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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 $[Ca^{2+}]_i$ mobilization and dense granule release, fibrinogen binding, fibronectin adhesion assay, and clot retraction. Our data showed that CXB suppressed collagen-induced human platelet aggregation, $[Ca^{2+}]_i$ 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, Ca^{2+} mobilization, αIIb/β3 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 $C\gamma_2$ hydrolyzes phosphatidylinositol 4,5-bisphosphate (PIP_2) into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol [1, 2]. IP_3 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 I_2 which elevates cyclic AMP (cAMP) and cyclic GMP (cGMP)

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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 $\alpha IIb/\beta_3$ activation, but its phosphorylation at Ser^{157} and at Ser^{239} leads to the inhibition of $\alpha IIb/\beta_3$ activation [7, 8]. In addition, the cAMP/cGMP-dependent kinases phosphorylate substrate protein, inositol 1, 4, 5-triphosphate receptor type I (IP_3RI) phosphorylation [9]. The action of IP_3RI is inhibited by its phosphorylation at Ser^{1756}, IP_3RI phosphorylation involves in inhibition of $[Ca^{2+}]_i$ 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 platelet agonists (collagen and thrombin). Cayman chemical (Ann Arbor, MI, USA) supplied U46619, cAMP EIA kit and cGMP enzyme immunoassay (EIA) kit, thromboxane B_2 assay kit. Cell signaling (Beverly, MA, USA) supplied the lysis buffer and antibodies against phospho-VASP (Ser^{157}), phospho-VASP (Ser^{239}), phospho-inositol-3-phosphate receptor type I (Ser^{1756}), phospho-$cPLA_2$ (Ser^{505}), phospho-$p38^{MAPK}$, phospho-Akt (Ser^{473}), 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 (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 $CaCl_2$ 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 $CaCl_2$. 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 $[Ca^{2+}]_i$ values.

Measurement of thromboxane B_2

Since thromboxane A_2 (TXA_2) is unstable and transforms into thromboxane B_2 (TXB_2) quickly, 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 with ELISA reader (TECAN, Salzburg, Austria) using TXB_2 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 $CaCl_2$ 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 were treated with fibrinogen (30 μg/mL, Alexa Flour 488-conjugated) at 37 °C for 5 min. 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 CXB and $CaCl_2$ (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.

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 $CaCl_2$, 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 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 (TXA_2 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 (IC_{50}) 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 $[Ca^{2+}]_i$ mobilization, IP_3RI phosphorylation and serotonin secretion

Intracellular ion concentration ($[Ca^{2+}]_i$) plays essential factor for platelet activation, thus we focused the effect of CXB on antagonistic activity of Ca^{2+}. As shown in Fig. 2a, $[Ca^{2+}]_i$ 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 $[Ca^{2+}]_i$ levels (Fig. 2a). Next, we investigated calcium mobilization associated signaling molecule, inositol 1, 4, 5-triphosphate receptor type I (IP_3RI) phosphorylation. As shown in Fig. 2b,

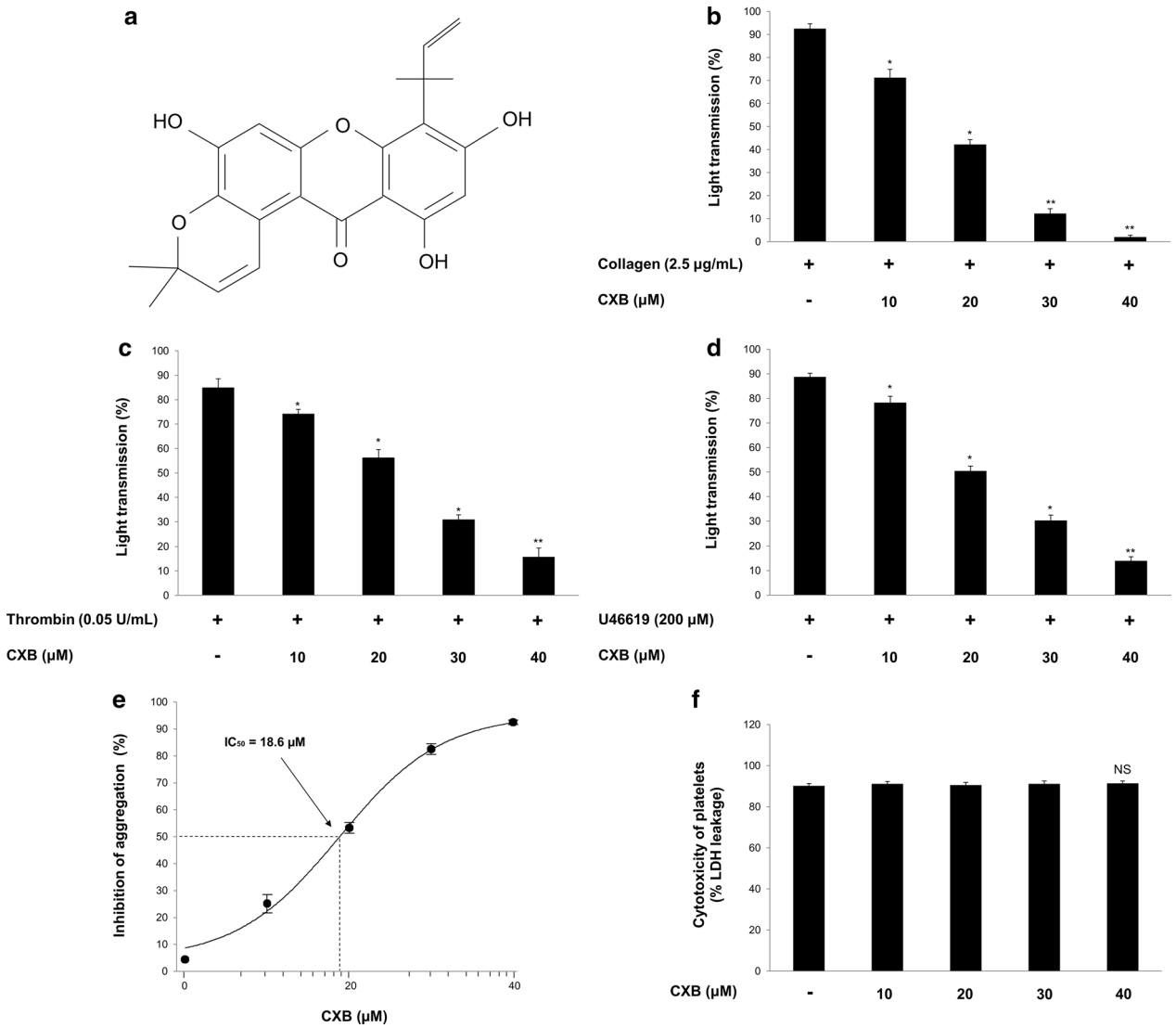


Fig. 1 Chemical structure and effects of cudraxanthone B on platelet aggregation. **a** Chemical structure of cudraxanthone B. **b** Effect of cudraxanthone B on collagen-induced human platelet aggregation. **c** Effect of cudraxanthone B on thrombin-induced human platelet aggregation. **d** Effect of cudraxanthone B on U46619-induced human platelet aggregation. **e** Half maximal inhibitory concentration (IC_{50}) value of cudraxanthone B in collagen-induced human platelet aggregation. **f** Effect of cudraxanthone B on cytotoxicity. Platelet aggregation and cytotoxicity were carried out as described in Sect. "Materials and methods". 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

CXB (30 to 40 μM) increased IP_3RI phosphorylation at Ser^{1756} in collagen-stimulated human platelet aggregation. This results mean that the decrease of $[Ca^{2+}]_i$ level by CXB is due to IP_3RI 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 TXB_2 and $cPLA_2$ and $p38^{MAPK}$ dephosphorylation

Collagen induced human platelet suspension increased TXA_2 (determined as TXB_2) levels to 45.2 ± 2.1 ng/10^8 platelets. However, CXB inhibited TXA_2 production dose-dependently (Fig. 2d). For identification of inhibitory effect of CXB on TXA_2 production, Next, we investigated TXA_2 production associated signaling molecule. The $cPLA_2$ has been reported to play key role in arachidonic acid release in human platelets. As shown

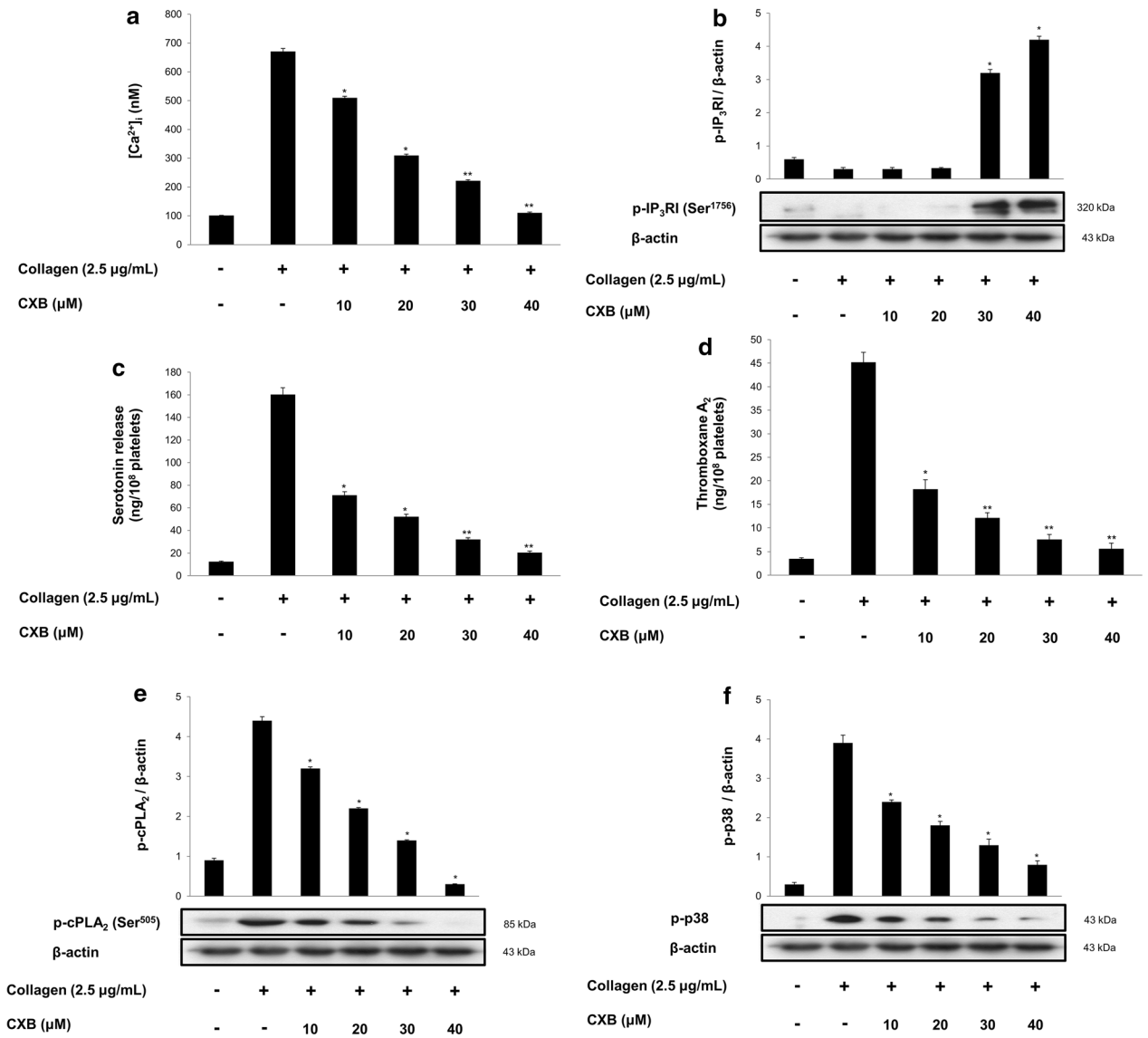


Fig. 2 Effects of cudraxanthone B on $[Ca^{2+}]_i$ mobilization, Serotonin release, TXA_2 generation and associated signaling molecules. **a** Effect of cudraxanthone B on collagen-induced $[Ca^{2+}]_i$ mobilization. **b** Effect of cudraxanthone B on collagen-induced IP_3RI (Ser^{1756}) phosphorylation. **c** Effect of cudraxanthone B on collagen-induced serotonin release. **d** Effect of cudraxanthone B on collagen-induced TXA_2 generation. **e** Effect of cudraxanthone B on collagen-induced $cPLA_2$ (Ser^{505}) phosphorylation. **f** Effect of cudraxanthone B on collagen-induced $p38^{MAPK}$ phosphorylation. Measurement of $[Ca^{2+}]_i$ mobilization, serotonin release, TXA_2 generation, Western blot was performed as described in Sect. "Materials and methods". The data are expressed as the mean ± standard deviation (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the collagen-stimulated human platelets

in Fig. 2e the $cPLA_2$ was phosphorylated at Ser^{505} by collagen, but CXB significantly inhibited $cPLA_2$ phosphorylation dose-dependently. It is well kwon that the $cPLA_2$ activity is achieved by mitogen-activated protein kinases p38 ($p38^{MAPK}$) and the $p38^{MAPK}$ also being activated through phosphorylation. As shown in Fig. 2f, collagen increased $p38^{MAPK}$ phosphorylation, but CXB inhibited collagen-elevated $p38^{MAPK}$ phosphorylation dose-dependently.

Inhibitory effects of CXB on fibrinogen binding to integrin αIIb/β_3 and fibronectin adhesion

Next, we investigated fibrinogen binding to αIIb/β_3, 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

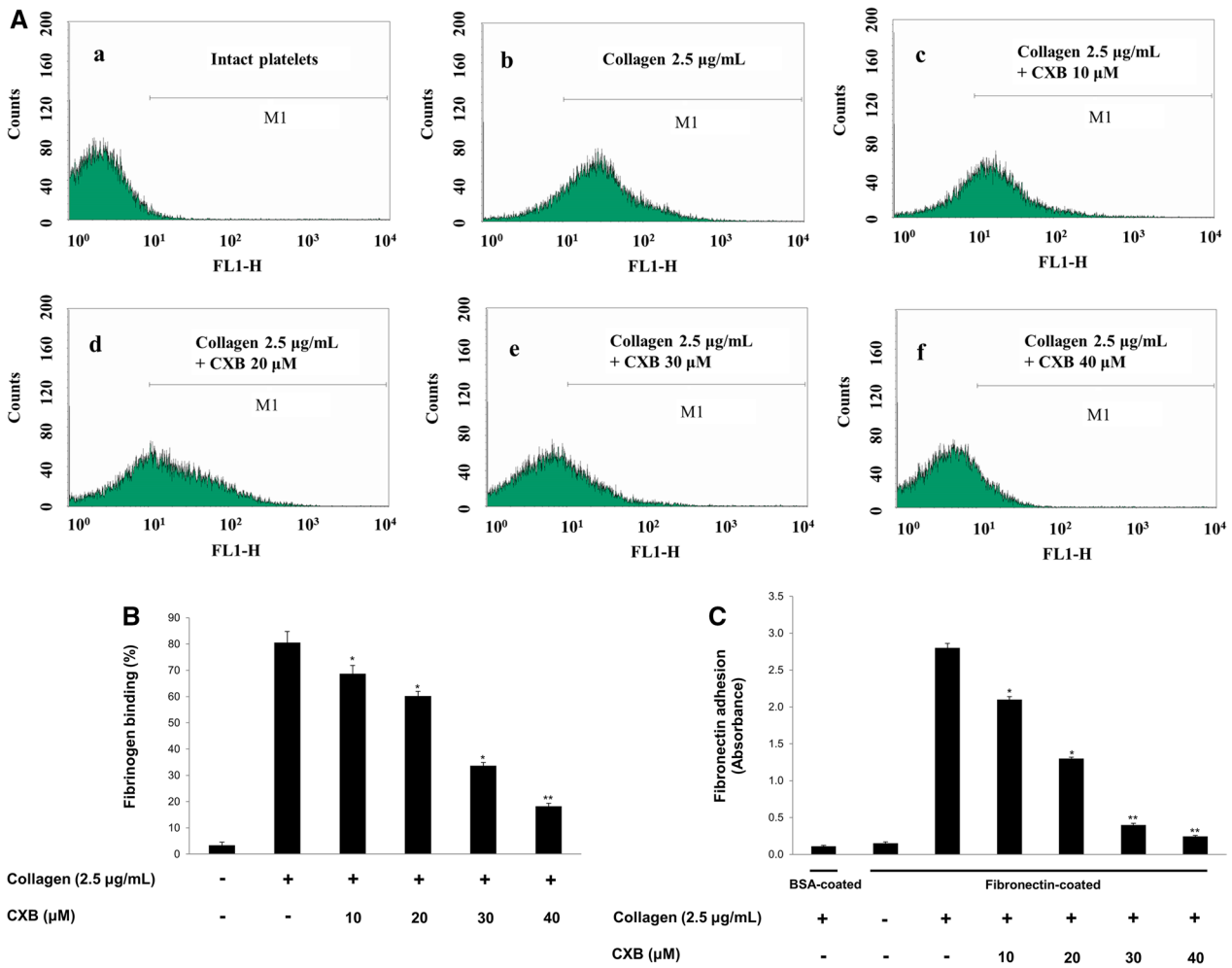


Fig.3 Effects of cudraxanthone B on fibrinogen binding to αIIb/β3 and Fibronectin adhesion. **A** The flow cytometry histograms on fibrinogen binding. **B** Effect of cudraxanthone B on collagen-induced fibrinogen binding (%). **C** Effect of cudraxanthone B on collagen-induced fibronectin adhesion. Measurement of fibrinogen binding and fibronectin adhesion was carried out as described in Sect. "Materials and methods". The data are expressed as the mean ± standard deviation (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the collagen-stimulated human platelets

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/β_3 activation (Fig. 3A, C), we investigated the effect of CXB on VASP Ser^{157} phosphorylation in collagen-stimulated platelets. CXB upregulated VASP Ser^{157} and VASP Ser^{239} phosphorylation significantly (Fig. 4a, b). Akt phosphorylation has been known as a positive signaling in αIIb/β_3 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% compare with

(See figure on next page.)

Fig. 4 Effects of cudraxanthone B on VASP, Akt phosphorylation, cyclic nucleotides and clot retraction. **a** Effect of cudraxanthone B on collagen-induced VASP (Ser^{157}) phosphorylation. **b** Effect of cudraxanthone B on collagen-induced VASP (Ser^{239}) phosphorylation. **c** Effect of cudraxanthone B on collagen-induced Akt (Ser^{473}) phosphorylation. **d** Effect of cudraxanthone B on collagen-induced cAMP production. **e** Effect of cudraxanthone B on collagen-induced cGMP production. **f** Photographs of fibrin clot (**g**) Effect of cudraxanthone B on thrombin-retracted fibrin clot (%). Measurement of Western blot and cyclic nucleotides level and quantification of fibrin clot retraction were performed as described in Sect. "Materials and methods". The data are expressed as the mean ± standard deviation (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the collagen-stimulated human platelets. $^{\dagger}p < 0.05$ versus the unstimulated human PRP, $^{\#}p < 0.05$ versus the thrombin-stimulated human PRP

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.

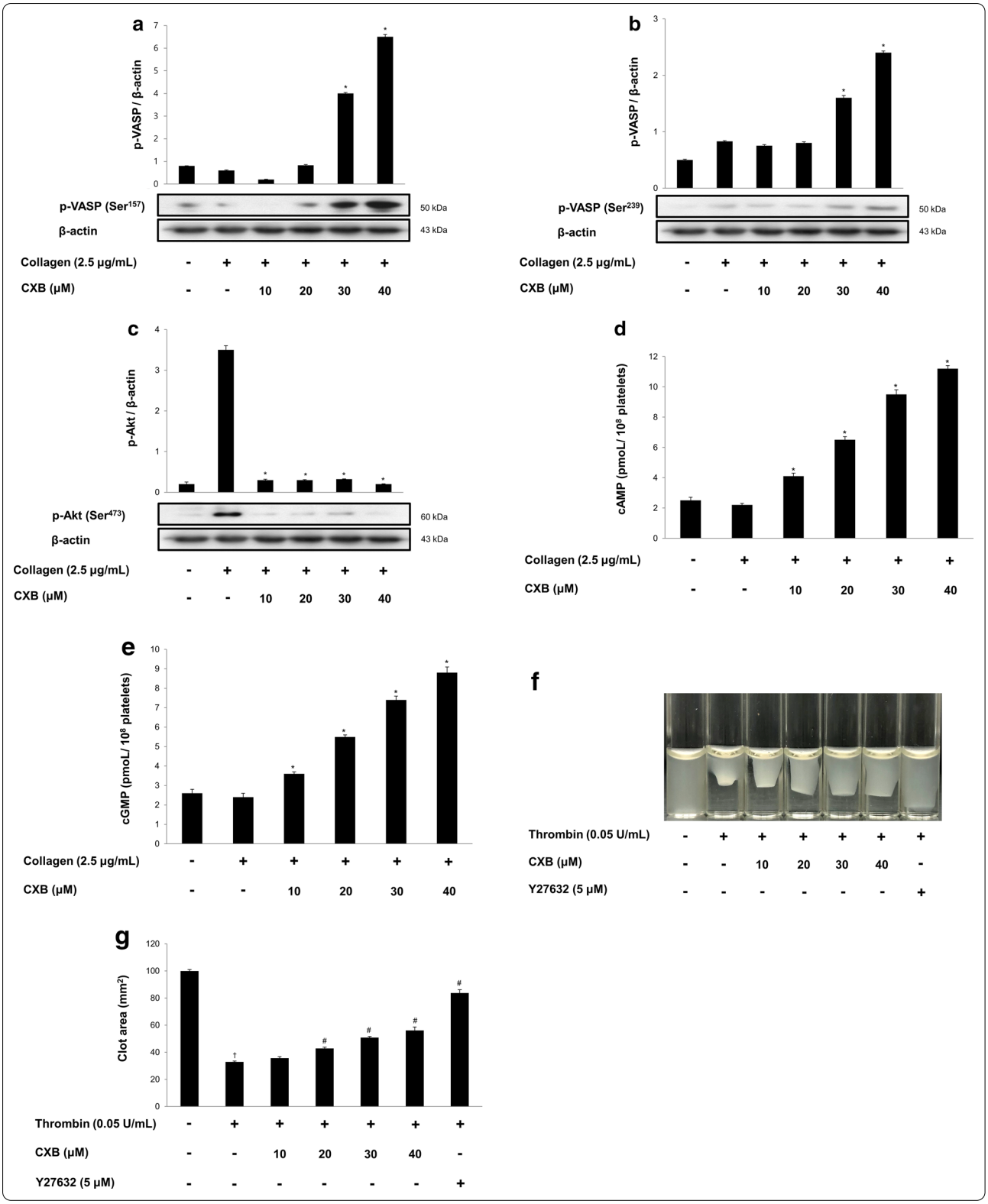
Discussion

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 cudraxanthone 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 Ca^{2+} mobilization, serotonin release, fibrinogen binding, fibronectin adhesion and associated signaling molecules.

CXB suppressed collagen-induced $[Ca^{2+}]_i$ level (Fig. 2a) through elevated IP_3RI (Ser^{1756}) phosphorylation (Fig. 2b) and affected serotonin release (Fig. 2c). TXA_2 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. $cPLA_2$ is Ca^{2+}-dependent enzyme and can hydrolyze membrane phospholipids to release arachidonic acid. Upon stimulation by agonists, $cPLA_2$ is translocated from cytosol to membrane in the presence of intracellular Ca^{2+} and phosphorylated at $cPLA_2$ at Ser^{505} by $p38^{MAPK}$ for full catalytic activity [21]. In addition, $p38^{MAPK}$ is also activated through phosphorylation and can phosphorylate $cPLA_2$ [22]. As shown in Fig. 2a, CXB inhibited $[Ca^{2+}]_i$ level, thus, we investigated whether CXB inhibits its TXA_2 production and dephosphorylation of $cPLA_2$ and $p38^{MAPK}$. CXB inhibited the phosphorylation of $p38^{MAPK}$ and $cPLA_2$ dose-dependently (Fig. 2e, f), which suppressed the TXA_2 generation (Fig. 2d). An important indicator in evaluating components or substances for platelet inhibitory activity is the generation of TXA_2 because TXA_2 acts as a autacoid that activates and aggregates other platelets. Therefore, substances that inhibit the production of TXA_2 are usefully used as antiplatelet substances, and for example, substances such as aspirin and ozagrel are known [23, 24].

The $\alpha IIb/\beta_3$ is the most plentiful integrin on platelet surface. The activation of $\alpha IIb/\beta_3$ 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 $[Ca^{2+}]_i$ 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 (Ser^{157}, Ser^{239}) and IP_3RI (Ser^{1756}), 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 $[Ca^{2+}]_i$ 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 Ca^{2+} by phosphorylation of IP_3RI (Ser^{1756}) and suppression of αIIb/β3 affinity by phosphorylation VASP (Ser^{157}, Ser^{239}) 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


a
p-VASP / β-actin
p-VASP (Ser157)
β-actin
50 kDa
43 kDa
Collagen (2.5 μg/mL)
CXB (μM)
b
p-VASP / β-actin
p-VASP (Ser239)
β-actin
50 kDa
43 kDa
Collagen (2.5 μg/mL)
CXB (μM)
c
p-Akt / β-actin
p-Akt (Ser473)
β-actin
60 kDa
43 kDa
Collagen (2.5 μg/mL)
CXB (μM)
d
cAMP (pmoL/ 10⁸ platelets)
Collagen (2.5 μg/mL)
CXB (μM)
e
cGMP (pmoL/ 10⁸ platelets)
Collagen (2.5 μg/mL)
CXB (μM)
f
Thrombin (0.05 U/mL)
CXB (μM)
Y27632 (5 μM)
g
Clot area (mm²)
Thrombin (0.05 U/mL)
CXB (μM)
Y27632 (5 μM)

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 adenylyl 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 reported to have therapeutic effects on thrombosis [27]. In fact, PDE inhibitors (cilostazol, dipyridamole) 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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