whether cudranaxanthone B (CXB) has antiplatelet effect. CXB significantly blocked various agonists-elevated human platelet aggregation. Among agonists, CXB potently inhibited collagen-induced platelet aggregation. Therefore, we checked Ca2⁺ mobilization, serotonin release, fibrinogen binding, fibroectin adhesion and associated signaling molecules.

CXB suppressed collagen-induced [Ca2⁺]i level (Fig. 2a) through elevated IP₃RI (Ser1756) phosphorylation (Fig. 2b) and affected serotonin release (Fig. 2c). TXA₂ is generated by agonists-stimulated platelets, and acts as a positive promotor on circulatory platelets, which is connected to the intensification of platelet mediated thrombus. cPLA₂ is Ca2⁺-dependent enzyme and can hydrolyze membrane phospholipids to release arachidonic acid. Upon stimulation by agonists, cPLA₂ is translocated from cytosol to membrane in the presence of intracellular Ca2⁺ and phosphorylated at cPLA₂ at Ser505 by p38MAPK for full catalytic activity [21]. In addition, p38MAPK is also activated through phosphorylation and can phosphorylate cPLA₂ [22]. As shown in Fig. 2a, CXB inhibited [Ca2⁺]i level, thus, we investigated whether CXB inhibits TXA₂ production and dephosphorylation of cPLA₂ and p38MAPK. CXB inhibited the phosphorylation of p38MAPK and cPLA₂ dose-dependently (Fig. 2e, f), which suppressed the TXA₂ generation (Fig. 2d). An important indicator in evaluating components or substances for platelet inhibitory activity is the generation of TXA₂ because TXA₂ acts as a autacoid that activates and aggregates other platelets. Therefore, substances that inhibit the production of TXA₂ are usefully used as antiplatelet substances, and for example, substances such as aspirin and ozagrel are known [23, 24].

The αIIb/β₃ is the most plentiful integrin on platelet surface. The activation of αIIb/β₃ leads to a rapid binding to adhesion molecules. CXB downregulated αIIb/β3 activity affecting fibrinogen binding and fibronectin adhesion (Fig. 3a–c) through upregulation of phosphorylation of VASP (Fig. 4a, b) and downregulation of Akt (Fig. 4c). Intracellular CAMP and cGMP are regulated by the balance between cyclic nucleotide-producing enzymes, adenylate/guanylate cyclase, and hydrolyzing enzymes, phosphodiesterases. These cyclic nucleotides can regulate αIIb/β3 activity and [Ca2⁺]i level through dependent kinases, protein kinase A and protein kinase G. In our study, CXB showed increased CAMP and cGMP level (Fig. 4d, e) and these cyclic nucleotides can affect the phosphorylation of VASP (Ser157, Ser239) and IP₃RI (Ser1756), which downregulates platelet function.

The thrombin-induced clot retraction is a final step to repair of the damaged portion of the blood vessel. Activated platelets accumulate in the injured blood vessel and develop into a fibrin-platelet complex. This complex seals up at the damaged vessel and starts to retract. The interaction between αIIb/β3 and fibrin is a key role for the clot formation. In addition to αIIb/β3, calpain is also known to help the function of αIIb/β3. Calpain, a calcium-dependent cysteine protease, has been implicated in the αIIb/β3-mediated signaling pathway [25]. Therefore, downregulated [Ca2⁺]i level by CXB also implicated in inhibitory effect of clot traction. As shown in Fig. 4f, CXB inhibited the thrombin-induced clot retraction dose-dependently. These data mean that downregulation of Ca2⁺ by phosphorylation of IP₃RI (Ser1756) and suppression of αIIb/β3 affinity by phosphorylation VASP (Ser157, Ser239) facilitates delay of clot retraction. We compare the effects of isoderrone and steppogenin with CXB, CXB showed strong inhibitory effect on αIIb/β3 affinity. Our previous studies of isoderrone and steppogenin, these molecules showed weak inhibition of fibrinogen binding to αIIb/β3 and we forecast that the difference is
achieved by Akt dephosphorylation (Fig. 4c). Therefore, CXB showed a clear inhibitory effect on clot retraction compared to the previous two substances. Therefore, we found that CXB is a potent antithrombotic drug. Taken together, these results show that the antiplatelet effect of CXB is due to the inhibition of cAMP and cGMP level. The cAMP and cGMP are known to depend on the activation of adenyl cyclase and guanylyl cyclase or cyclic nucleotide phosphodiesterase (PDE) [26]. Since, in platelet aggregation, the level of cyclic nucleotides increases from the inhibition of PDE activity, PDE inhibitors have been used as antiplatelet materials to increase cyclic nucleotides production [28]. Therefore, it is thought that CXB could be developed as an antiplatelet agent through increasing cyclic nucleotides.

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Authors’ contributions
Conception and design of the experiment: HK, MHR. Performance of the experiment: JHS, MI. Analysis and arrangement of the data: HWK, JHS, MI. Funding acquisition: HWK. Writing original draft: JHS, MI. All authors read and approved the final manuscript.

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Availability of data and materials
All data generated or analyzed during the present study are included in this published article.

Competing interest
The authors declare no conflict of interest.

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