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Artocarpesin acts on human platelet aggregation through inhibition of cyclic nucleotides and MAPKs

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Abstract

The cardiovascular diseases (CVDs) are becoming a critical threat to our lives in these years. It is now widely accepted that platelets play an important role in cardiovascular disease as they have a fundamental role in thrombosis. Therefore, many drugs or natural substances have been developed to treat CVDs. *Cudrania tricuspidata* is a regional plant containing various constituents, such as xanthones, flavonoids, organic acids, and polysaccharides. It has been widely used in East Asia as an important ethnomedicine for the treatment of many diseases such as eczema, mumps, tuberculosis and acute arthritis. Therefore, we evaluated antiplatelet effects using artocarpesin isolated from *C. tricuspidata*. Confirmation of the antiplatelet function of artocarpesin was made according to the following analyzes. Artocarpesin inhibited collagen-induced human platelet aggregation, calcium mobilization, glycoprotein IIb/IIIa activation and thrombin-induced clot retraction through the regulation of associated signaling molecules. Artocarpesin increased the phosphorylation of vasodilator-stimulated phosphoprotein (VASP) and inositol 1, 4, 5-triphosphate receptor I (IP_3RI). On the other hand, the phosphorylation of cytosolic phospholipase A_2 ($cPLA_2$), mitogen-activated protein kinases p38, JNK and phosphoinositide 3-kinase (PI3K)/Akt decreased. Thus, the study highlights that artocarpesin has an inhibitory effect on platelet activity and thrombus formation, showing its potential value in preventing platelet-induced cardiovascular disease.

Keywords: Artocarpesin, Ca^{2+} mobilization, Serotonin secretion, αIIb/β3 affinity, Clot retraction, cAMP and cGMP

Background

In normal circulation of blood, damaged vascular wall shows collagen fibers and interact with circulatory platelets to start hemostasis [1, 2]. After platelet activation, phosphatidylinositol 4,5-bisphosphate hydrolyzes into inositol 1,4,5-trisphosphate (IP_3) and IP_3 induced calcium mobilization affecting granule release [3–5]. These signaling cascades are called "inside-out signaling" and activated platelets occurs structural change of glycoprotein IIb/IIIa (αIIb/β3). The signaling mechanism induced by activated αIIb/β3 is called "outside-in signaling pathway". During inside-out signaling, endogenous enzyme produces thromboxane A_2 affecting circulatory platelets [6–8]. Therefore, since platelets cause hemostasis and thrombosis, it is important to balance activity [9] and there is a need to develop various substances to inhibit activities to reduce CVDs [10].

Cyclic-adenosine monophosphate (cAMP) and cyclic-guanosine monophosphate (cGMP) are the best known mechanisms related to platelet inhibitory activity. Vasodilator-stimulated phosphoprotein (VASP) and inositol 1, 4, 5-triphosphate receptor type I (IP_3RI) are major substrates of protein kinase A and protein kinase G and VASP contributes to $\alpha IIb/\beta_3$ affinity and IP_3RI affects $[Ca^{2+}]_i$ mobilization. It has been reported that cAMP/cGMP-dependent kinases are involved in $\alpha IIb/\beta_3$ activation and $[Ca^{2+}]_i$ mobilization [11–13].

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Cudrania tricuspidata has been investigated various substances and biological activities. In this study, we searched for a new substance from *C. tricuspidata*. We have confirmed the effects of isoderrone and steppogenin in previous studies [14, 15]. In addition, it has been reported that root extract of *C. tricuspidata* inhibited rat platelet aggregation [16]. In order to prevent cardiovascular diseases, it is necessary to find various substances. Thus, we investigated a more diverse material in *C. tricuspidata* and found artocarpesin.

Methods

Chemicals and reagents

Artocarpesin was purchased from ChemFaces (Wuhan, China). Collagen was purchased form Chrono-Log Co. (Havertown, PA, USA). Fura 2-AM (2-acetoxymethyl) and alexa fluor 488-conjugated fibrinogen were obtained from Invitrogen (Eugene, OR, USA). Serotonin ELISA kit was purchased from Labor Diagnostika Nord GmbH and CO. (Nordhorn, Germany). Bicinchoninic acid protein assay kit was purchased form Pierce Biotechnology (IL, USA). Cayman chemical (Ann Arbor, MI, USA) offered thromboxane B_2 assay kit, cAMP, cGMP enzyme immunoassay kit. Cell signaling (Beverly, MA, USA) supplied the lysis buffer and antibodies against phospho-p38, phosphor-JNK (1/2), phospho-VASP (Ser^{157}), phospho-VASP (Ser^{239}), phospho-cPLA$_2$ (Ser^{505}), phosphor-PI3K (Tyr^{458}), phospho-Akt (Ser^{473}), phospho-inositol-3-phosphate receptor type I (Ser^{1756}), phosphor-PLC$_{\gamma 2}$ (Tyr^{759}), β-actin, and anti-rabbit secondary antibody. Fibronectin-coated cell adhesion kit as procured from Cell Biolabs (San Diego, CA, USA).

Human platelets suspension

Human platelet-rich plasma (PRP) was obtained from Korean Red Cross Blood Center (Suwon, Korea). The platelets were then washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 5.5 mM glucose, and 1 mM Na_2EDTA), and resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM $NaHCO_3$, 0.36 mM NaH_2PO_4, 0.49 mM $MgCl_2$, 5.5 mM glucose, 0.25% gelatin). All experiments were approved by the Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Korea) (PIRB-P01-201,812–31-007). The platelet suspension was adjusted to 5×10^8/mL concentration according to the previous research [17, 18].

Platelet aggregation

For in vitro platelet aggregation, human platelets suspension (10^8/mL) was pre-incubated for 3 min in presence or absence of artocarpesin along with 2 mM $CaCl_2$ at 37 °C, then collagen (2.5 μg/mL) was added for stimulation. Dimethyl sulfoxide solution (0.1%) was used to dissolve the artocarpesin. Platelet aggregation was measured for 7 min under stirring condition. The change in light transmission is converted into the aggregation rate (%). Platelet aggregation was monitored using an aggregometer (Chrono-Log, Havertown, PA, USA).

Cytotoxicity measurement

Cytotoxicity of artocarpesin was conducted through lactate dehydrogenase leakage assay. Human platelets (10^8/mL) was incubated with artocarpesin (40 to 100 μM) for 1 h and centrifuged at 12,000 g. The supernatant was used to detect the lactate dehydrogenase using ELISA reader (TECAN, Salzburg, Austria).

Calcium mobilization

The PRP added Fura 2-AM (5 μM) was incubated for 60 min. After incubation, PRP washed with washing buffer. After washing step, platelets were suspended using suspending buffer and the platelets ware adjusted to 5×10^8/mL concentration. The Fura 2-AM loaded platelet suspension was pre-incubated with artocarpesin (40 to 100 μM) for 3 min at 37 °C then added collagen (2.5 μg/mL). The calcium mobilization was measured using a spectro-fluorometer (Hitachi F-2700, Tokyo, Japan) and Grynkiewicz method was used for calculate the $[Ca^{2+}]_i$ values [19].

Measurement of thromboxane B_2 production

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

Serotonin release detection

Platelet aggregation was conducted for 7 min at 37 °C with artocarpesin, then reaction cuvette place onto ice in order to terminate serotonin release for 3 min. After termination, the reaction mixture was centrifuged and the supernatant was used. The serotonin was detected using ELISA reader (TECAN, Salzburg, Austria).

Western blotting analysis

After platelet aggregation, platelets are dissolved using lysis buffer. The amount of dissolved protein was calculated and proteins (15 μg) were divided by 8% SDS-PAGE. After electrophoresis, proteins are transferred onto membranes and treated primary (1:1000) and secondary antibodies (1:10,000). Western blotting was performed using the same sample separated after the platelet

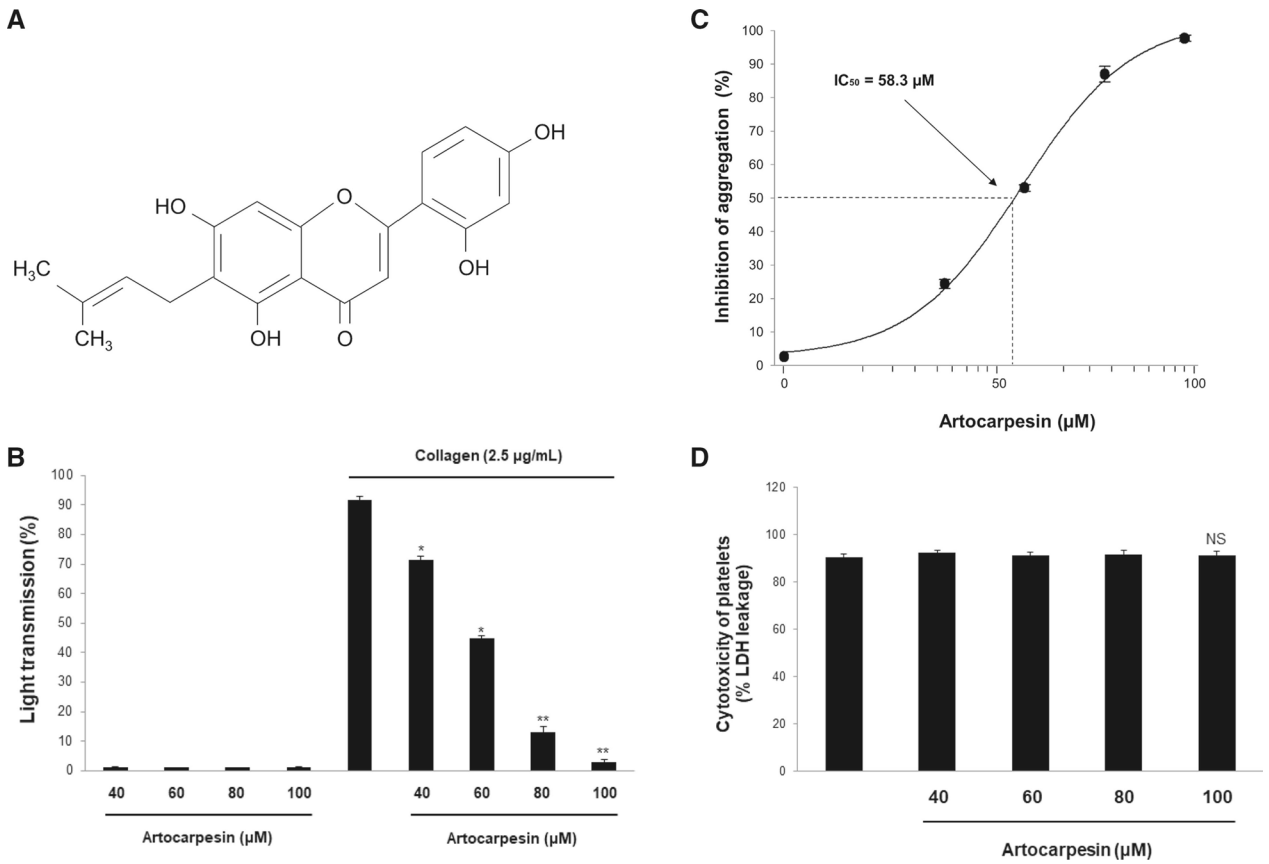

Fig. 1 Effects of artocarpesin on platelet aggregation, half maximal inhibitory concentration, and cytotoxicity. **A** Chemical structure of artocarpesin. **B** Effect of artocarpesin on collagen-induced human platelet aggregation. **C** Half maximal inhibitory concentration (IC_{50}) value of artocarpesin in collagen-induced human platelet aggregation. **D** Effect of artocarpesin 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

aggregation experiment. Western blotting analysis was conducted by using the Quantity One, Ver. 4.5 (BioRad, Hercules, CA, USA).

Fibrinogen binding to αIIb/β3

After platelet aggregation for 7 min, the reaction mixture was incubated with alexa flour 488-conjugated fibrinogen for 5 min. After incubation, 0.5% paraformaldehyde was added to fix the binding between platelet integrin and fibrinogen marker. All procedures of fibrinogen binding assay were conducted in the dark condition. The binding assay was measured using flow cytometry (BD Biosciences, San Jose, CA, USA), and results were presented by the CellQuest software (BD Biosciences).

Fibronectin adhesion assay

Human platelets (10^8/mL) was placed in fibronectin coated wells (bovine serum albumin coated well is used as a negative control) and incubated with artocarpesin in the presence of collagen (2.5 μg/mL) for 1 h at 37 °C. After incubation, wells were washed using PBS buffer and added cell stain solution for 10 min. After that, extraction solution was added and each extraction was measured by ELISA reader (TECAN, Salzburg, Austria).

Platelet-mediated fibrin clot retraction

Human platelet-rich plasma (300 μL) was incubated with artocarpesin for 30 min at 37 °C, and clot retraction was triggered by adding thrombin (0.05 U/mL). After reacting for 15 min, pictures of fibrin clot were taken using a digital camera. Image J Software (v1.46) was used to calculate the clot area (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

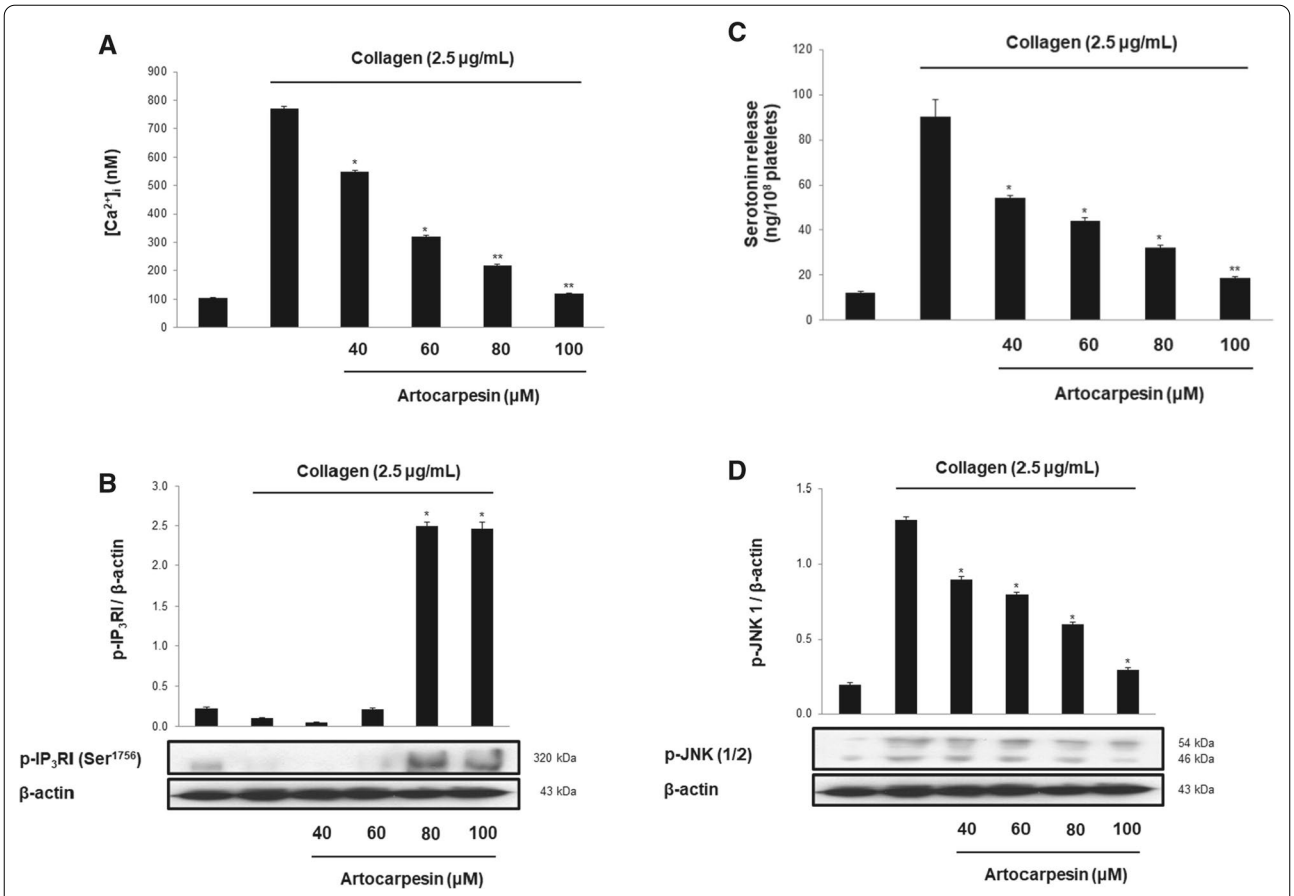

Fig. 2 Effects of artocarpesin on $[Ca^{2+}]_i$ mobilization, Serotonin release, TXA_2 generation, and IP_3RI and JNK (1/2) phosphorylation. **A** Effect of artocarpesin on collagen-induced $[Ca^{2+}]_i$ mobilization. **B** Effect of artocarpesin on collagen-induced IP_3RI phosphorylation. **C** Effect of artocarpesin on collagen-induced serotonin release. **D** Effect of artocarpesin on collagen-induced JNK (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

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 artocarpesin on human platelets aggregation and cytotoxicity

Platelets suspension was incubated with various concentrations of artocarpesin (40 to 100 μM, Fig. 1A) without stimulation of collagen for 7 min, but the light transmission was not changed (Fig. 1B). However, collagen-induced platelet aggregation treated with artocarpesin (40 to 100 μM) was decreased dose-dependently and half maximal inhibitory concentration (IC_{50}) was 58.3 μM (Fig. 1C). To investigate the cytotoxicity, we used lactate dehydrogenase-based assay. As shown in Fig. 1D, artocarpesin (40 to 100 μM) did not affect cytotoxicity.

Effects of artocarpesin on $[Ca^{2+}]_i$ mobilization, IP_3RI phosphorylation, serotonin secretion, JNK phosphorylation

Intracellular calcium concentration ($[Ca^{2+}]_i$) is a crucial essential factor for platelet aggregation and activation. Increased calcium activates myosin light chain phosphorylation and can affect granule release, thus we focused on $[Ca^{2+}]_i$ mobilization. As shown in Fig. 2A, $[Ca^{2+}]_i$ mobilization were elevated from 105.2 ± 0.6 nM to 770.6 ± 8.4 nM by collagen (2.5 μg/mL). However, artocarpesin dose-dependently reduced the increased $[Ca^{2+}]_i$ levels. To confirm the $[Ca^{2+}]_i$ mobilization regulation, we investigated Ca^{2+} control signaling molecule, inositol 1, 4, 5-triphosphate receptor type I (IP_3RI). As shown in Fig. 2B, artocarpesin (80 to 100 μM) increased IP_3RI phosphorylation in collagen-induced human platelets. This result means that the decrease of $[Ca^{2+}]_i$ level by artocarpesin is due to change of IP_3RI. Next, we

examined whether artocarpesin affect serotonin release in δ-granules. As shown in Fig. 2C, artocarpesin dose-dependently inhibited collagen-stimulated serotonin secretion. The JNK1 is involved in platelet secretion [20], thus we investigated the change of JNK (1/2) phosphorylation by artocarpesin. As shown in Fig. 2D, artocarpesin decreased JNK1 phosphorylation in collagen-induced human platelets. These results suggest that inhibition of granule release by artocarpesin is achieved by inhibiting release-related signal regulators, IP_3RI and JNK1.

Effects of artocarpesin on fibrinogen binding to integrin αIIb/β_3, fibronectin adhesion and VASP phosphorylation and PI3K/Akt dephosphorylation

Next, we investigated αIIb/β_3 activation, leading integrin-mediated outside-in signaling. Collagen elevated the αIIb/β_3 activation, with a binding rate of 82.5 ± 3.1% (Fig. 3A, B). However, artocarpesin inhibited the binding force of fibrinogen dose-dependently (Fig. 3A, B). The αIIb/β3 can also interact with fibronectin. Therefore, we examined whether artocarpesin affect fibronectin

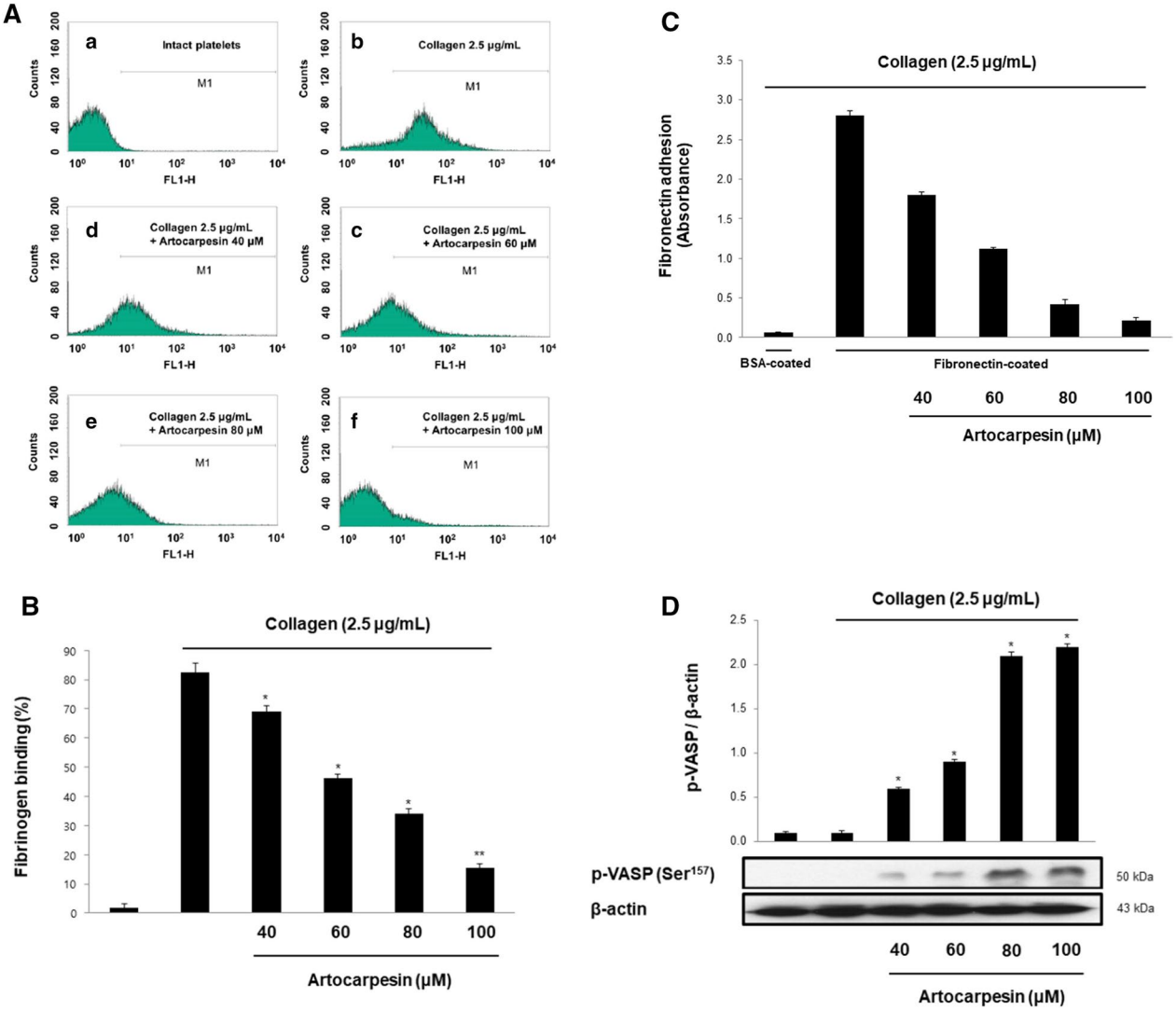

Fig. 3 Effects of artocarpesin on fibrinogen binding to αIIb/β3, fibronectin adhesion and VASP and PI3K/Akt phosphorylation. **A** The flow cytometry histograms on fibrinogen binding. **B** Effects of artocarpesin on collagen-induced fibrinogen binding (%). **C** Effects of artocarpesin on collagen-induced fibronectin adhesion. **D** Effect of artocarpesin on collagen-induced VASP (Ser^{157}) phosphorylation. **E** Effect of artocarpesin on collagen-induced VASP (Ser^{239}) phosphorylation. **F** Effect of artocarpesin on collagen-induced PI3K (Tyr^{458}) phosphorylation. **G** Effect of artocarpesin on collagen-induced Akt (Ser^{473}) phosphorylation. Measurement of fibrinogen binding, fibronectin adhesion, and Western blot was carried out as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n = 4). $^{*}p < 0.05$, $^{**}p < 0.01$ versus the collagen-stimulated human platelets

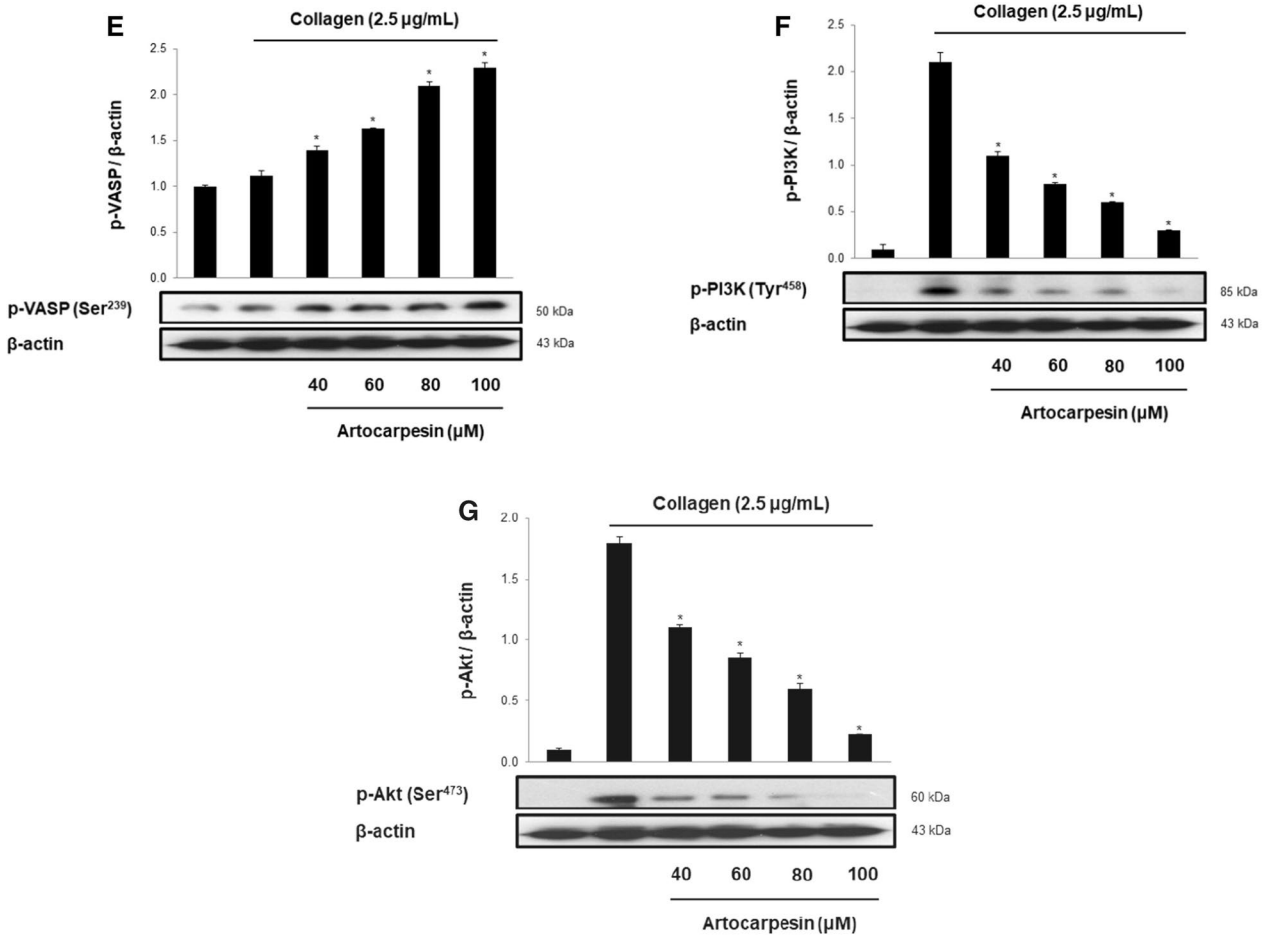

Fig. 3 continued

adhesion. As shown in Fig. 3C, fibronectin adhesion was suppressed by artocarpesin dose-dependently. Since we confirmed that artocarpesin inhibits fibrinogen binding and fibronectin adhesion, we investigated which signaling molecules were affected by artocarpesin.

It is well known that phosphorylated VASP (Ser^{157}, Ser^{239}) acts as a negative signaling in $\alpha IIb/\beta_3$ and phosphorylated phosphoinositide 3-kinase (PI3K)/Akt has been known as a positive signaling in $\alpha IIb/\beta_3$ [21, 22]. Thus, we examined whether artocarpesin affects its phosphorylation. Collagen-induced VASP phosphorylation was increased by artocarpesin dose-dependently (Fig. 3D, E) but, PI3K/Akt phosphorylation was suppressed by artocarpesin dose-dependently (Fig. 3F, G). These results mean that the decrease of $\alpha IIb/\beta_3$ affinity by artocarpesin is due to VASP (Ser^{157}, Ser^{239}) phosphorylation and PI3K (Tyr^{458})/Akt (Ser^{473}) dephosphorylation.

Measurement thromboxane A_2 production, dephosphorylation of $cPLA_2$, p38 and cyclic nucleotides

We investigated TXA_2 production associated signaling molecule. Collagen (2.5 μg/mL) stimulated human platelet produced TXA_2 (determined as TXB_2) from 1.2 ± 0.2 nM to 48.0 ± 0.2 ng/10^8 platelets. However, artocarpesin inhibited TXA_2 production dose-dependently (Fig. 4A). Thus, we investigated TXA_2 associated signaling molecules, cytosolic phospholipase A_2 ($cPLA_2$) and p38 mitogen-activated protein kinases (p38). As shown in Fig. 4B and C, the $cPLA_2$ and p38 are phosphorylated by collagen, but artocarpesin inhibited $cPLA_2$ and p38 phosphorylation dose-dependently. These results mean that the decrease of TXA_2 production by artocarpesin is due to $cPLA_2$ and p38 dephosphorylation.

Next, we investigated the production of cAMP and cGMP in platelets. As shown in Fig. 4D and E, the

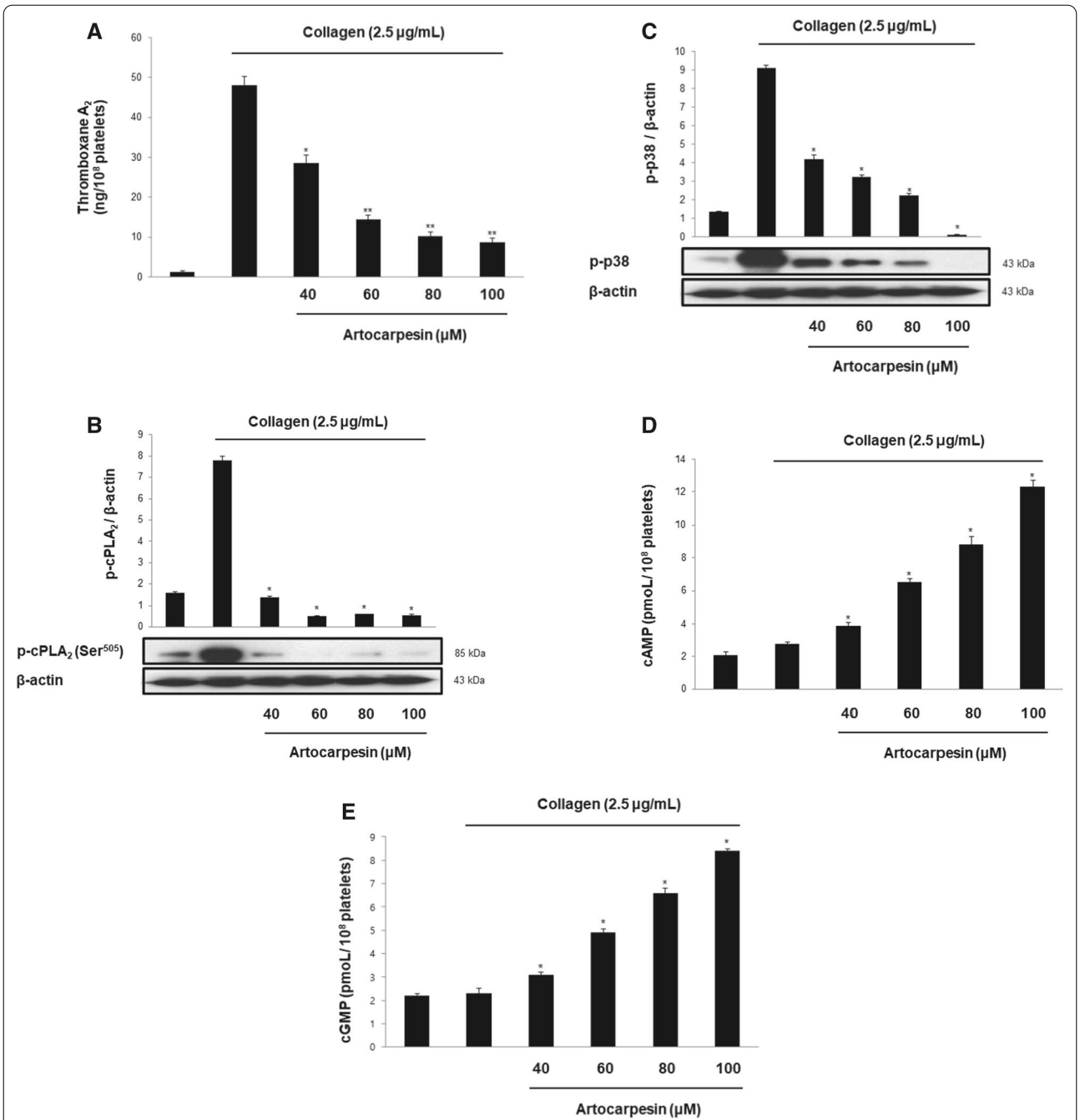

Fig. 4 Effects of artocarpesin on TXA_2 generation, $cPLA_2$, p38phosphorylation and cyclic nucleotides. **A** Effects of artocarpesin on collagen-induced TXA_2 generation. **B** Effect of artocarpesin on collagen-induced $cPLA_2$ phosphorylation. **C** Effect of artocarpesin on collagen-induced p38 phosphorylation. **D** Effect of artocarpesin on collagen-induced cAMP production. **E** Effect of artocarpesin on collagen-induced cGMP production. Measurement of TXA_2 generation, Western blot and cyclic nucleotides 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

production of cAMP and cGMP was increased by artocarpesin dose-dependently. These results mean that artocarpesin can increase cAMP and cGMP level in human platelet and activate cAMP/cGMP dependent signaling pathways affecting $[Ca^{2+}]_i$ mobilization and αIIb/β3 activation. Therefore, it is considered that the antiplatelet effect of artocarpesin is caused by the increase of cAMP and cGMP and the decrease of p38 and JNK1 phosphorylation. The effect of PKA and PKG, which are submolecules of cAMP and cGMP, on MAPKs has not yet

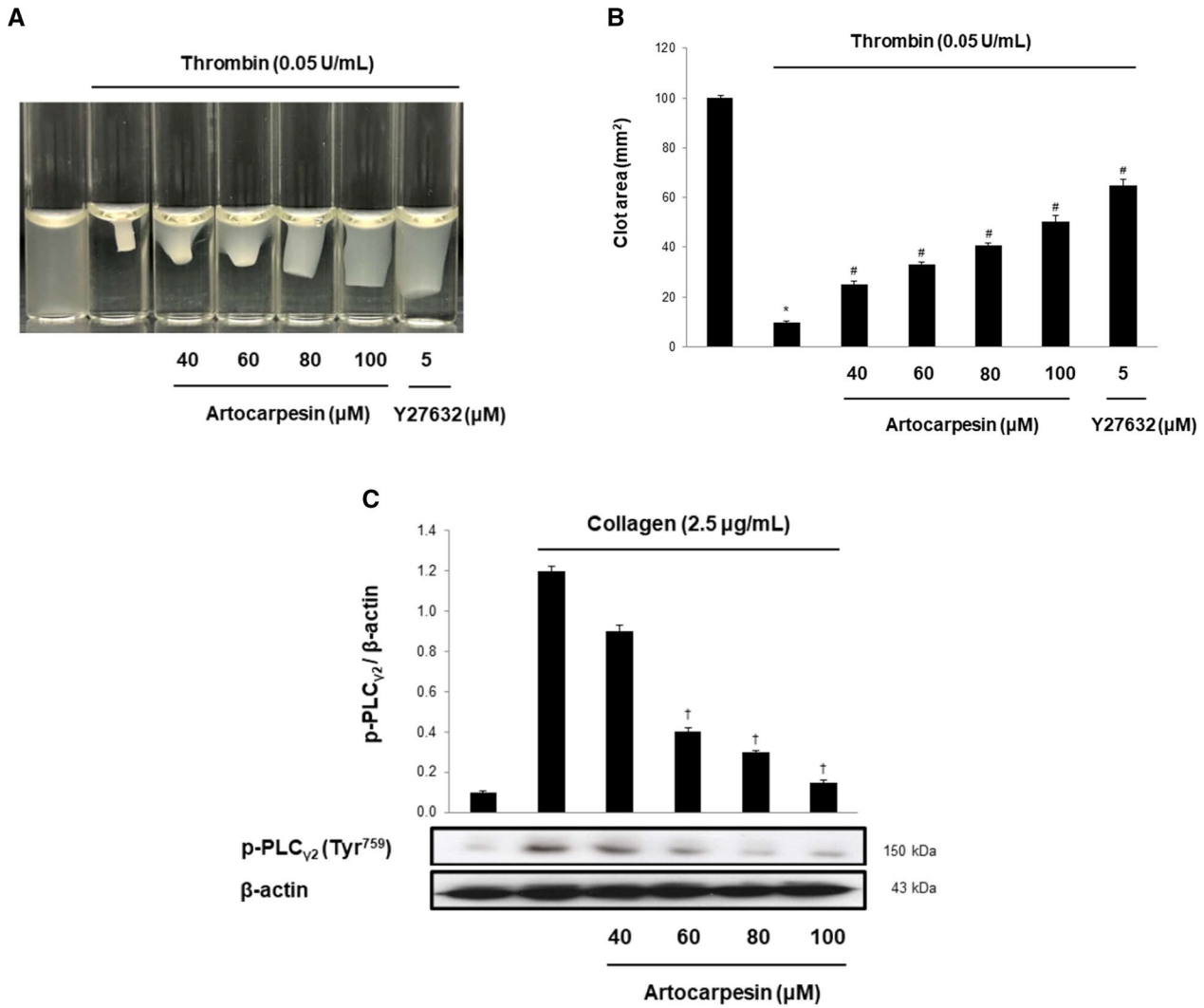

Fig. 5 Effects of artocarpesin on fibrin clot retraction and $PLC_{\gamma 2}$ phosphorylation. **A** Photographs of fibrin clot **B** Effects of artocarpesin on thrombin-retracted fibrin clot (%). **C** Effect of artocarpesin on collagen-induced $PLC_{\gamma 2}$ (Tyr^{759}) phosphorylation. Quantification of fibrin clot retraction and Western blot 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, $^{\#}p < 0.05$ versus the thrombin-stimulated human PRP. $^{\dagger}p < 0.05$ versus the collagen-stimulated human platelets

been clearly identified. Therefore, artocarpesin is thought to exhibit antiplatelet effect by inhibiting each of the two pathways.

Effects of artocarpesin on clot retraction and $PLC_{\gamma 2}$ phosphorylation

$[Ca^{2+}]_i$ mobilization leads inside-out signaling pathway and activated integrin αIIb/β3 facilitates outside-in signaling pathway which trigger various actions in platelets such as spreading, granule secretion, adhesion and clot retraction. Therefore, we examined the inhibitory effects of artocarpesin on clot retraction. Figure 5A and B shows thrombin-induced fibrin clot formation and contraction. Thrombin induced platelet rich plasma was contracted with an inhibition rate of 90.3% compare with unstimulated platelet rich plasma. However, the retraction was suppressed by artocarpesin (40 to 100 μM) dose-dependently, with inhibitory degrees of 74.9, 67.1, 59.2 and 50.0%, respectively, compared with unstimulated platelet rich plasma (Fig. 5B). αIIbβ3 is an important medium for causing clot retraction. Activated αIIbβ3 triggers tyrosine phosphoryation of β3 integrin tail and activates

Table 1 Human platelet aggregation screening by phytochemicals in *Cudrania tricuspidata*

Treatment	Aggregation (%)
Collagen (2.5 μg/mL)	91.8 ± 1.3
Cudraxanthone D	
40 μM	91.1 ± 1.1
60 μM	91.2 ± 1.2
80 μM	90.2 ± 1.1
100 μM	92.2 ± 0.5
Cudraflavanone B	
40 μM	91.0 ± 0.7
60 μM	91.5 ± 0.5
80 μM	89.6 ± 1.2
100 μM	89.4 ± 1.1
Alboctalol	
40 μM	89.7 ± 1.3
60 μM	90.7 ± 0.8
80 μM	91.3 ± 0.9
100 μM	91.6 ± 1.4
Isolupalbigenin	
40 μM	92.3 ± 1.5
60 μM	91.7 ± 1.2
80 μM	89.7 ± 1.4
100 μM	90.0 ± 0.8
Xanthone V1a	
40 μM	88.0 ± 1.5
60 μM	91.0 ± 1.1
80 μM	90.3 ± 1.5
100 μM	92.3 ± 1.4
Cudraflavone B	
40 μM	91.7 ± 0.5
60 μM	90.0 ± 1.1
80 μM	91.7 ± 1.3
100 μM	92.0 ± 1.5
Shuterin	
40 μM	90.3 ± 1.5
60 μM	92.1 ± 0.8
80 μM	88.1 ± 1.1
100 μM	90.4 ± 1.2
Artocarpesin	
40 μM	71.5 ± 1.3
60 μM	44.8 ± 1.0*
80 μM	13.0 ± 2.2*
100 μM	3.0 ± 0.8**

Results are expressed as % of aggregation induced by collagen

The data are expressed as the mean ± standard deviation (n = 4)

*$p < 0.05$, **$p < 0.01$ versus the collagen-stimulated human platelets

phospholipase $C_{\gamma 2}$ ($PLC_{\gamma 2}$). The $PLC_{\gamma 2}$ has been reported to be crucial for spreading action of platelets and mediating clot retraction [23]. Therefore, we examined whether artocarpesin affects the phosphorylation of $PLC_{\gamma 2}$. As shown in Fig. 5C, collagen elevated $PLC_{\gamma 2}$ phosphorylation was suppressed by artocarpesin dose-dependently.

Discussion

C. tricuspidata is widespread throughout East Asia and used in ethnomedicine. In China, *C. tricuspidata* have been used as herbal teas for a long time. In Korea, *C. tricuspidata* have been widely used as traditional medicine and it has been reported that *C. tricuspidata* have various physiological activities including inflammation, diabetes, obesity, and tumor [24]. With respect to antiplatelet effect, isoderrone, steppogenin and cudratricusxanthone A were found to have an inhibitory effect. [14, 15, 25]. Thus, we searched new anti-platelet substances from *C. tricuspidata* and we investigated 8 single compounds such as alboctalol, cudraxanthone D, cudraflavanon B, isolupalbigenin, xanthone V1a, cudraflavone B, shuterin, and artocarpesin (Table 1). Artocarpesin potently inhibited collagen-induced platelet aggregation. Therefore, we checked Ca^{2+} mobilization, serotonin release, $\alpha IIb/\beta_3$ affinity, clot retraction and associated signaling molecules.

Artocarpesin suppressed $[Ca^{2+}]_i$ level and serotonin release through IP_3RI (Ser^{1756}) phosphorylation (Fig. 2B) and dephosphorylation of JNK1 (Fig. 2D). The activation of $\alpha IIb/\beta_3$ leads to a rapid binding to fibrinogen and fibronectin and triggers outside-in signaling. Our results clarified that artocarpesin downregulated αIIb/β3 activity (Fig. 3A, C) through upregulation of phosphorylation of VASP (Fig. 3D, E) and downregulation of PI3K/Akt phosphorylation (Fig. 3F, G). Artocarpesin also suppressed TXA_2 production through dephosphorylation of $cPLA_2$ and p38 dose-dependently (Fig. 4B, C). Intracellular cAMP and cGMP are strong negative molecules and regulated by enzymes such as cyclic adenylate/guanylate cyclase, and phosphodiesterases [26]. These cyclic nucleotides inhibit αIIb/β3 affinity and $[Ca^{2+}]_i$ mobilization. In our study, artocarpesin increased cAMP and cGMP concentration Fig. 4D and E and these cyclic nucleotides can elevate the phosphorylation of VASP (Ser^{157}, Ser^{239}) and IP_3RI (Ser^{1756}).

The interaction between αIIb/β3 and causes the clot formation [5]. Therefore, we investigated that whether artocarpesin affect thrombin-induced fibrin clot retraction. As shown in Fig. 5A, artocarpesin strongly suppressed the retraction. This result is achieved through inhibition of Ca^{2+} mobilization, thromboxane A_2 production and αIIb/β3 inactivation.

This effect of artocarpesin was similar to the previously studied substances cudraxanthone B and euchrestaflavanone A [27, 28]. All three substances showed antiplatelet effect by increasing cyclic nucleotides. Therefore, *C. tricuspidata* has potential as an antithrombotic agent.

We checked these results through signaling molecules such as IP_3RI, JNK1, VASP, PI3K/Akt, $cPLA_2$ and p38. We revealed that the inhibitory effects of artocarpesin on anti-platelet activities and anti-thrombus functions are due to the elevated cyclic nucleotides and dephosphoryalation of MAPKs. Through the all experimental results, we believe that artocarpesin is valuable as a potential treatment for cardiovascular diseases and pulmonary hypertension [29]. As evidence, PDE inhibitors (cilostazol, dipyridamole) have been reported to have therapeutic effects on thrombosis to increase cyclic nucleotides production [30, 31]. *C. tricuspidata* has potential as an antithrombotic substance and phytochemicals in *C. tricuspidata* may have synergistic effects. Therefore the synergistic effect of *C. tricuspidata*-derived ingredients will be carried out in future studies. If an optimal synergistic effect is found, it is thought that effective functional food can be developed by including it in the *C. tricuspidata* extract. Preprint of this paper is available at research square [32].

Abbreviations

CVDs: Cardiovascular diseases; VASP: Vasodilator-stimulated phosphoprotein; IP_3RI: Inositol 1, 4, 5-triphosphate receptor I; MAPKs: Mitogen-activated protein kinases; cAMP: Cyclic adenosine monophosphate; cGMP: Cyclic guanosine monophosphate; TXA_2: Thromboxane A_2; $PLC\gamma_2$: Phospholipase $C\gamma_2$; PI3K: Phosphoinositide 3-kinase; $[Ca^{2+}]_i$: Intracellular calcium concentration.

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Author contributions

Conception and design of the experiment: MHR, HWK. Performance of the experiment: JHS, HWK. Analysis and arrangement of the data: JHS, MI, YYL, HWK. Contribution of reagents, materials, and tool: JHS, HWK. Contribution of manuscript preparation: MHR, HWK. 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.

Declarations

Ethics approval and consent to participate

All experiments were approved by the Public Institutional Review Board at the National Institute for Bioethics Policy (Seoul, Korea) (PIRB-P01-201812–31-007). All authors agreed to participate in the study and submit the manuscript to Applied Biological Chemistry.

Consent for publication

All authors have approved the manuscript and agree with its publication to Applied Biological Chemistry.

Competing interests

The authors declare no conflict of interest.

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