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Pharmacological actions of morusinol on modulation of platelet functions *via* integrin αIIb/β_3 signaling

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Abstract *Morus alba*, a popular medicinal plant belonging to the family Moraceae, has long been used commonly in traditional medicine and has various physiological activities, including anti-diabetic, anti-microbial, diuretic, anti-oxidant, and anti-cancer activities. Morusinol was isolated from the root bark of *M. alba*; however, its biological effects have not yet been reported. Therefore, we examined the inhibitory effects of morusinol on human platelet aggregation, Ca^{2+} mobilization, and αIIb/β_3 activity. Our data showed that collagen-induced human platelet aggregation was inhibited by morusinol without cytotoxicity. In this study, we examined whether morusinol inhibits platelet aggregation through the regulation of integrin αIIb/β_3 and its associated signaling molecules. We observed that morusinol inhibited αIIb/β_3 activation by regulating vasodilator-stimulated phosphoprotein, phosphatidylinositol-3 kinase, Akt (protein kinase B), and glycogen synthase kinase-3α/β. These results show that morusinol inhibited fibronectin adhesion, fibrinogen binding, and clot retraction. Taken together, morusinol shows strong antiplatelet and anti-clot retraction effects and is a potential therapeutic drug candidate to prevent platelet-related thrombosis and cardiovascular disease.

Keywords αIIb/β_3 activity · Clot retraction · *Morus Alba* · Morusinol

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Introduction

Morus alba has been used as a traditional medicine for cough, asthma, bronchitis, edema, insomnia, diabetes, and wound healing [1]. *M. alba* leaves contain rutin, quercetin, and apigenin as bioactive constituents, and many flavones have been isolated from the root bark as active components. Furthermore, albafuran, albanol, calystegin, morusin, moranoline, morusinol, and kuwanol isolated from *M. alba* exhibit pharmacological action [2]. *M. alba* has various physiological activities, including antidiabetic, antimicrobial, diuretic, antioxidant, and anticancer [1-3].

Platelets play a fundamental role in hemostasis and thrombosis, and antiplatelet drugs are effective in reducing thrombosis in patients with cardiovascular disease. However, various side effects of antiplatelet drugs have been reported, and although various antiplatelet drugs have been developed, the mortality rate of cardiovascular diseases has not decreased [4]. Therefore, it is necessary to discover new antiplatelet drugs with fewer side effects. Medicinal plants have advantages of being in use for a long time with proven stability. The leaf extracts of *M. alba* have shown antiplatelet effect on rat platelets [5,6]. In addition, antiplatelet effect of morusinol isolated from *M. alba* has been previously reported. Various agonist-induced aggregation of rabbit platelets was inhibited by morusinol; however, its inhibitory mechanism has not been elucidated. Therefore, we examined the inhibitory effect of morusinol on aggregation of human platelets and regulation of various signaling molecules.

Integrin αIIb/β_3 plays a key role in various platelet aggregation processes. Various physiological agonists such as collagen, ADP, and thrombin can activate phospholipase C_β and phospholipase C_γ, which hydrolyze phosphatidylinositol 4,5-bisphosphate into inositol 1,4,5-trisphosphate (IP_3) and diacylglycerol. IP_3 triggers the release of Ca^{2+} from intracellular stores *via* IP_3 receptor (IP_3R) present on the surface of the endoplasmic reticulum. Elevated $[Ca^{2+}]_i$ levels facilitate myosin light chain and pleckstrin phosphorylation to trigger granule release and activate αIIb/β_3 [7]. These agonist-induced signaling events, called inside-out signaling,

cause conformational changes in glycoproteins IIb/IIIa (integrin αIIb/β_3). Activated αIIb/β_3 is essential for platelet aggregation, and adhesive proteins such as fibrinogen, fibronectin, and von Willebrand factor adhere to αIIb/β_3. Activated αIIb/β_3 binds to other platelets *via* fibrinogen. This interaction between platelets and fibrinogen regulates platelet aggregation and clot retraction [8]. Therefore, the αIIb/β_3-mediated signaling pathway is known as outside-in signaling. In this study, we focused on the effect of morusinol on αIIb/β_3 activation.

Materials and Methods

Materials

Morusinol was procured from ChemFaces (Wuhan, China), and human platelet-rich plasma (PRP) was obtained from the Korean Red Cross Blood Center, Suwon. Phospho-IP_3R, Phospho-vasodilator-stimulated phosphoprotein (VASP), Phospho-phosphatidylinositol-3 kinase (PI_3K), Phospho-Akt (S473 and T308), Phospho-glycogen synthase kinase-3 (GSK-3)α/β, and β-actin were purchased from Cell Signaling Technology (Beverly, MA, USA). Fura 2-AM (2-acetoxymethyl) and Alexa Fluor 488-conjugated fibrinogen were obtained from Invitrogen (Eugene, OR, USA). Collagen and thrombin were procured from Chrono-Log Co. (Havertown, PA, USA). Cyclic adenosine monophosphate (cAMP) enzyme immunoassay kit was obtained from Cayman Chemical (Ann Arbor, MI, USA). Serotonin enzyme-linked immunosorbent assay (ELISA) kit was purchased from Labor Diagnostika Nord GmbH and Co. (Nordhorn, Germany).

Human platelet aggregation analysis

Human platelets were prepared by washing with washing buffer and then resuspending in suspension buffer. The platelet concentration was adjusted to 10^8/mL, which was in accordance with a previous study [9]. Morusinol was dissolved in 0.1% dimethyl sulfoxide, and platelet suspension was pre-incubated with different concentrations of morusinol (50, 75, 100, and 150 μM) at 37 °C for 2 min. Collagen was used for the platelet aggregation assay, and the experiment was carried out for 5 min using an aggregometer (Chrono-Log, Havertown, PA, USA).

Cytotoxicity

To investigate the potential cytotoxicity of morusinol, human platelet suspension (10^8/mL) was incubated with different concentrations of morusinol (50, 75, 100, and 150 μM) for 5 min at 37 °C. The mixture was then centrifuged at 12,000 × *g* to separate the supernatant, and an ELISA kit (TECAN, Salzburg, Austria) was used to measure the levels of platelet lactate dehydrogenase.

Determination of fibrinogen binding activity

To investigate the binding of fibrinogen to αIIb/β_3, platelet aggregation was induced using a fibrinogen dye (Alexa Fluor 488). After stimulating collagen-induced platelet aggregation with different concentrations of morusinol (50, 75, 100, and 150 μM) for 3 min, reaction tubes were incubated with fibrinogen dye for 30 min and fixed with 0.5% paraformaldehyde, which was then analyzed using flow cytometry (BD Biosciences, San Jose, CA, USA) to determine the extent of fibrinogen binding to αIIb/β_3.

Determination of fibronectin adhesion activity

To examine the effect of morusinol on platelet adhesion, platelet suspension (10^8/mL) was incubated with different concentrations of morusinol (50, 75, 100, and 150 μM) for 15 min at 37 °C. Platelet suspension was then added to fibronectin-coated wells and stimulated with collagen (2.5 μg/mL) for 30 min at 37 °C. After completion of the reaction, the wells were washed with phosphate-buffered saline, and cell staining solution was added and incubated for 10 min. After the staining step, an extraction solution was added to extract the supernatant, which was then transferred to a 46-well microtiter plate. The plate was read at 560 nm using an ELISA reader (TECAN) to determine the extent of platelet adhesion.

Measurement of thrombin-induced clot retraction time

To investigate the effect of morusinol on fibrin clot retraction, human PRP (300 μL) was incubated with morusinol for 30 min at 37 °C. The clot reaction was initiated by adding thrombin (0.05 U/mL) and allowed to react for 15 min. Digital images of fibrin clots were captured, and Image J (v1.46) (National Institutes of Health, USA) was used to convert these images to clot area for further analysis.

Immunoblotting analysis

Following platelet aggregation, the reaction was stopped by adding lysis buffer, and proteins were quantified using BCA protein assay kit. The proteins were then separated by electrophoresis and transferred onto PVDF membranes. The membranes were incubated with a primary antibody overnight at 4 °C, followed by incubation with a secondary antibody for 2 h at room temperature. Detection was performed in a dark room, and the bands were converted into graphs using the Quantity One program (Bio-Rad, Hercules, CA, USA) for further analysis.

Ca^{2+} mobilization analysis

To measure $[Ca^{2+}]_i$, the Grynkiewicz method [10] was used. First, human PRP was pre-incubated with Fura 2-AM (5 μM) for 30 min and washed with washing buffer. Then, platelet suspension (10^8/mL) was incubated with morusinol (50, 75, 100, and 150 μM) for 5 min at 37 °C and stimulated with collagen (2.5 μg/mL). Ca^{2+} analysis was performed using a fluorescence spectrophotometer (F-2700; Hitachi, Tokyo, Japan).

Measurement of serotonin release

To evaluate the effect of morusinol on serotonin release, platelet suspension (10^8/mL) was pre-incubated with various concentrations of morusinol (50, 75, 100, and 150 μM) at 37 ºC for 5 min, followed by stimulation with collagen (2.5 μg/mL). After platelet aggregation, the reaction solution was centrifuged, and the amount of serotonin released into the supernatant was determined using a serotonin ELISA kit. The final absorbance was measured using an ELISA reader (TECAN).

Measurement of cAMP

Platelet suspension (10^8/mL) was pre-incubated with morusinol for 5 min at 37 ºC. The platelets were then stimulated with collagen (2.5 μg/mL) for 5 min, and platelet aggregation was stopped by adding 80% ice-cold ethanol. The supernatant was collected after centrifugation at 500×*g* and used for determining cAMP levels using an ELISA kit and ELISA reader (TECAN).

Data analysis

The data are expressed as the mean ± standard deviation; the number of observations varied between different groups. To determine significant differences among the groups, analysis of variance (ANOVA) was performed, and the Tukey-Kramer method was used for post-hoc comparisons. Statistical analysis was conducted using the SPSS 21.0.0.0 software (SPSS, Chicago, IL, USA), and $p < 0.05$ was considered statistically significant.

Results

Effect of morusinol on platelet aggregation and evaluation of cytotoxicity

This study aimed to investigate the effect of morusinol (Fig. 1A) on platelet aggregation induced by collagen (2.5 μg/mL). Because platelet aggregation leads to a transparent suspension with increased light transmittance, we converted light transmittance of platelet suspension to an aggregation rate (%) to evaluate antiplatelet effect of morusinol. The results showed that morusinol effectively inhibited collagen-induced platelet aggregation (Fig. 1B) without any cytotoxicity (Fig. 1C). The Inhibition rate of morusinol is 19.5% at 50 μM, 42.7% at 75 μM, 83.4 % at 100 μM, and 97.4% at 150 μM. Although 150 μM is a very high concentration, it was used to confirm maximal inhibitory activity.

Effect of morusinol on fibrinogen binding and fibronectin adhesion

We examined the effect of morusinol on fibrinogen binding to αIIb/β_3, which is a crucial step for platelet-platelet interaction. The results showed that morusinol reduced the binding of fibrinogen to αIIb/β_3, as demonstrated by the decreased binding force (Fig. 2A). Moreover, morusinol inhibited the adhesion of collagen-stimulated platelet suspension to fibronectin-coated wells, indicating reduced activity of αIIb/β_3 (Fig. 2C). These findings suggest that morusinol can modulate the function of αIIb/β_3, an important regulator of platelet function.

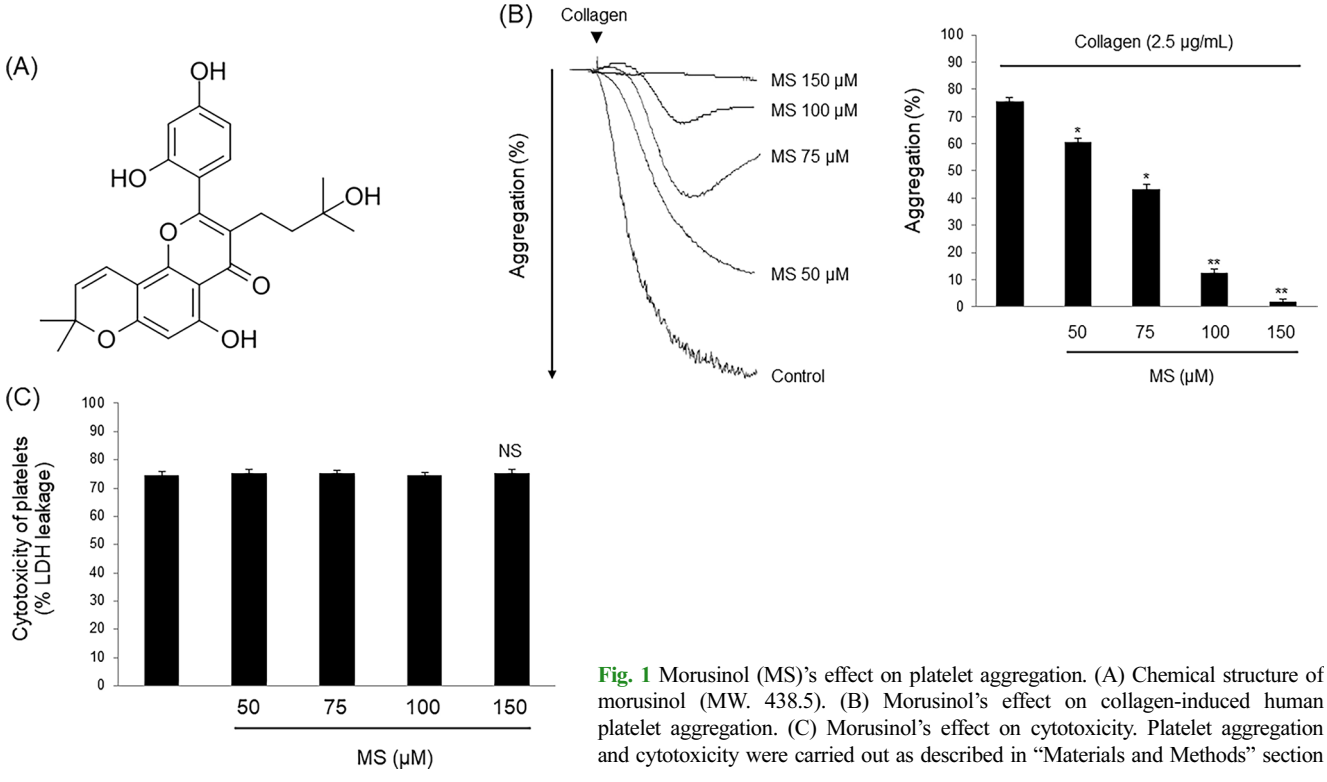

Fig. 1 Morusinol (MS)'s effect on platelet aggregation. (A) Chemical structure of morusinol (MW. 438.5). (B) Morusinol's effect on collagen-induced human platelet aggregation. (C) Morusinol's effect on cytotoxicity. Platelet aggregation and cytotoxicity were carried out as described in "Materials and Methods" section

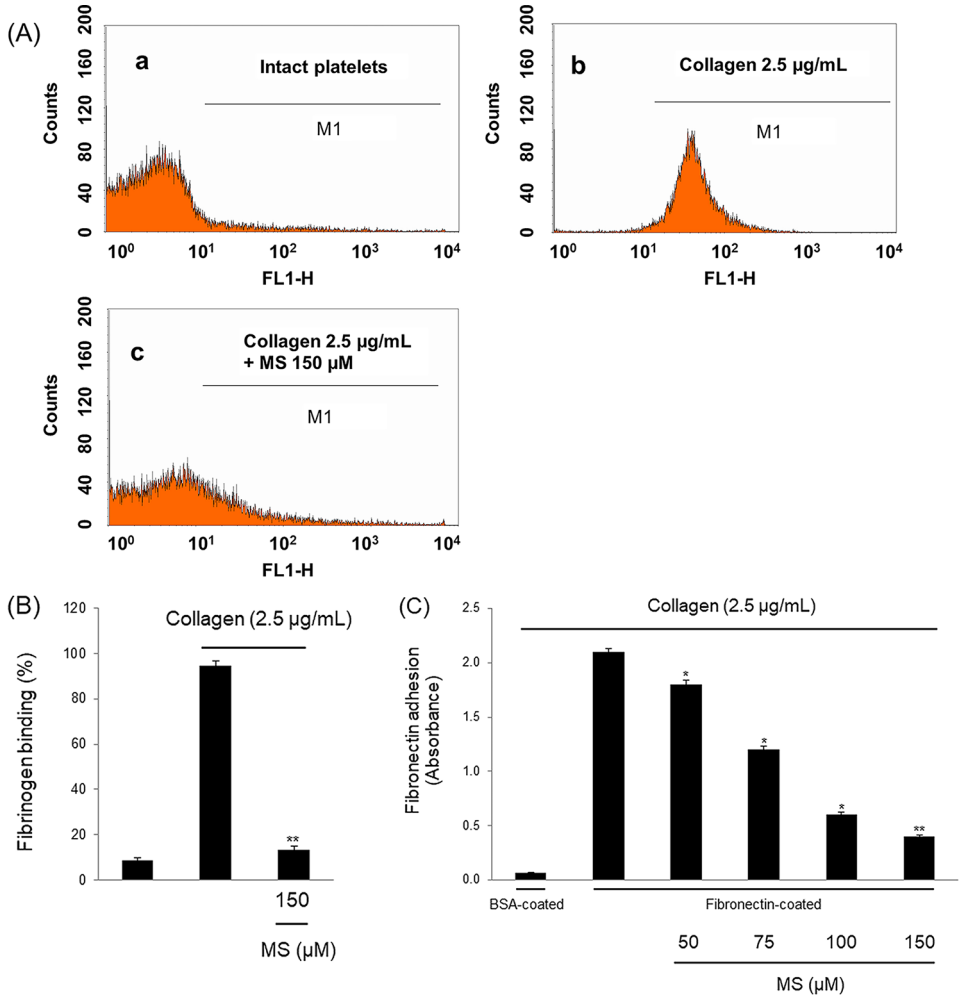

Fig. 2 Morusinol (MS)'s effect on fibrinogen binding to αIIb/β_3, and fibronectin adhesion. (A) The flow cytometry histograms on fibrinogen binding. (B) Morusinol's effect on collagen-induced fibrinogen binding (%). (C) Morusinol's effect on collagen-induced fibronectin adhesion. All experiments were 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

Effect of morusinol on clot retraction

This study further examined the effect of morusinol on fibrin clot retraction. During primary hemostasis, platelets form a hemostatic plug to prevent bleeding due to damaged blood vessels. Concurrently, fibrinogen is converted into fibrin *via* activation of blood coagulation factors, thereby strengthening the hemostatic plug. This process results in the constriction of hemostatic plug, leading to repair of damaged blood vessels. Thrombin was used as an agonist for the clot retraction test because it stimulates platelets and produces fibrin. This study aimed to determine the effect of morusinol on hemostatic plug formation produced by primary and secondary hemostasis. The results showed that morusinol delayed clot formation (Fig. 3A). Y27632 was used as positive control for this experiment.

Effect of morusinol on phosphorylation and dephosphorylation

This study investigated the signaling factors that play a role in integrin αIIb/β_3 binding. These factors include PI_3K/Akt/GSK-3α/β and VASP, which are essential regulators of αIIb/β_3 activation, platelet spreading, and adhesion [11-13]. The study findings indicated that morusinol significantly reduced the phosphorylation of PI3K/Akt/GSK-3 and increased the phosphorylation of VASP (Ser^{157}) (Fig. 3C).

Effect of morusinol on Ca^{2+} regulation and phosphorylation of IP_3R, ERK, and p38

The study then examined the impact of morusinol on calcium mobilization and phosphorylation of signaling molecules that control calcium secretion. The results indicated that collagen stimulation led to an increase in intracellular calcium levels, whereas morusinol suppressed this increase in a dose-dependent manner (Fig. 4A). The study also found that IP_3R, which is located in the endoplasmic reticulum, is a crucial regulator of Ca^{2+} levels, and its phosphorylation by cAMP/cyclic guanosine mono-

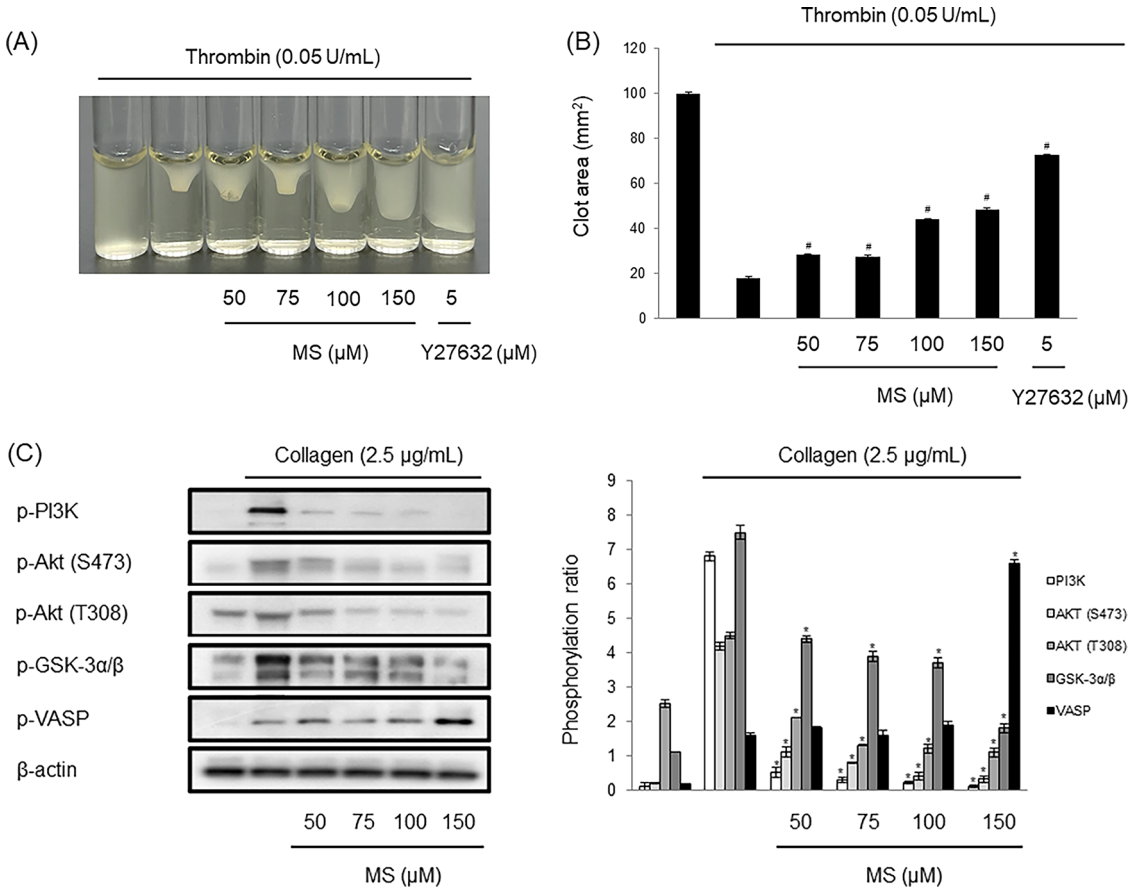

Fig. 3 Morusinol (MS)'s effect on fibrin clot retraction and PI_3K/Akt/GSK-3α/β, VASP phosphorylation. (A) Photographs of fibrin clot. (B) Morusinol's effect on thrombin-retracted fibrin clot (%). (C) Morusinol's effect on collagen-induced PI_3K/Akt/GSK-3α/β and VASP phosphorylation. Quantification of 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 thrombin-stimulated human PRP. $*p$ <0.05 versus the collagen-stimulated human platelets

phosphate (cGMP)-dependent kinases inhibits Ca^{2+} mobilization. The results showed that morusinol increased IP_3R phosphorylation induced by collagen (Fig. 4B). Researchers have also investigated the effects of morusinol on extracellular signal-regulated kinase (ERK1/2) and p38, the two signaling molecules that regulate platelet calcium, thromboxane A_2 synthesis, and platelet aggregation [14,15]. The results revealed that morusinol inhibited the phosphorylation of ERK2 and p38 (Fig. 4B).

Effect of morusinol on serotonin release and cAMP production

The effects of morusinol on serotonin release and cAMP production in platelets were investigated. After collagen stimulation, phosphorylation of myosin light chain and pleckstrin promote the release of platelet granules. The study findings showed that morusinol inhibited serotonin release (Fig. 5A).

Under normal conditions, vascular endothelial cells release prostacyclin and nitric oxide, which activate adenylyl cyclase and guanylyl cyclase, resulting in the production of cAMP and cGMP. These cyclic nucleotides stimulate protein kinase A and protein kinase G to phosphorylate substrates, leading to the inhibition of platelet adhesion, granule release, and aggregation [16,17]. VASP and IP_3R are the two major substrates of cAMP and cGMP, and increased cAMP and cGMP levels can inhibit $[Ca^{2+}]_i$ mobilization and $\alpha IIb/\beta_3$ activation. We confirmed that morusinol increased cAMP levels in human platelets (Fig. 5B).

Discussion

Morusinol suppressed collagen-stimulated human platelet aggregation (Fig. 1B). Next, we investigated its effect on $\alpha IIb/\beta_3$ activation. Agonists stimulate signaling cascade called the inside-out signaling, which facilitates integrin activation, leading to structural changes in $\alpha IIb/\beta_3$. These conformational changes activate $\alpha IIb/\beta_3$, which promotes platelet aggregation. The $\alpha IIb/\beta_3$-mediated action is called the outside-in signaling pathway, and it is very important for fibrin-platelet interactions. Therefore, we analyzed signaling molecules that contribute to the activation of $\alpha IIb/\beta_3$ and the effect of morusinol on $\alpha IIb/\beta_3$-mediated thrombogenic response. In our previous study, mulberroside C has similar results to morusinol.

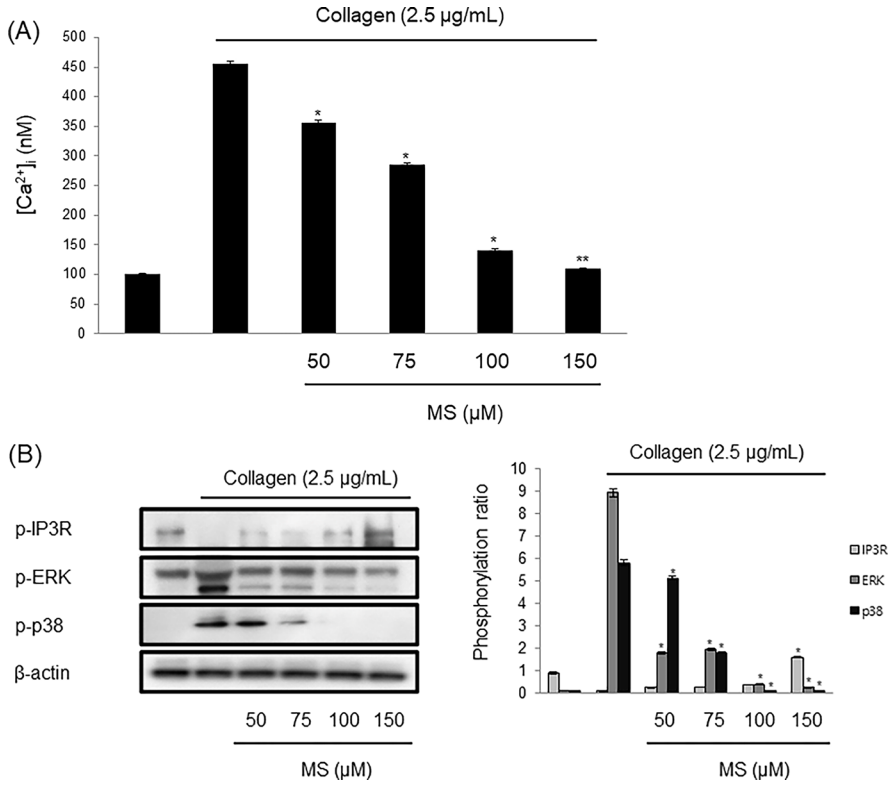

Fig. 4 Morusinol (MS)'s effect on $[Ca^{2+}]_i$ mobilization, and IP_3RI/ERK/p38 phosphorylation (A) Morusinol's effect on collagen-induced $[Ca^{2+}]_i$ mobilization. (B) Morusinol's effect on collagen-induced IP_3RI/ERK/p38 phosphorylation. All experiments were performed as described in "Materials 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

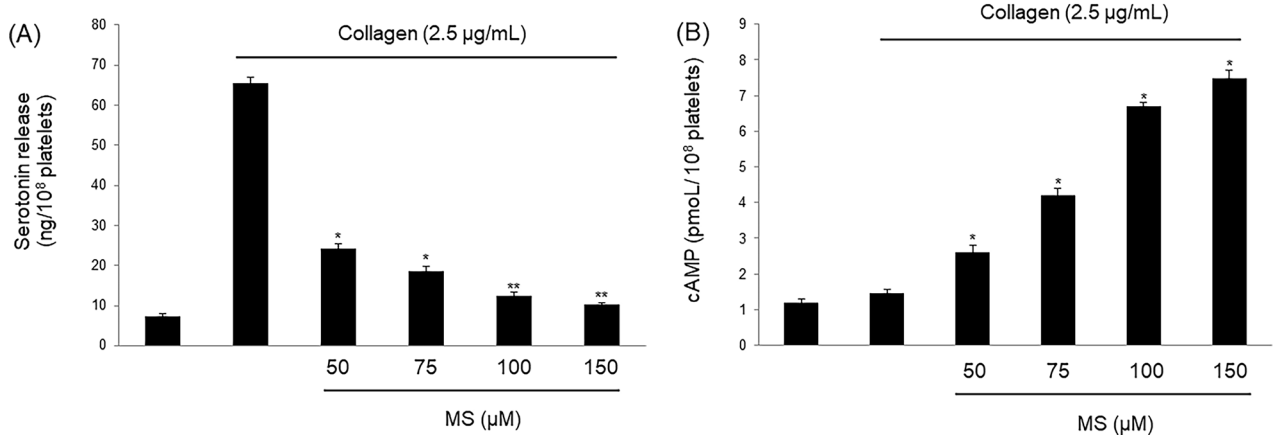

Fig. 5 Morusinol (MS)'s effect on serotonin release and cAMP production. (A) Morusinol's effect on serotonin release. (B) Morusinol's effect on collagen-induced cAMP production. All experiments were performed as described in "Materials and Methods" section. The data are expressed as the mean ± standard deviation (n =4). *p <0.05, **p <0.01 versus the collagen-stimulated human platelets

Mulberroside C is an active ingredient of *Morus alba* and inhibited collagen-induced $[Ca^{2+}]_i$ mobilization, thromboxane A_2 production and p-selectin expression [18].

As shown in Fig. 2A-C, morusinol suppressed fibrinogen binding to $\alpha IIb/\beta_3$ and fibronectin adhesion. Next, we evaluated whether morusinol inhibits antithrombotic action. Integrin $\alpha IIb/\beta_3$ forms a hemostatic plug through interaction with other platelets; however, if this action is overactive, it can produce blood clots, leading to cardiovascular disease. Thus, it is important to evaluate whether a natural product can inhibit blood clots. As shown in Fig. 3A and 3B, morusinol strongly inhibited clot retraction.

PI_3K and Akt are well-known signaling molecules that facilitate

αIIb/β_3 activation [19]. Recent studies have suggested that GSK-3 functions as a negative regulator of platelet function, but the underlying mechanism has not been examined in detail. GSK-3 is reported to be a downstream signaling molecule of Akt that phosphorylates GSK [20]. GSK-3 is a Ser/Thr kinase, which is inhibited by the phosphorylation of an N-terminal Ser residue (GSK-3α at Ser^{21}, GSK-3β at Ser^{9}). In addition, wortmannin (phosphatidylinositol 3-kinase inhibitor) and Ro31-8220 (protein kinase C inhibitor) have been reported to decrease GSK-3 phosphorylation [21]. Therefore, inhibition of GSK phosphorylation is thought to inhibit platelet activity. We analyzed the effect of morusinol on the phosphorylation of signaling molecules (PI_3K, Akt [Ser^{473} and Thr^{308}], and GSK-3α/β) related to αIIb/β_3 activation. As shown in Fig. 3C, morusinol strongly inhibited the collagen-induced phosphorylation of PI_3K, Akt (Ser^{473} and Thr^{308}), and GSK-3α/β. Therefore, it was confirmed that morusinol inhibited αIIb/β_3 activation through inhibition of phosphorylation of PI_3K, Akt, and GSK-3α/β. In addition, VASP is a major substrate that contributes to the regulation of actin filament dynamics and focal adhesions; however, its phosphorylation leads to the inhibition of actin filament elongation and αIIb/β_3 activation. As shown in Fig. 3C, morusinol strongly elevated collagen-induced phosphorylation of VASP (Ser^{157}).

Next, we investigated whether morusinol reduces $[Ca^{2+}]_i$ levels through phosphorylation of IP_3R. IP_3R is a well-known substrate of PKA, and IP_3R phosphorylation leads to inhibition of Ca^{2+} mobilization. Therefore, phosphorylation of IP_3R is useful for evaluating the Ca^{2+}-antagonistic effect of natural substances. As shown in Fig. 4A and B, morusinol inhibited $[Ca^{2+}]_i$ mobilization and increased IP_3R phosphorylation. Next, we examined whether morusinol affects δ-granule release. As shown in Fig. 5A, morusinol inhibited collagen-stimulated serotonin secretion.

Finally, we investigated the effect of morusinol on cAMP production. Various substrates of cAMP-dependent protein kinase A have been reported in platelets. Representative substrates are actin binding protein, caldesmon, G-protein α_{13} subunit, glycoprotein Ib β subunit, heat shock protein 27, inositol 1,4,5-trisphosphate receptor (IP_3 receptor), Rap1b, and phosphodiesterase 3 (PDE_3) [17]. These cyclic nucleotides can block αIIb/β_3 activity and $[Ca^{2+}]_i$ mobilization [22,23]. In contrast, elevated cAMP levels are degraded by PDEs. For example, dipyridamole and cilostazol are approved therapies for the prevention of stroke. Dipyridamole elevates cAMP levels in platelets, and cilostazol is a specific inhibitor of PDE_3 [24]. Therefore, substances that inhibit cAMP have potential for the treatment of cardiovascular diseases. In this study, we examined whether morusinol increases cAMP levels, and confirmed that morusinol elevated cAMP levels in human platelets (Fig. 5B). We also confirmed the effect of morusinol on cGMP, but morusinol did not affect the concentration of cGMP. VASP, a substrate for cAMP and cGMP, has two phosphorylation sites. It has been reported that Ser^{157} is dependent on cAMP and Ser^{239} is affected by cGMP [13]. In our experimental results, morusinol phosphorylated the Ser^{157} position of VASP because it only increased cAMP. Phosphorylation of Ser^{239} of VASP by morusinol was not increased (Data not shown).

Our study had some limitations. It was an *in vitro* study and did not involve *ex vivo* or *in vivo* evaluations. Moreover, since the study was not conducted *in vivo*, the findings cannot be extrapolated to the effects of morusinol in the human body. However, based on our experimental results, we observed that morusinol inhibited human platelet aggregation, αIIb/β_3 activation, and clot retraction through regulation of signaling molecules and cAMP levels. Various studies on natural products and cardiovascular diseases are being conducted. A representative natural product is ginseng and looking at the review papers on ginseng and cardiovascular diseases [25], it can be seen that studies from various perspectives should be accompanied. Therefore, our study needs to be further expanded, and we think that morusinol has potential for cardiovascular disease.

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Conflict of interest The authors declare no conflict of interest.

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