

Article

In Vitro Antiplatelet Activity of Mulberroside C through the Up-Regulation of Cyclic Nucleotide Signaling Pathways and Down-Regulation of Phosphoproteins

Hyuk-Woo Kwon ¹, Dong-Ha Lee ^{2,3}, Man Hee Rhee ⁴ and Jung-Hae Shin ^{5,*}

¹ Department of Biomedical Laboratory Science, Far East University, Eumseong 27601, Korea; kwonhw@kdu.ac.kr

² Department of Biomedical Laboratory Science, Namseoul University, Cheonan 31020, Korea; dhlee@nsu.ac.kr

³ Molecular Diagnostics Research Institute, Namseoul University, Cheonan 31020, Korea

⁴ Laboratory of Physiology and Cell Signaling, College of Veterinary Medicine, Kyungpook National University, Daegu 41566, Korea; rheemh@knu.ac.kr

⁵ Department of Biomedical Laboratory Science, Catholic Kwandong University, Gangneung 25601, Korea

* Correspondence: mlsjshin@cku.ac.kr; Tel.: +82-33-649-7431

Citation: Kwon, H.-W.; Lee, D.-H.; Rhee, M.H.; Shin, J.-H. In vitro Antiplatelet Activity of Mulberroside C through The Up-Regulation of Cyclic Nucleotide Signaling Pathways and Down-Regulation of Phosphoproteins. *Genes* **2021**, *12*, 1024. <https://doi.org/10.3390/genes12071024>

Academic Editors: Tomas Laursen and Bruna Marques dos Santos

Received: 26 April 2021

Accepted: 29 June 2021

Published: 30 June 2021

Publisher's Note: MDPI stays neutral with regard to jurisdictional claims in published maps and institutional affiliations.



Copyright: © 2021 by the authors. Licensee MDPI, Basel, Switzerland. This article is an open access article distributed under the terms and conditions of the Creative Commons Attribution (CC BY) license (<http://creativecommons.org/licenses/by/4.0/>).

Abstract: Physiological agonists trigger signaling cascades, called “inside-out signaling”, and activated platelets facilitate adhesion, shape change, granule release, and structural change of glycoprotein IIb/IIIa (α IIb/ β 3). Activated α IIb/ β 3 interacts with fibrinogen and begins second signaling cascades called “outside-in signaling”. These two signaling pathways can lead to hemostasis or thrombosis. Thrombosis can occur in arterial and venous blood vessels and is a major medical problem. Platelet-mediated thrombosis is a major cause of cardiovascular disease (CVD). Therefore, controlling platelet activity is important for platelet-mediated thrombosis and cardiovascular diseases. In this study, focus on *Morus alba* Linn, a popular medicinal plant, to inhibit the function of platelets and found the containing component mulberroside C. We examine the effect of mulberroside C on the regulation of phosphoproteins, platelet-activating factors, and binding molecules. Agonist-induced human platelet aggregation is dose-dependently inhibited by mulberroside C without cytotoxicity, and it decreased Ca^{2+} mobilization and p-selectin expression through the upregulation of inositol 1, 4, 5-triphosphate receptor I (Ser^{1756}), and downregulation of extracellular signal-regulated kinase (ERK). In addition, mulberroside C inhibited thromboxane A_2 production, fibrinogen binding, and clot retraction. Our results show antiplatelet effects and antithrombus formation of mulberroside C in human platelets. Thus, we confirm that mulberroside C could be a potential phytochemical for the prevention of thrombosis-mediated CVDs.

Keywords: mulberroside C; intracellular calcium; glycoprotein IIb/IIIa; granule secretion; clot retraction

1. Introduction

Many studies have been conducted on health and longevity, and new agents that are beneficial for human health can be challenging to identify. Among various candidates, medicinal plants are major constituents of alternative medicines that lead to new drugs [1]. Cardiovascular diseases (CVDs), in particular, affect human health. Various factors have been identified as causative of cardiovascular disease; however, the role of platelets is putatively very important. Platelets play a fundamental role in thrombosis, and the available antiplatelet drugs effectively reduce thrombosis in patients with CVDs. However, some side effects of these drugs have been reported [2]. Therefore, to develop new antiplatelet drugs, we focused on the antiplatelet activity of various medicinal plants.

M. alba Linn, a popular medicinal plant belonging to the family Moraceae, has long been used in traditional medicine in many Asian countries [3]. Regarding the effects of *M. alba* extracts for reducing thrombosis, it has been reported that the *M. alba* leaves extract inhibits rat platelets and showed synergistic effects with *Schisandra chinensis* [4,5]. In addition, morusinol isolated from *M. alba* inhibits agonist-induced rabbit platelets [6]. Therefore, we examined the antiplatelet effects of mulberroside C isolated from *M. alba* [7].

When endothelial walls are injured, collagen can bind to integrin $\alpha 2\beta 1$ and glycoprotein VI of platelets. These interactions lead to the inside-out signaling pathways. During platelet activation, phospholipase $C\gamma 2$ breaks down phosphatidylinositol 4,5-bisphosphate into inositol diacylglycerol and 1,4,5-trisphosphate (IP_3), and released IP_3 binds to the IP_3 receptor on the endoplasmic reticulum [8]. These signaling pathways facilitate Ca^{2+} mobilization, α - and δ -granule secretion, and platelet aggregation. The p-selectin is located inside the α -granule, and during inside-out signaling, p-selectin is re-expressed on the platelet surface through α -granule release [9]. Another mechanism that regulates calcium is an influx from extracellular spaces. Depletion of Ca^{2+} in the endoplasmic reticulum facilitates the influx of extracellular Ca^{2+} , which is controlled by extracellular signal-regulated kinases [10].

Another platelet activator is thromboxane A_2 (TXA_2). The mitogen-activated protein kinase p38 ($p38^{MAPK}$) is an essential mediator for the activation of cytosolic phospholipase A_2 ($cPLA_2$) through phosphorylation at Ser⁵⁰⁵ [11]. Activated $cPLA_2$ hydrolyzes the plasma membrane and releases arachidonic acid, which is synthesized as TXA_2 by cyclooxygenase and TXA_2 synthase [12]. TXA_2 acts as an autacoid that activates other platelets and facilitates thrombosis [13].

The most well-known substances for the inhibitory action of platelets are cyclic nucleotides, cyclic adenosine monophosphate (cAMP), and cyclic guanosine monophosphate (cGMP) [14]. In normal blood circulation, endothelial cells release nitric oxide and prostaglandin I_2 , which maintain the inactive platelet condition. Two platelet-inactivating substances act on platelets, increasing cAMP and cGMP levels. Vasodilator-stimulated phosphoprotein (VASP) and inositol 1,4,5-triphosphate receptor type I (IP_3RI) are major targets of cAMP and cGMP. These signaling molecules can affect $\alpha IIb/\beta_3$ activity and Ca^{2+} mobilization in platelets [15]. Therefore, medical plants that regulate cyclic nucleotides can be used for platelet-mediated thrombosis.

In this study, we examined whether mulberroside C inhibits platelet aggregation and thrombus formation through the concentration of cyclic nucleotides and associated signaling molecules.

2. Materials and Methods

2.1. Materials

Mulberroside C (Figure 1) was provided by ChemFaces (Wuhan, China). Mouse monoclonal to CD62P (P-selectin) antibody was purchased from Biolegend (San Diego, CA, USA). Fura 2-AM (2-acetoxymethyl) and Alexa Fluor 488-conjugated fibrinogen were obtained from Invitrogen (Eugene, OR, USA). The BCA protein assay kit was obtained from Pierce Biotechnology (Rockford, IL, USA). The serotonin ELISA kit was provided by Labor Diagnostika Nord GmbH and Co. (Nordhorn, Germany). Collagen, U46619, and thrombin were purchased from Chrono-Log Co. (Havertown, PA, USA). The thromboxane B_2 assay kit, cAMP, cGMP enzyme immunoassay kit, and ATP assay kit were obtained from Cayman Chemical (Ann Arbor, MI, USA). Cell signaling (Beverly, MA, USA) supplied antiphospho- $p38^{MAPK}$, antiphospho-ERK (1/2), antiphospho-VASP (Ser¹⁵⁷), antiphospho-VASP (Ser²³⁹), antiphospho- $cPLA_2$ (Ser⁵⁰⁵), antiphospho-PI3K (Tyr⁴⁵⁸), antiphospho-Akt (Ser⁴⁷³), antiphospho-inositol-3-phosphate receptor type I (Ser¹⁷⁵⁶), antiphospho- $PLC\gamma 2$ (Tyr⁷⁵⁹), anti- β -actin, and antirabbit secondary antibodies.

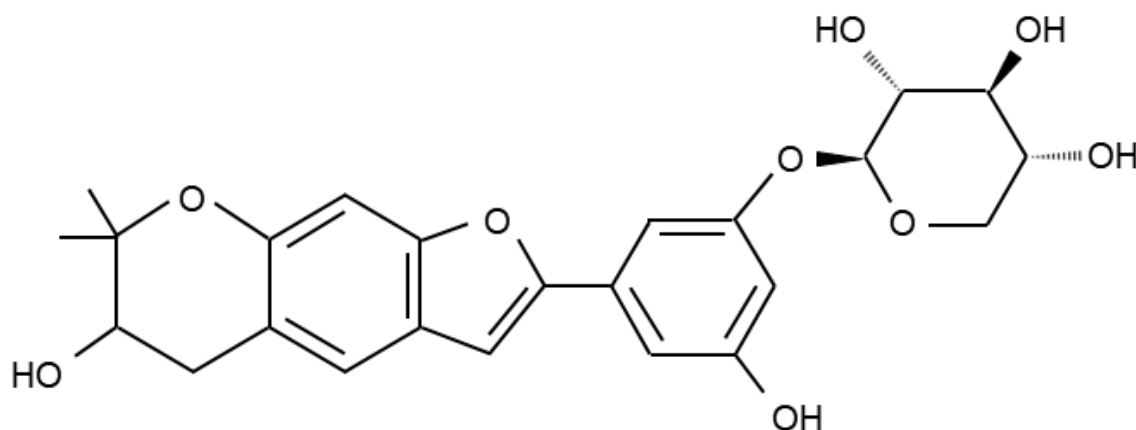


Figure 1. Chemical structure of mulberroside C (MW. 458.46) isolated from *M. alba*.

2.2. Preparation of Suspension of Human Platelets

Human platelet-rich plasma (PRP) was obtained from the Korean Red Cross Blood Center (Suwon, Korea). Experiments are conducted on human PRP isolated from 4 independent PRP probes. The platelets were then washed twice with washing buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 5.5 mM glucose, and 1 mM Na₂EDTA), and resuspended in suspension buffer (138 mM NaCl, 2.7 mM KCl, 12 mM NaHCO₃, 0.36 mM NaH₂PO₄, 0.49 mM MgCl₂, 5.5 mM glucose, 0.25% gelatin). All experiments were approved by the Catholic Kwandong University Institutional Review Board (CKU-20-01-0109). The platelet suspension was adjusted to a concentration of 5×10^8 /mL cells/mL according to a previous study [16].

2.3. In Vitro Platelet Aggregation Assay

Platelet suspensions (10^8 /mL) were preincubated for 2 min with mulberroside C (50–150 μ M), and then agonists (collagen, thrombin, and U46619) are added for aggregation. Dimethyl sulfoxide (DMSO) is used to dissolve mulberroside C, and the final concentration of DMSO is 0.1% (not interfere with the platelet aggregation). Platelet aggregation was measured for five minutes at 37 °C under stirring conditions. An aggregometer (Chrono-Log, Havertown, PA, USA) is a device for measuring light transmission. The platelet suspension is turbid, but becomes transparent when platelets are aggregated. Therefore, we analyze the change in light transmission, and the change in the light transmission was reported as the aggregation rate.

2.4. Cytotoxicity Analysis

A lactate dehydrogenase leakage assay was conducted for cytotoxicity. Human platelets (10^8 /mL) were incubated with mulberroside C (50–150 μ M) for 20 min and centrifuged at 12,000 $\times$ g. Lactate dehydrogenase was detected in the supernatant using an ELISA reader (TECAN, Salzburg, Austria).

2.5. Ca²⁺ Mobilization Analysis

The Fura 2-AM (5 μ M) and PRP mixture were preincubated at 37 °C for 60 min, and then the platelets (10^8 /mL) were washed with washing buffer as previously described. After the washing step, platelets were suspended in suspension buffer and preincubated with or without G-derrone for 3 min at 37 °C. The platelets were stimulated with collagen (2.5 μ g/mL) in the presence of 2 mM CaCl₂. A spectrofluorometer (Hitachi F-2700, Tokyo, Japan) was used to measure Fura 2-AM fluorescence according to the Grynkiewicz method [17] to calculate the Ca²⁺ values.

2.6. Serotonin and ATP Analysis

Platelet aggregation was conducted with mulberroside C (50–150 μ M) for 6 min at 37 °C. The reactant is transferred to a new centrifuge tube and briefly centrifuged at 500 $\times$ g, and the supernatant was used for serotonin and ATP detection. Serotonin was detected using an ELISA reader (TECAN, Salzburg, Austria), and ATP release was measured with a Synergy HT Microplate Reader (BioTek Instruments, Winooski, VT, USA).

2.7. Flow Cytometric Determination of P-Selectin Release

Human platelet suspension (10⁸/mL) was preincubated with mulberroside C (50–150 μ M) and then stimulated with collagen (2.5 μ g/mL). After platelet aggregation, the reaction tube was incubated with Alexa Fluor 488 antihuman CD62P under dark conditions. Next, the platelets were washed twice with ice-cold PBS and fixed with 0.5% paraformaldehyde. The p-selectin expression was measured using flow cytometry and CellQuest software (BD Biosciences, San Diego, CA, USA).

2.8. Thromboxane B₂ Analysis

Because thromboxane A₂ (TXA₂) is quickly converted to thromboxane B₂ (TXB₂), TXA₂ generation was measured by TXB₂. After platelet aggregation with mulberroside C (50–150 μ M), the reaction tube was terminated by adding indomethacin (0.2 mM) and centrifugation of the supernatant. TXB₂ was detected using an ELISA reader (Tecan, Salzburg, Austria).

2.9. Immunoblotting Assay

Platelet aggregation was lysed by adding lysis buffer. The platelet lysates were measured using a BCA protein assay kit (Pierce Biotechnology, IL, USA). After electrophoresis (8% SDS-PAGE), proteins (12 μ g) were transferred onto membranes and treated with primary (1:1000) and secondary antibodies (1:10,000). Western blotting analysis was conducted using the Quantity One program (Bio-Rad, Hercules, CA, USA).

2.10. Flow Cytometric Determination of α IIb/ β 3 Activation

For the experiment of fibrinogen binding to α IIb/ β 3, platelet aggregation was conducted with mulberroside C (50–150 μ M) for 5 min. After aggregation, the reaction tube was incubated with fibrinogen (Alexa Fluor 488-conjugated) for 15 min and fixed with 0.5% paraformaldehyde. Since α IIb/ β 3 of activated platelets binds very strongly to fibrinogen, fluorescent fibrinogen was used as an indicator for the fibrinogen binding experiment. Flow cytometry was used to measure binding (BD Biosciences, San Jose, CA, USA).

2.11. Measurement of cAMP and cGMP

Washed human platelets (10⁸/mL) were preincubated with mulberroside C (50–150 μ M) for 2 min in the presence of 2 mM CaCl₂, then stimulated with collagen (2.5 μ g/mL) for 5 min at 37 °C for platelet aggregation. The aggregation was terminated by the addition of 80% ice-cold ethanol. The reactant is transferred to a new centrifuge tube and briefly centrifuged at 500 $\times$ g, and the supernatant was used for cAMP and cGMP detection. cAMP and cGMP were measured using EIA kit with ELISA reader (TECAN, Salzburg, Austria).

2.12. Fibrin Clot Retraction

For the fibrin clot retraction experiment, platelets were reacted with thrombin (0.05 U/mL). Human platelet-rich plasma (300 μ L) was incubated with mulberroside C (50–150 μ M) for 30 min at 37 °C, and the clot reaction was triggered by thrombin. After reacting for 15 min, pictures of fibrin clots were taken using a digital camera. Image J (v1.46) was used to convert the clot area (National Institutes of Health, Bethesda, MD, USA).

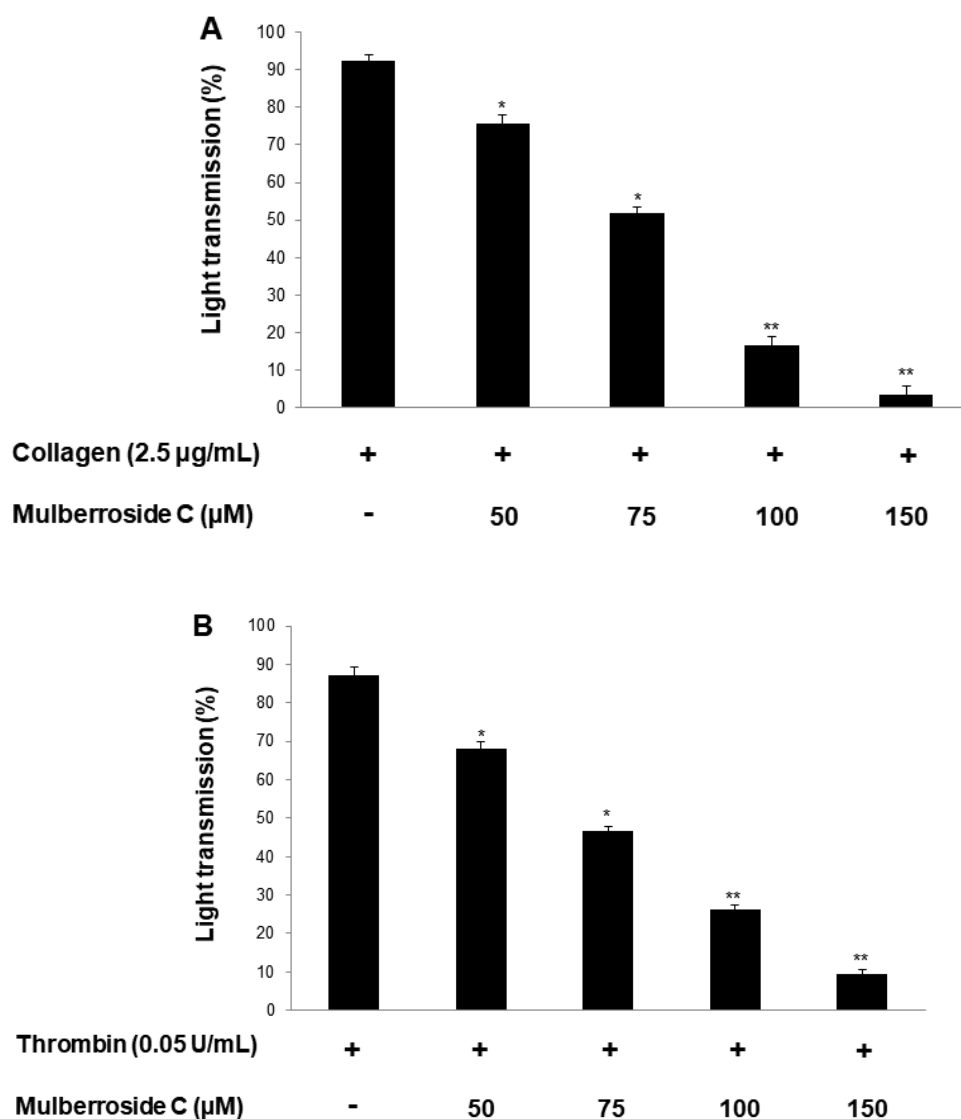
2.13. Data Analyses

All data are presented as the mean $\pm$ standard deviation with various numbers of observations. To determine major differences among groups, analysis of variance was performed, followed by the Tukey-Kramer method. SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA) was used for statistical analysis, and $p < 0.05$ was considered statistically significant.

3. Results

3.1. Mulberroside C Blocks Platelet Aggregation and Cytotoxicity

Platelet suspensions ($10^8/\text{mL}$) were preincubated for 2 min with mulberroside C (50–150 μM , Figure 1). Three agonists (collagen, thrombin, U46619) are used for platelet aggregation assay. Mulberroside C inhibited all agonist-induced platelet aggregation (Figure 2A–C) without cytotoxicity (Figure 2D); among them, collagen-induced platelets treated with mulberroside C (50, 75, 100, and 150 μM) were the most strongly inhibited (17.9%, 43.9%, 81.8% and 96.2%). Half-maximal inhibitory concentration (IC_{50}) is the most widely used measure of a drug's efficacy. The IC_{50} of mulberroside C was 77.3 in collagen-induced human platelet (Figure 2E).



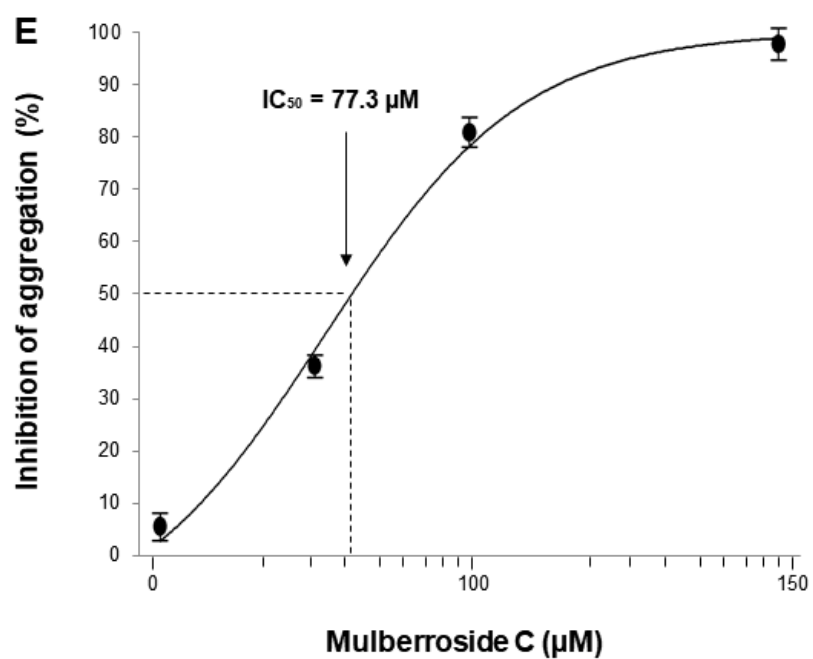
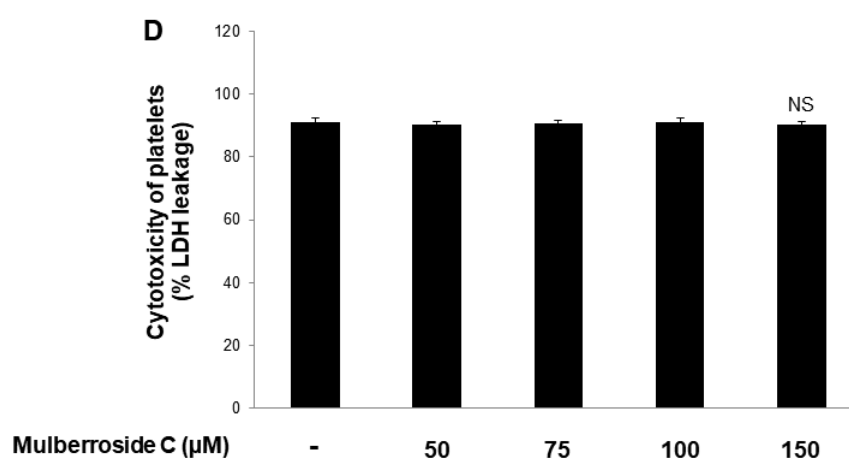
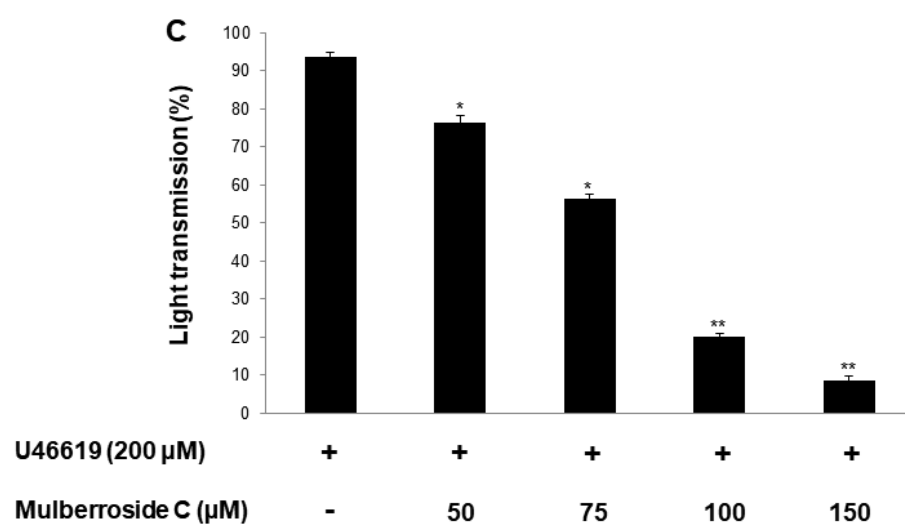
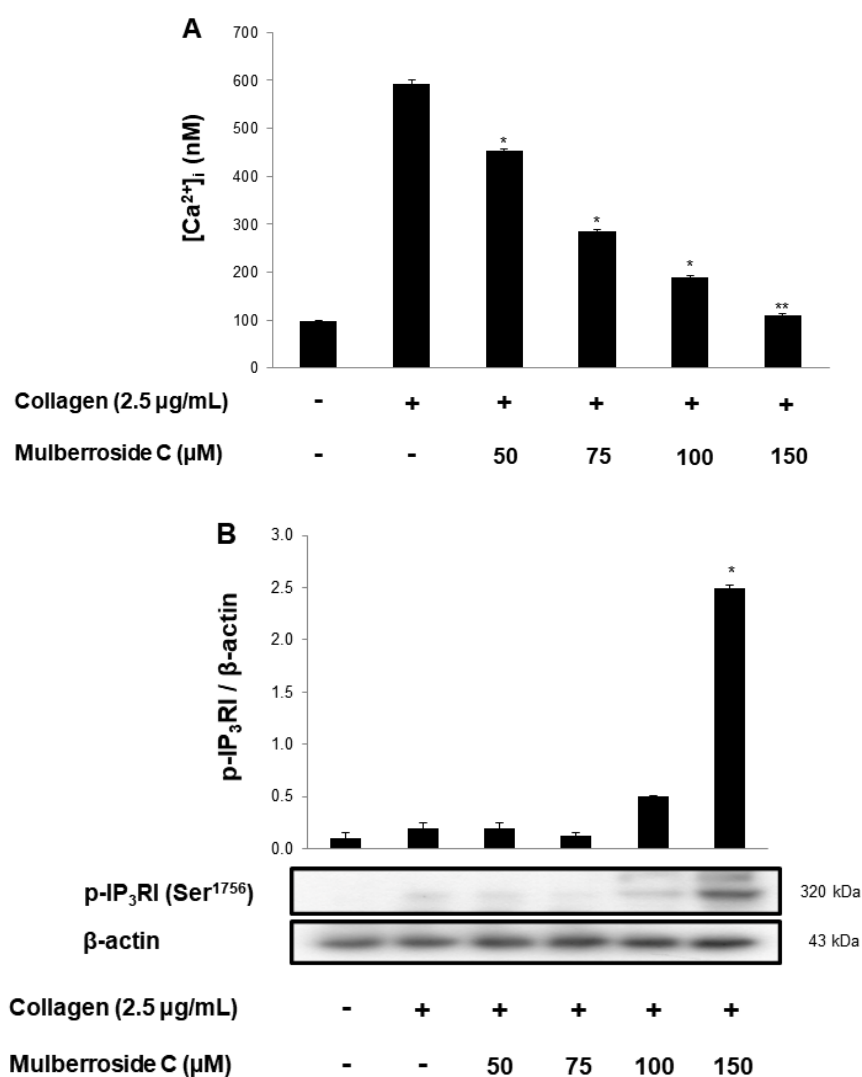


Figure 2. Effects of mulberroside C on platelet aggregation, half-maximal inhibitory concentration, and cytotoxicity (A) Effect of mulberroside C on collagen-induced human platelet aggregation; (B) Effect of mulberroside C on thrombin-induced human platelet aggregation; (C) Effect of mulberroside C on U46619-induced human platelet aggregation; (D) Effect of mulberroside C on cytotoxicity; (E) Half-maximal inhibitory concentration (IC₅₀) value of mulberroside C in collagen-induced human platelet aggregation. Platelet aggregation and cytotoxicity were carried out as described in Section 2. The data are expressed as the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$, ** $p < 0.01$ versus each agonist-stimulated human platelets. NS, not significant.

3.2. Mulberroside C Blocks Ca^{2+} Levels and IP₃RI-, ERK-Phosphorylation

Next, we investigated the phosphorylation of calcium mobilization. As shown in Figure 3A, calcium level ($[Ca^{2+}]_i$) in cytosol was elevated to 98.2 ± 0.5 nM by collagen (2.5 μ g/mL). However, mulberroside C (50–150 μ M) reduced the collagen-induced increase in $[Ca^{2+}]_i$ levels (Figure 3A). It has been reported that if cAMP/cGMP-dependent kinases phosphorylate inositol 1, 4, 5-triphosphate receptor type I (IP₃RI), $[Ca^{2+}]_i$ mobilization is inhibited [14]. Another pathway is Ca^{2+} influx from extracellular spaces, which increases the $[Ca^{2+}]_i$ level. Depletion of the intracellular Ca^{2+} level in the endoplasmic reticulum by collagen is known to trigger Ca^{2+} influx, which is facilitated by ERK [10]. Therefore, we investigated whether these two signaling molecules and mulberroside C (50 to 150 μ M) increased IP₃RI phosphorylation and decreased ERK phosphorylation (Figure 3B,C).



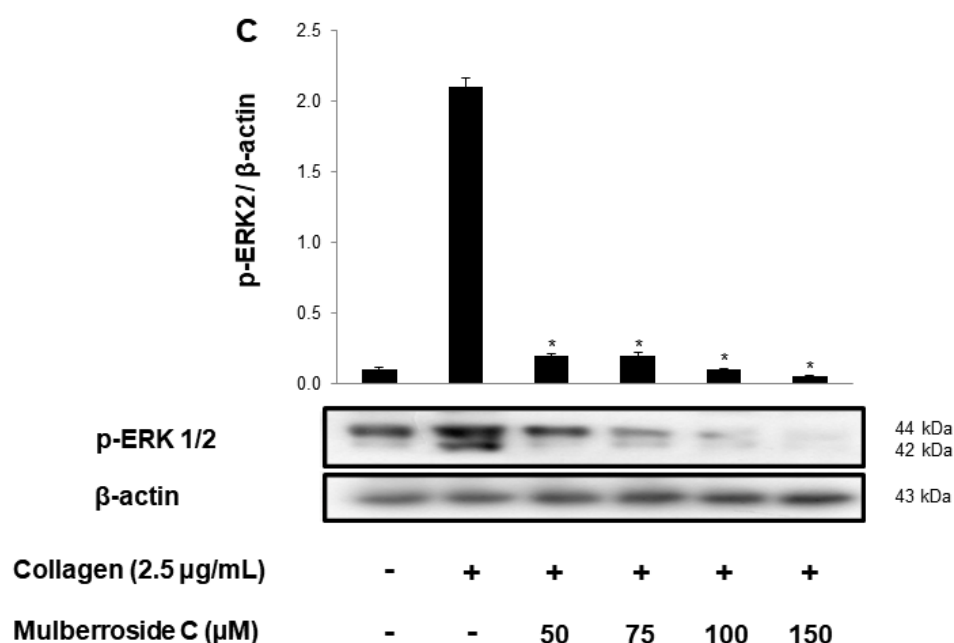
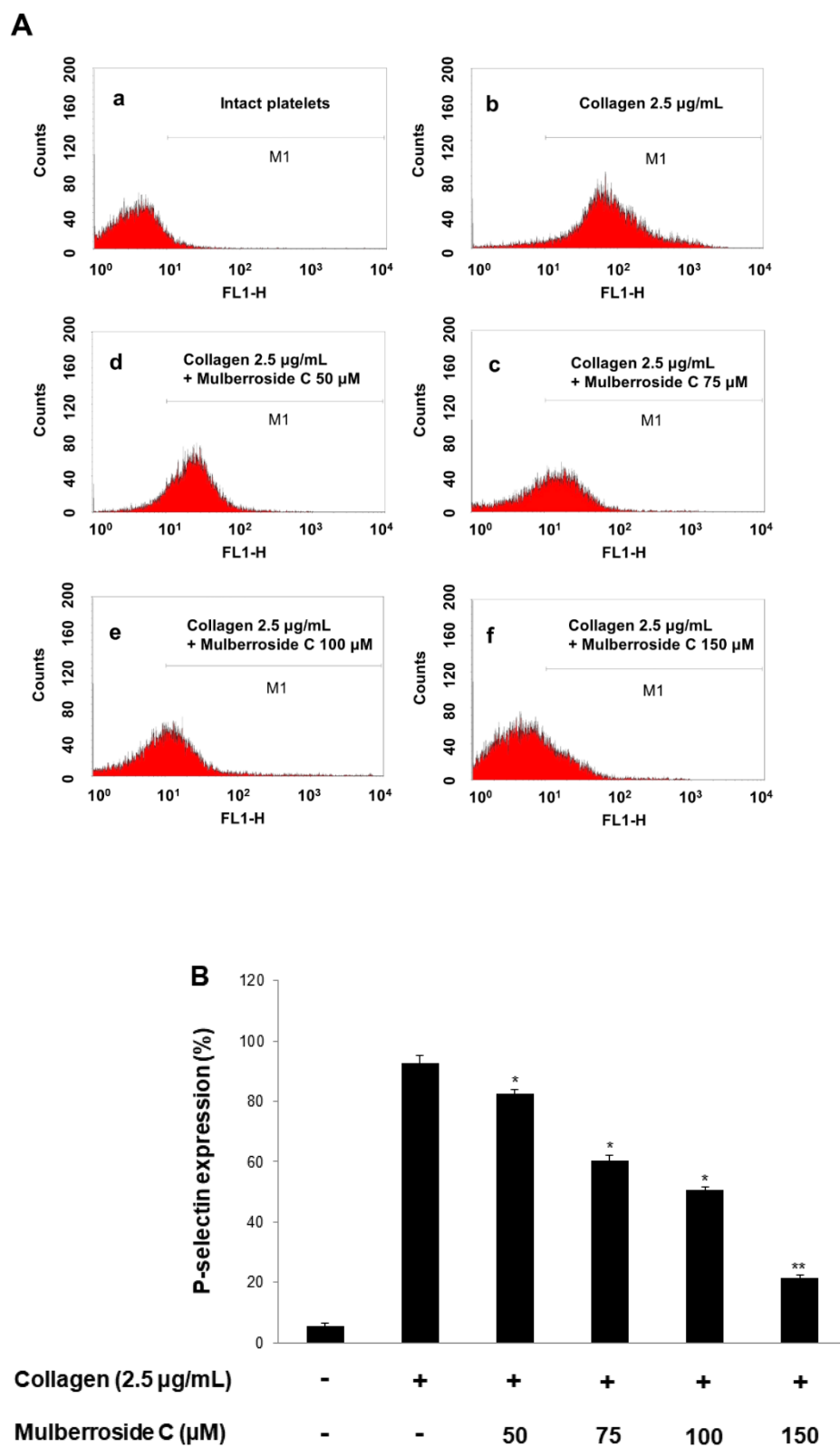


Figure 3. Effects of mulberroside C on Ca^{2+} mobilization, IP_3RI and ERK phosphorylation (A) Effect of mulberroside C on collagen-induced Ca^{2+} mobilization; (B) Effect of mulberroside C on collagen-induced IP_3RI phosphorylation; (C) Effect of mulberroside C on collagen-induced ERK phosphorylation. Measurement of Ca^{2+} mobilization, Western blot was performed as described in Section 2. The data are expressed as the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$, ** $p < 0.01$ versus the collagen-stimulated human platelets.

3.3. Mulberroside C Blocks P-Selectin Expression and Serotonin Release

Next, we investigated whether mulberroside C is involved in granule release in human platelets. Increased calcium level in cytosol activates myosin light chain and pleckstrin phosphorylation to trigger granule release (δ -granule and α -granule). Because mulberroside C decreased $[\text{Ca}^{2+}]_i$ levels through IP_3RI phosphorylation and ERK dephosphorylation, we investigated granule release. As shown in Figure 4A, collagen elevated the expression of p-selectin (Figure 4A,B); however, mulberroside C (50–150 μM) inhibited collagen-induced p-selectin expression in a dose-dependent manner (Figure 4A,B). Next, we also determined whether δ -granule release was regulated by mulberroside C. As shown in Figure 4C,D, collagen increased serotonin release, and ATP release was suppressed by mulberroside C (50–150 μM).



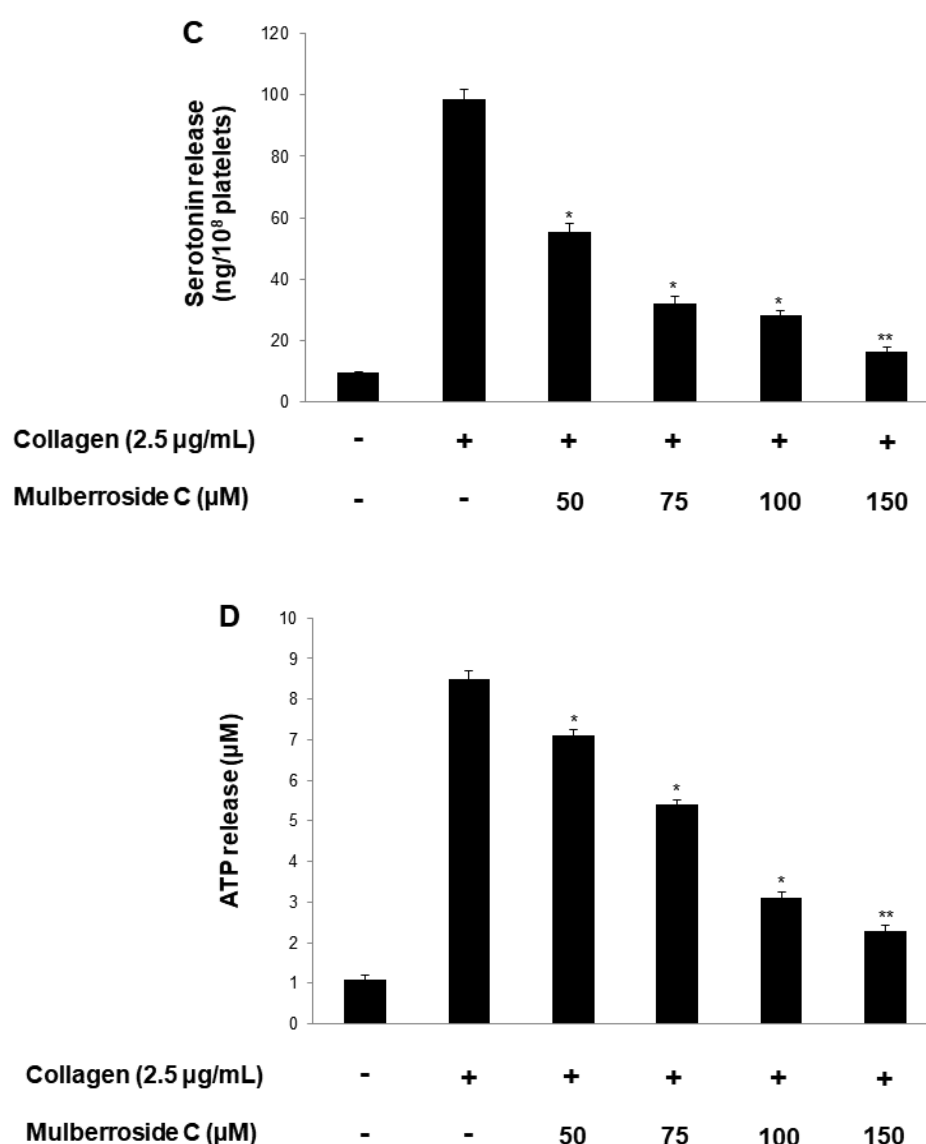


Figure 4. Effects of mulberroside C on p-selectin expression, serotonin, ATP release. (A) The flow cytometry histograms on p-selectin expression; (B) Effects of mulberroside C on collagen-induced p-selectin expression (%); (C) Effects of mulberroside C on collagen-induced serotonin release; (D) Effects of mulberroside C on collagen-induced ATP release. Determination of p-selectin expression, serotonin, ATP was carried out as described in Section 2. The data are expressed as the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$, ** $p < 0.01$ versus the collagen-stimulated human platelets.

3.4. Mulberroside C Blocks Thromboxane B₂ Production, and Dephosphorylation of cPLA₂, p38^{MAPK}

TXA₂ stimulates platelet activation increased TXA₂ may play a role in the pathogenesis of myocardial infarction, stroke, and atherosclerosis. As shown in Figure 5A, collagen (2.5 μg/mL) stimulation elevated the TXA₂ concentration to 66.2 ± 1.8 ng/10⁸ platelets. However, mulberroside C (50–150 μM) inhibited TXA₂ production dose-dependently (Figure 5A). To identify the inhibitory action of mulberroside C on TXA₂ production, we investigated cPLA₂ and mitogen-activated protein kinase p38 (p38^{MAPK}). cPLA₂ hydrolyzes arachidonic acid from the membrane, and the released arachidonic acid transforms to TXA₂. During the transformation, the p38^{MAPK} elevates the enzyme activity of cPLA₂. As shown in Figure 5B,C, mulberroside C suppressed cPLA₂ and p38^{MAPK} phosphorylation in a dose-dependent manner.

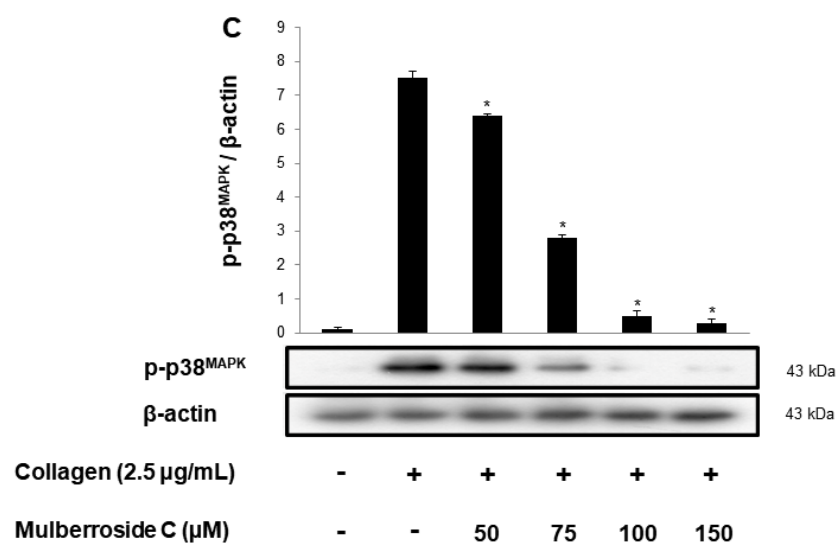
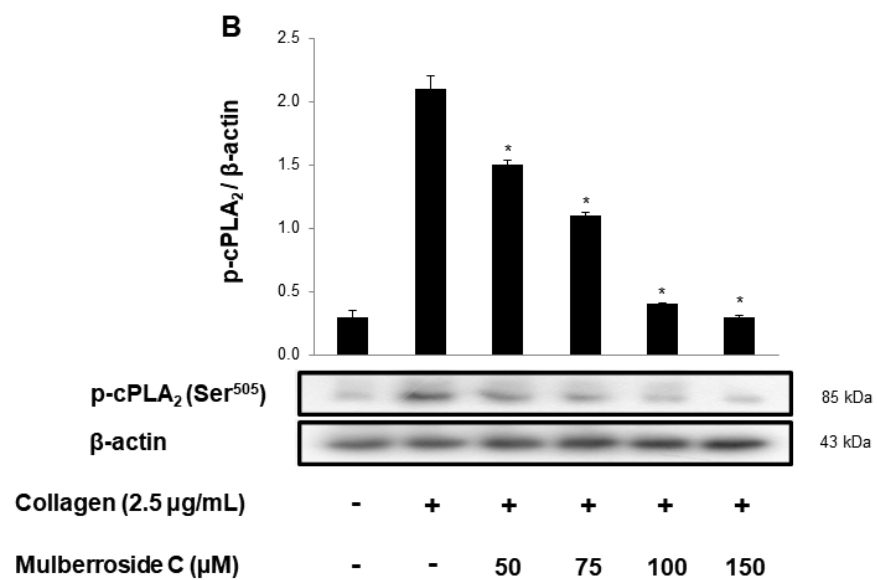
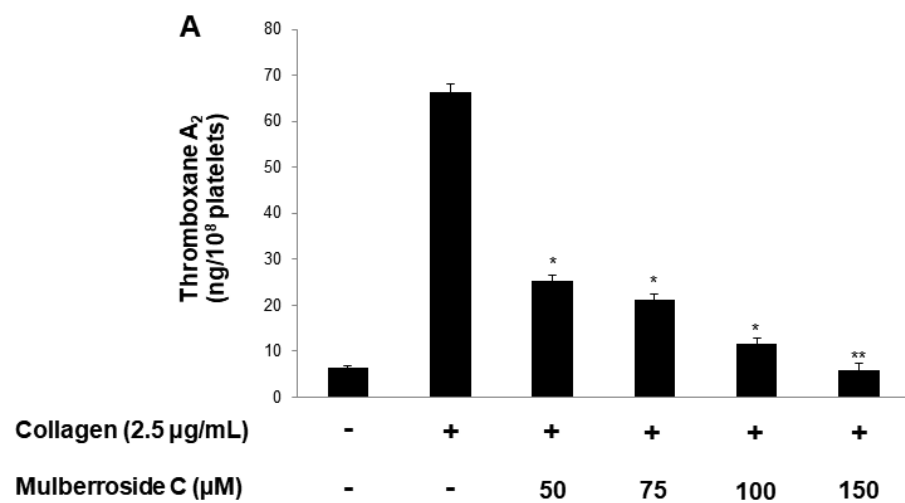
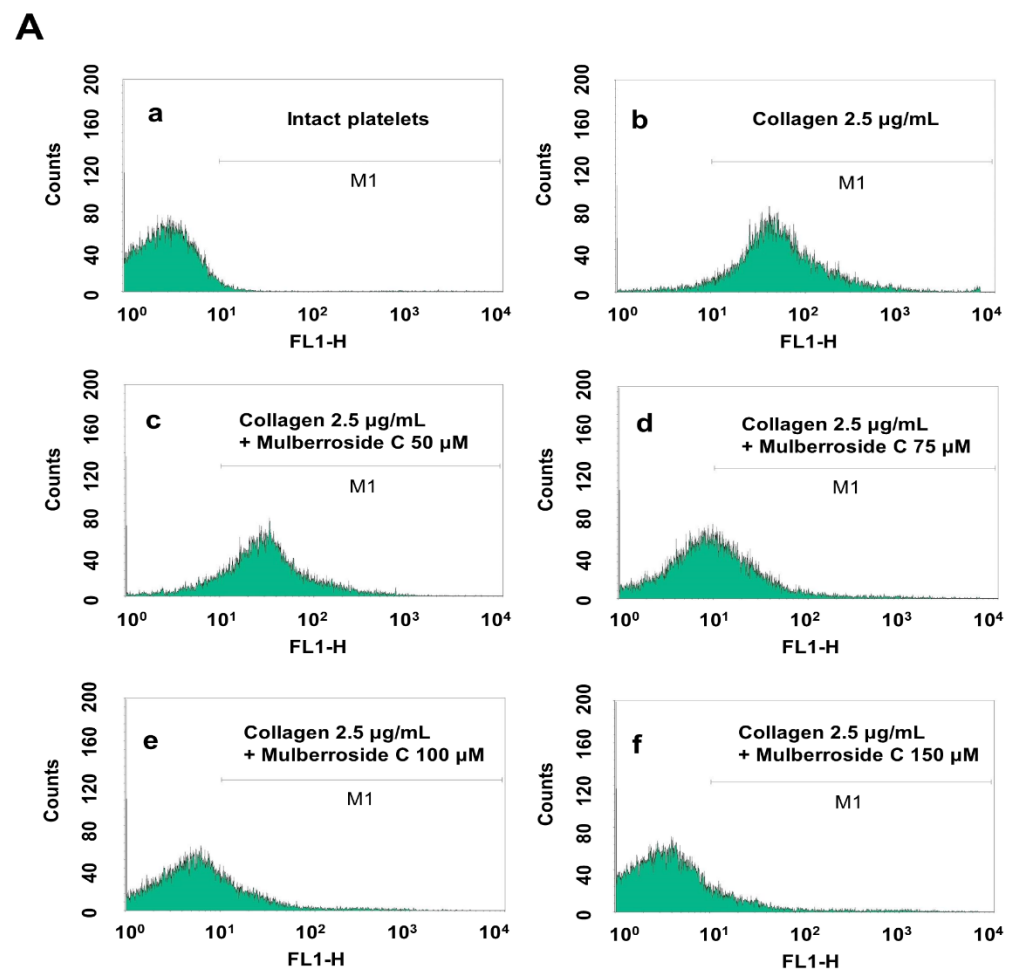
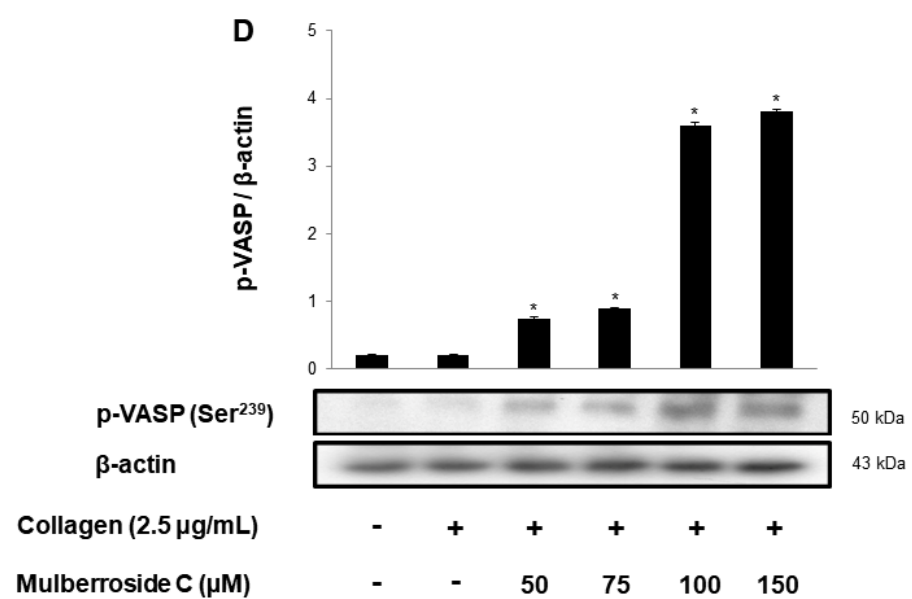
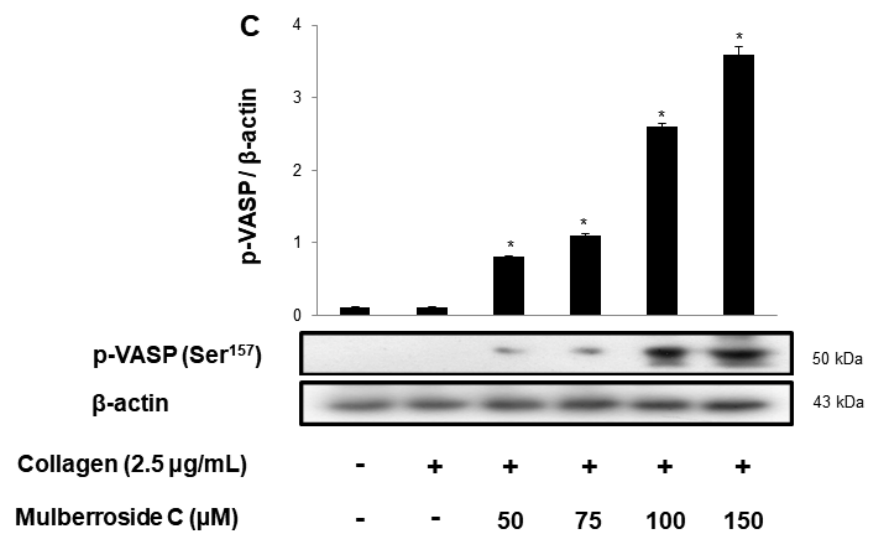
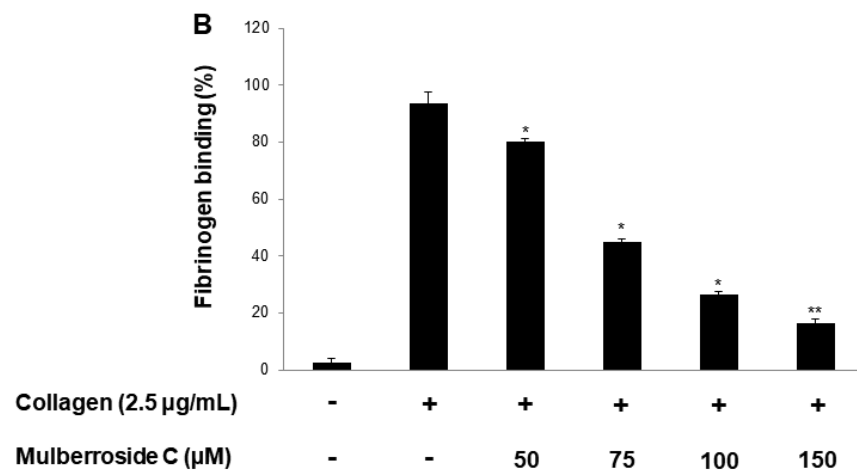


Figure 5. Effects of mulberroside C on TXA₂ production, cPLA₂, p38 phosphorylation (A) Effects of mulberroside C on collagen-induced TXA₂ generation; (B) Effect of mulberroside C on collagen-induced cPLA₂ phosphorylation; (C) Effect of mulberroside C on collagen-induced p38 phosphorylation. Measurement of TXA₂ generation and Western blot was performed as described in Section 2. The data are expressed as the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$, ** $p < 0.01$ versus the collagen-stimulated human platelets.

3.5. Mulberroside C Blocks Platelet Binding to Fibrinogen by Limiting α IIb/ β 3 Affinity and VASP, PI3K/Akt Dephosphorylation

α IIb/ β 3 is essential for platelet adhesion, binding, and spreading. Collagen-induced fibrinogen binding to α IIb/ β 3, and its binding rate was $93.7 \pm 3.9\%$ (Figure 6A). However, mulberroside C significantly decreased conformational change of α IIb/ β 3 (Figures 5B and 6A(c–f)). Next, we investigated α IIb/ β 3 associated signaling molecules, vasodilator-stimulated phosphoprotein (VASP), and PI3K/Akt. VASP regulates actin, but its phosphorylation by kinases suppressed the α IIb/ β 3 affinity [18,19]. Furthermore, phosphoinositide 3-kinase (PI3K) and Akt are the most important mediators in human platelets, leading to adhesive function, platelet spreading, and α IIb/ β 3 activation [20,21]. Our data showed that mulberroside C significantly upregulated VASP phosphorylation (Ser¹⁵⁷, Ser²³⁹) (Figure 6C,D) and downregulated PI3K/Akt phosphorylation (Figure 6E,F).





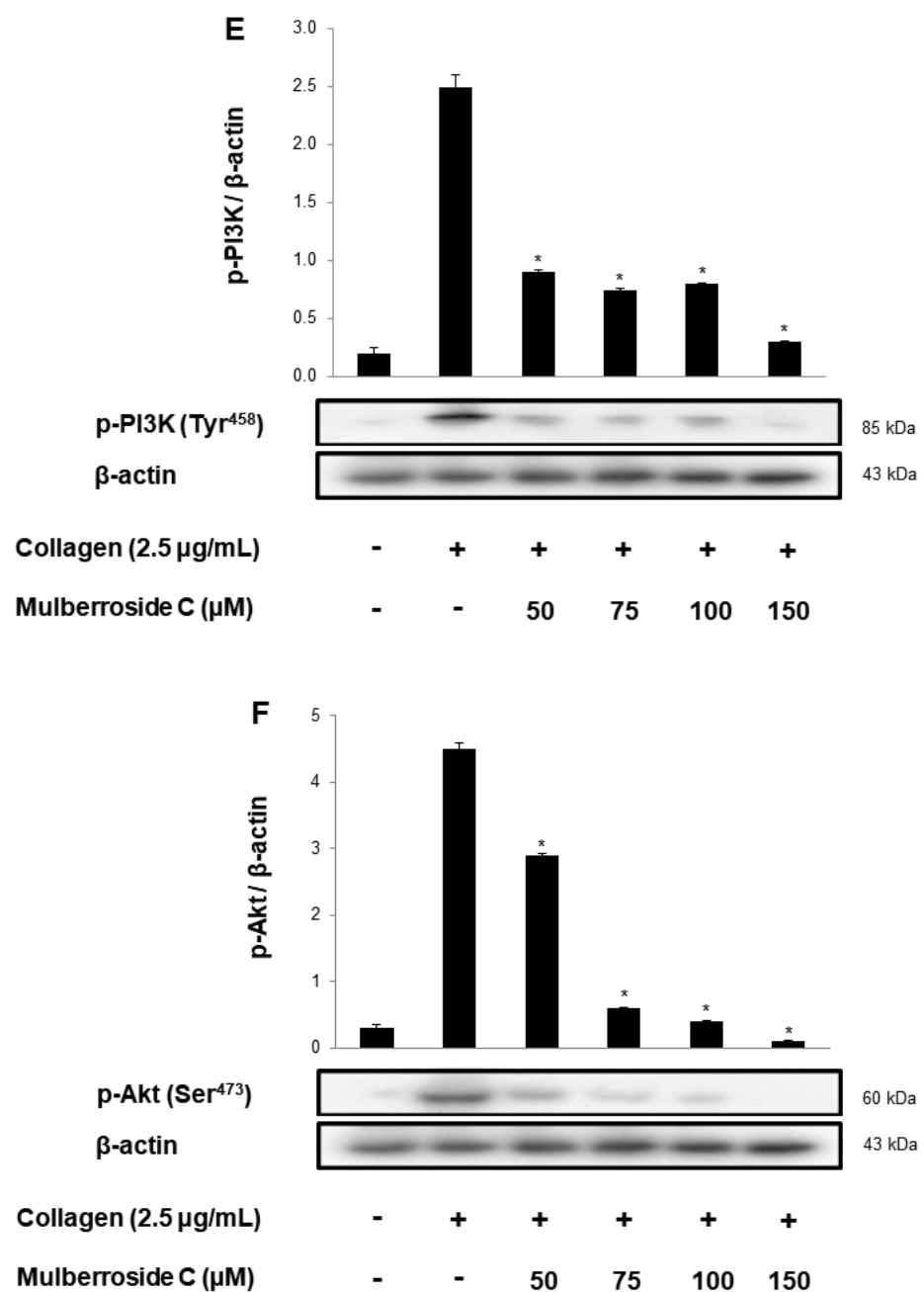
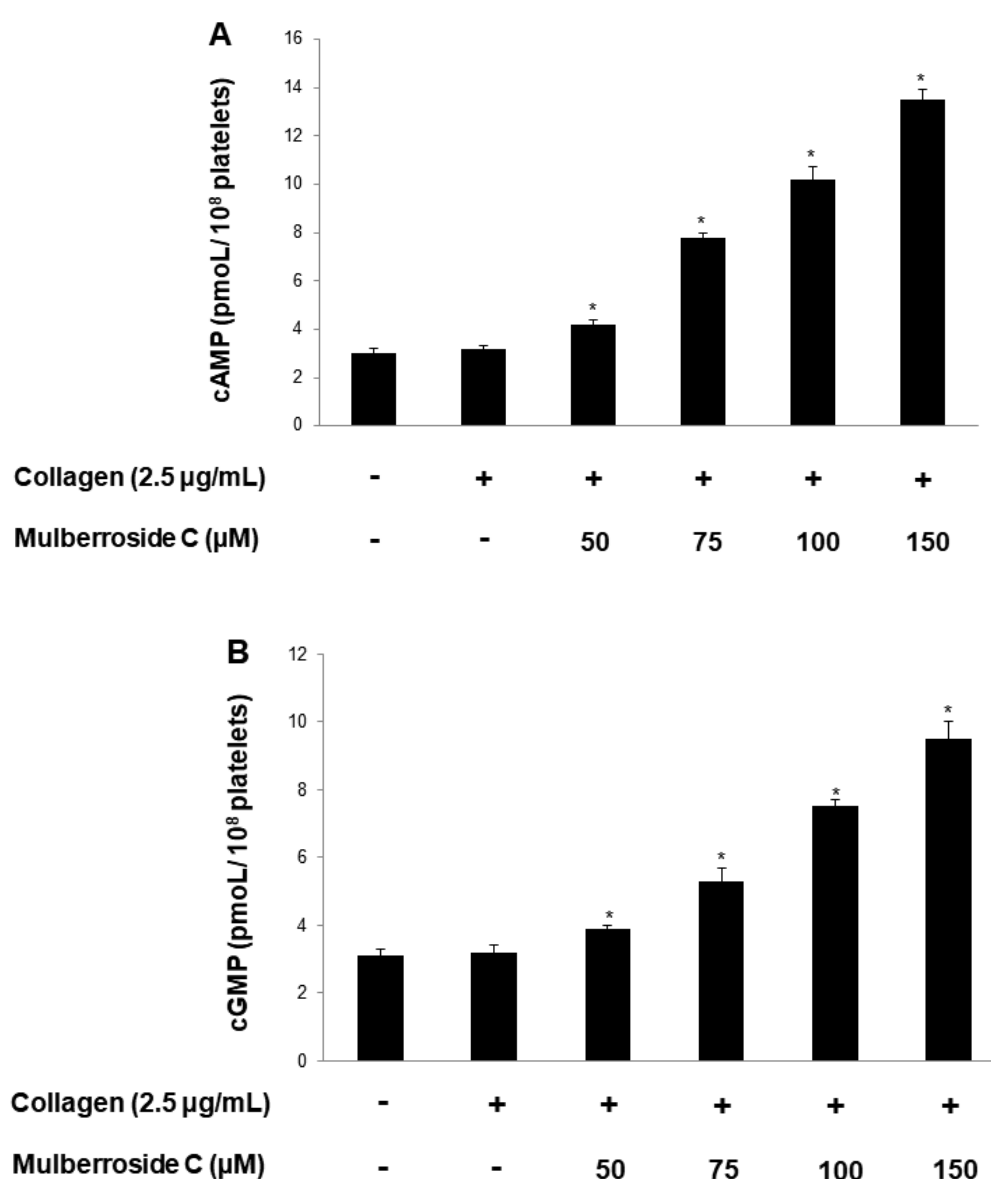
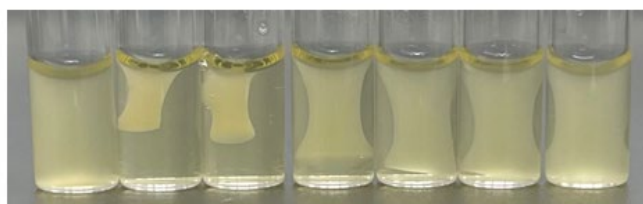


Figure 6. Effects of mulberroside C on fibrinogen binding to α IIb/ β 3, VASP, and PI3K/Akt phosphorylation (A) The flow cytometry histograms on fibrinogen binding; (B) Effects of mulberroside C on collagen-induced fibrinogen binding (%); (C) Effect of mulberroside C on collagen-induced VASP (Ser¹⁵⁷) phosphorylation; (D) Effect of mulberroside C on collagen-induced VASP (Ser²³⁹) phosphorylation; (E) Effect of mulberroside C on collagen-induced PI3K (Tyr⁴⁵⁸) phosphorylation; (F) Effect of mulberroside C on collagen-induced Akt (Ser⁴⁷³) phosphorylation. Measurement of fibrinogen binding, fibronectin adhesion, and Western blot was carried out as described in Section 2. The data are expressed as the mean $\pm$ standard deviation ($n = 4$). * $p < 0.05$, ** $p < 0.01$ versus the collagen-stimulated human platelets.

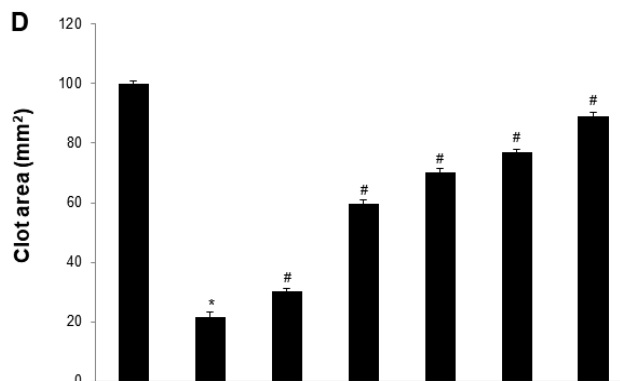
3.6. Mulberroside C Elevated Cyclic Nucleotides and Limits Thrombin-Induced Clot Retraction and Phospholipase C γ_2 Phosphorylation

Next, we investigated cyclic adenosine monophosphate (cAMP) and cyclic guanosine monophosphate (cGMP) concentration in human platelets. As shown in Figure 7A,B, mulberroside C significantly increased cAMP and cGMP levels and more strongly increased cGMP levels than cAMP levels. Taken together, we investigated whether mulberroside C can affect fibrin clot retraction. As shown in Figure 7C, thrombin-stimulated PRP was contracted, and the clot is formed. However, mulberroside C (50–150 μ M) effectively delayed clot formation (Figure 7D). Activated α IIb β 3 triggers tyrosine phosphorylation of β 3 integrin tail and activates phospholipase C γ_2 (PLC γ_2). PLC γ_2 has been reported to be essential for platelet spreading and clot retraction [22]. As shown in Figure 7C, collagen elevated PLC γ_2 phosphorylation was suppressed by mulberroside C (Figure 7E).

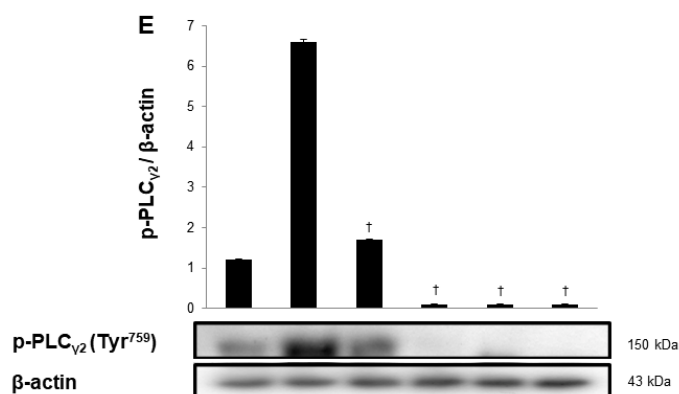


C

Thrombin (0.05 U/mL)	-	+	+	+	+	+	+
Mulberroside C (μM)	-	-	50	75	100	150	-
Y27632 (5 μM)	-	-	-	-	-	-	+

D

Thrombin (0.05 U/mL)	-	+	+	+	+	+	+
Mulberroside C (μM)	-	-	50	75	100	150	-
Y27632 (5 μM)	-	-	-	-	-	-	+

E

Collagen (2.5 μg/mL)	-	+	+	+	+	+
Mulberroside C (μM)	-	-	50	75	100	150

Figure 7. Effects of mulberroside C on cAMP and cGMP concentration, fibrin clot retraction, and PLCγ2 phosphorylation; (A) Effect of mulberroside C on collagen-induced cAMP production; (B) Effect of mulberroside C on collagen-induced cGMP production; (C) Photographs of fibrin clot; (D) Effects of mulberroside C on thrombin-retracted fibrin clot (%); (E) Effect of mulberroside C on collagen-induced PLCγ2 (Tyr759) phosphorylation. Quantification of fibrin clot retraction and Western blot, cAMP and cGMP was performed as describe in Section 2. 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. $+ p < 0.05$ versus the collagen-stimulated human platelets.

4. Discussion

M. alba Linn (*M. alba*), known as white mulberry, belongs to the family Moraceae and is commonly regarded as a food source for silkworms. *M. alba* is widespread throughout East Asia and is used in traditional remedies as a diuretic, antiheadache, expectorant, and antidiabetic agent, and almost all parts of the plant are used in traditional medicine. Several compounds have been isolated from various parts of *M. alba*, including bark, fruits, and leaves [3]. With regards to cardiovascular diseases, administration of *M. alba* (500 mg/kg body weight) has shown cardioprotective effects in myocardial infarcted rats [23] and vascular protection effects [24]. In prior research investigating the effects of *M. alba* effects on platelets, *M. alba* extract inhibited in vitro rat platelet aggregation [4,5], and morusinol isolated from *M. alba* inhibited in vitro rabbit platelet aggregation, thromboxane B₂ formation, and in vivo ferric chloride-induced thrombosis [6]. Because the *M. alba* extract showed antiplatelet effects, we searched for a new candidate and confirmed that mulberroside C has antiplatelet effects.

Mulberroside C suppressed collagen-, thrombin-, and U46619 (TXA₂ analog) induced human platelet aggregation in a dose-dependent manner (Figure 2A–C). Next, we confirmed that mulberroside C suppressed $[Ca^{2+}]_i$ levels. $[Ca^{2+}]_i$ levels are regulated by Ca^{2+} mobilization from the endoplasmic reticulum and Ca^{2+} influx from the extracellular space. It is well known that Ca^{2+} mobilization is activated by collagen and is inhibited by IP₃RI phosphorylation. In addition, ERK phosphorylation has been reported to affect calcium influx. Therefore, we investigated whether mulberroside C inhibited $[Ca^{2+}]_i$ levels and confirmed that mulberroside C can suppress $[Ca^{2+}]_i$ levels through the phosphorylation of IP₃RI and dephosphorylation of ERK (Figure 3B,C) and inhibited p-selectin expression, serotonin release, and ATP release (Figure 4A,C,D).

Next, we determined that mulberroside C suppressed TXA₂ release (Figure 4A). TXA₂ acts as a strong agonist, leading to hemostasis and thrombosis. We focused on the regulatory signaling molecules of TXA₂, such as cPLA₂ and p38^{MAPK}. Mulberroside C inhibited p38^{MAPK} and cPLA₂ phosphorylation (Figure 5B,C).

On the platelet surface, α IIb/ β 3 is the most important receptor for binding with fibrinogen and fibrin for hemostasis and thrombosis [25]. We investigated whether mulberroside C suppresses conformational change of α IIb/ β 3 by regulation of the VASP (Figure 6A,B). Mulberroside C suppressed α IIb/ β 3 affinity and increased VASP phosphorylation (Ser¹⁵⁷ and Ser²³⁹). Intracellular cAMP and cGMP are strong negative molecules that are regulated by enzymes, such as cyclic adenylylate/guanylate cyclase and phosphodiesterases. These cyclic nucleotides inhibit α IIb/ β 3 affinity and Ca^{2+} mobilization. In our study, mulberroside C increased cAMP and cGMP levels (Figure 4D,E), and these cyclic nucleotides can elevate the phosphorylation of VASP (Ser¹⁵⁷ and Ser²³⁹) and IP₃RI (Ser¹⁷⁵⁶). PI3K and Akt are the most important mediators in human platelets, leading to adhesive function, filopodia formation, platelet spreading, and α IIb/ β 3 activation [20,21]. Mulberroside C inhibited PI3K p85 (Tyr⁴⁵⁸) and Akt (Ser⁴⁷³) phosphorylation (Figure 6C,D) and increased cAMP and cGMP levels (Figure 7A,B). α IIb/ β 3 with fibrin is involved in fibrin clot formation, which plays an important role in thrombosis and hemostasis. The clot forms a clot plaque and gradually contracts formation. The contraction process is caused by the interaction between fibrin and α IIb/ β 3, and the internal signaling molecules of platelets. Therefore, we investigated whether mulberroside C affects thrombin-induced fibrin clot retraction. As shown in Figure 7C, mulberroside C strongly suppressed the retraction. Our study had some limitations, in that it was conducted in vitro and did not consider other factors in vivo. Moreover, since our study is not an in vivo study thus, it is difficult to prove its effectiveness on the human body. To resolve these questions, in vitro whole blood aggregation, animal tests (in vivo, ex vivo), and clinical trials in humans should be accompanied. However, since the present study did not deal with these experiments, it must

be conducted in a future study to prove the true antiplatelet effect of mulberroside C. However, based on the in vitro study, we suggest that mulberroside C has potential as a drug.

This study found that mulberroside C decreases calcium mobilization, fibrinogen binding to $\alpha\text{IIb}/\beta_3$, fibronectin adhesion, and thrombin-facilitated clot retraction by regulating the associated signaling molecules, such as IP_3RI , ERK, cPLA₂, p38, VASP, PI3K/Akt, and PLC γ_2 . Therefore, we suggest that mulberroside C from *M. alba* would be a useful compound for preventing thrombosis.

Author Contributions: Conception and design of the experiment: M.H.R. and J.-H.S.; Performance of the experiments: H.-W.K.; Analysis and arrangement of data: H.-W.K. and D.-H.L.; Contribution of reagents, materials, and tools: M.H.R.; Contribution of manuscript preparation: H.-W.K. and J.-H.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the Basic Science Research Program through the National Research Foundation of Korea (NRF) funded by the Ministry of Education (NRF-2020R111A1A01067709).

Institutional Review Board Statement: All experiments were approved by the Catholic Kwandong University Institutional Review Board (CKU-20-01-0109).

Informed Consent Statement: Not applicable.

Data Availability Statement: The data presented in this study are available on request from the corresponding author. The data are not publicly available due to privacy.

Acknowledgments: The authors would like to express their sincere gratitude to all the participants.

Conflicts of Interest: The authors declare no conflicts of interest.

References

- Atanasov, A.G.; Zotchev, S.B.; Dirsch, V.M.; Supuran, C.T. Natural products in drug discovery: Advances and opportunities. *Nat. Rev. Drug Discov.* **2021**, *20*, 1–17.
- Jackson, S.P. Arterial thrombosis-insidious, unpredictable and deadly. *Nat. Med.* **2011**, *17*, 1423–1436.
- Chan, E.W.C.; Lye, P.Y.; Wong, S.K. Phytochemistry, pharmacology, and clinical trials of *M. alba*. *Chin. J. Nat. Med.* **2016**, *14*, 17–30.
- Kim, D.S.; Ji, H.D.; Rhee, M.H.; Sung, Y.Y.; Yang, W.K.; Kim, S.H.; Kim, H.K. Antiplatelet activity of *M. alba* leaves extract, mediated via inhibiting granule secretion and blocking the phosphorylation of extracellular-signal-regulated kinase and akt. *Evid.-Based. Complement. Alt.* **2014**, doi:10.1155/2014/639548.
- Kim, D.S.; Irfan, M.; Sung, Y.Y.; Kim, S.H.; Park, S.H.; Choi, Y.H.; Kim, H.K. Schisandra chinensis and *M. alba* synergistically inhibit in vivo thrombus formation and platelet aggregation by impairing the glycoprotein VI pathway. *Evid.-Based. Complement. Alt.* **2017**, doi:10.1155/2017/7839658.
- Lee, J.J.; Yang, H.; Yoo, Y.M.; Hong, S.S.; Lee, D.; Lee, H.J.; Jeung, E.B. Morusinol extracted from *M. alba* inhibits arterial thrombosis and modulates platelet activation for the treatment of cardiovascular disease. *J. Atheroscler. Thromb.* **2012**, *6*, 1204020479.
- Du, J.; He, Z.D.; Jiang, R.W.; Ye, W.C.; Xu, H.X.; But, P.P.H. Antiviral flavonoids from the root bark of *M. alba* L. *Phytochemistry* **2003**, *62*, 1235–1238.
- Farndale, R.W. Collagen-induced platelet activation. *Blood Cell Mol. Dis.* **2006**, *36*, 162–165.
- Zarbock, A.; Polanowska-Grabowska, R.K.; Ley, K. Platelet-neutrophil-interactions: Linking hemostasis and inflammation. *Blood Rev.* **2007**, *21*, 99–111.
- Rosado, J.A.; Sage, S.O. The ERK cascade, a new pathway involved in the activation of store-mediated calcium entry in human platelets. *Trends Cardiovasc. Med.* **2002**, *12*, 229–234.
- Kramer, R.M.; Roberts, E.F.; Um, S.L.; Börsch-Haubold, A.G.; Watson, S.P.; Fisher, M.J.; Jakubowski, J.A. p38 mitogen-activated protein kinase phosphorylates cytosolic phospholipase A2 (cPLA2) in thrombin-stimulated platelets Evidence that proline-directed phosphorylation is not required for mobilization of arachidonic acid by cPLA2. *J. Biol. Chem.* **1996**, *271*, 27723–27729.
- Needleman, P.; Moncada, S.; Bunting, S.; Vane, J.R.; Hamberg, M.; Samuelsson, B. Identification of an enzyme in platelet microsomes which generates thromboxaneA2 from prostaglandin endoperoxides. *Nature* **1976**, *261*, 558–560.
- Smyth, E.M. Thromboxane and the thromboxane receptor in cardiovascular disease. *J. Clin. Lipidol.* **2010**, *5*, 209–219.
- Smolenski, A. Novel roles of cAMP/cGMP-dependent signaling in platelets. *J. Thromb. Haemost.* **2012**, *10*, 167–176.
- Schwarz, U.R.; Walter, U.; Eigenthaler, M. Taming platelets with cyclic nucleotides. *Biochem. Pharmacol.* **2001**, *62*, 1153–1161.

16. Shin, J.H.; Kwon, H.W.; Lee, D.H. Ginsenoside F4 inhibits platelet aggregation and thrombus formation by dephosphorylation of IP3RI and VASP. *J. Appl. Biol. Chem.* **2019**, *62*, 93–100.
17. Grynkiewicz, G.; Poenie, M.; Tsien, R.Y. A new generation of Ca²⁺ indicators with greatly improved fluorescence properties. *J. Biol. Chem.* **1985**, *260*, 3440–3450.
18. Laurent, V.; Loisel, T.P.; Harbeck, B.; Wehman, A.; Gröbe, L.; Jockusch, B.M.; Wehland, J.; Gertler, F.B.; Carlier, M.F. Role of proteins of the Ena/VASP family in actin-based motility of *Listeria monocytogenes*. *J. Cell Biol.* **1999**, *144*, 1245–1258.
19. Sudo, T.; Ito, H.; Kimura, Y. Phosphorylation of the vasodilator-stimulated phosphoprotein (VASP) by the anti-platelet drug, cilostazol, in platelets. *Platelets* **2003**, *4*, 381–390.
20. Chen, J.; De, S.; Damron, D.S.; Chen, W.S.; Hay, N.; Byzova, T.V. Impaired platelet responses to thrombin and collagen in AKT-1-deficient mice. *Blood* **2004**, *104*, 1703–1710.
21. Valet, C.; Severin, S.; Chicanne, G.; Laurent, P.A.; Gaits-Iacovoni, F.; Gratacap, M.P.; Payrastre, B. The role of class I, II and III PI 3-kinases in platelet production and activation and their implication in thrombosis. *Adv. Biol. Regul.* **2016**, *61*, 33–41.
22. Suzuki-Inoue, K.; Hughes, C.E.; Inoue, O.; Kaneko, M.; Cuyun-Lir, O.; Takafuta, T.; Watson, S.P.; Ozaki, Y. Involvement of Src kinases and PLC γ 2 in clot retraction. *Thromb. Res.* **2007**, *120*, 251–258.
23. Madhumitha, S.; Indhuleka, A. Cardioprotective effect of *M. alba* L. leaves in isoprenaline induced rats. *Int. J. Pharm. Sci. Res.* **2012**, *3*, 1475.
24. Panth, N.; Paudel, K.R.; Gong, D.S.; Oak, M.H. Vascular protection by ethanol extract of *M. alba* root bark: Endothelium-dependent relaxation of rat aorta and decrease of smooth muscle cell migration and proliferation. *Evid.-Based. Complement. Alt.* **2018**, doi:10.1155/2018/7905763.
25. Payrastre, B.; Missy, K.; Trumel, C.; Bodin, S.; Plantavid, M.; Chap, H. The integrin α IIb/ β 3 in human platelet signal transduction. *Biochem. Pharmacol.* **2000**, *60*, 1069–1074.