

Original Article

Morusinol Extracted from *Morus Alba* Inhibits Arterial Thrombosis and Modulates Platelet Activation for the Treatment of Cardiovascular Disease

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Morus alba (white mulberry) has been used in traditional Chinese medicine as an anti-headache, diuretic, expectorant, and anti-diabetic agent. In previous studies, extracts of *Morus alba* demonstrated favorable biological properties, such as antioxidant activity, suppression of lipoxygenase (LOX)-1, cytotoxicity against cancer cells, and inhibition of the invasion and migration of cancer cells.

Aim: This study further evaluated the effects of morusinol, a flavonoid derived from *Morus alba* root bark, on platelet aggregation and thromboxane B₂ (TXB₂) formation *in vitro* and thrombus formation *in vivo*.

Methods: The antiplatelet potential of morusinol was measured using *in vitro* rabbit platelet aggregation and TXB₂ formation assays. Arterial thrombus formation was investigated using an *in vivo* ferric chloride (FeCl₃)-induced thrombosis model.

Results: Morusinol significantly inhibited collagen- and arachidonic acid-induced platelet aggregation and TXB₂ formation in cultured platelets in a concentration-dependent manner. Thrombus formation was reduced by 32.1, 42.0, and 99.0% for collagen-induced TXB₂ formation, and 8.0, 24.1, and 29.2% for arachidonic acid-induced TXB₂ formation, with 5, 10, and 30 µg/mL morusinol, respectively. Moreover, oral morusinol (20 mg/kg) or aspirin (20 mg/kg) for three days significantly increased the time to occlusion *in vivo* by 20.3 ± 5.0 or 6.8 ± 2.9 min, respectively, compared with the control (1% CMC, carboxymethyl cellulose).

Conclusion: Taken together, these results indicate that morusinol may significantly inhibit arterial thrombosis *in vivo* due to antiplatelet activity. Thus, morusinol may exert beneficial effects on transient ischemic attacks or stroke via the modulation of platelet activation.

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Key words; Morusinol, Platelet aggregation, Thrombus formation, *Morus alba*

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Introduction

Thrombus formation is one of the most common causes of cardiovascular diseases, including transient ischemic attacks and stroke, and platelet aggregation as a result of thrombus formation is believed to play a key role in these processes¹. Commonly, plate-

lets play an important role in both physiological hemostatic and pathological thrombosis. Once blood vessels become damaged, a diverse array of adhesive ligands, such as collagen and von Willebrand factor (vWF), and soluble agonists, such as adenosine diphosphate (ADP) and thrombin, are exposed or generated at the injury site. These clotting molecules stimulate platelet adhesion, activation, and aggregation²⁾.

Thrombus formation is inseparably connected to the initiation of the coagulation cascade. The ultimate result is the production of fibrin. Following tissue injury, exposure of membrane-bound tissue factor (TF) is a crucial step in the coagulation process. Membrane-bound TF binds to blood coagulation factor VIIa (FVIIa) via an extracellular domain to activate the extrinsic coagulation pathway. The binary FVIIa/TF complex then generates activated blood coagulation factor Xa (FXa) via the intrinsic coagulation pathway³⁻⁵⁾. Generation of FIXa by the FVIIa/TF complex results in the formation of the Xnase complex following binding of FIXa to a non-enzymatic co-factor, activated factor VIII (FVIIIa). The Xnase complex, along with the FVIIa/TF complex, converts FX to activated factor X (FXa). FXa then assembles with activated factor V (FVa) into the prothrombinase complex, which is directly responsible for the formation of thrombin³⁻⁵⁾.

Collagens support the adhesion of platelets to an injury site through platelet membrane receptors glycoprotein VI (GPVI) and integrin $\alpha 2\beta 1$ ⁶⁾. The binding of collagen to GPVI and integrin $\alpha 2\beta 1$ induces platelet activation through a tyrosine kinase-dependent signaling pathway that involves spleen tyrosine kinase (Syk) and phospholipase C $\gamma 2$. This signaling pathway results in an increase in platelet cytosolic calcium, and the platelet shape changes from discoid to more spheroid with pseudopod projections, and granule release⁷⁻⁹⁾. Adhesion is partly dependent and aggregation is largely dependent on the release of the secondary agonists ADP and prostaglandin H₂/thromboxane A₂ (TXA₂) from platelet granules¹⁰⁻¹²⁾.

Morus alba (Moraceae; white mulberry tree) has been used in traditional Chinese medicine as an anti-headache, diuretic, expectorant, and anti-diabetic agent^{13, 14)}. Many parts of *Morus alba* have medicinal properties. For example, white mulberry leaves, an important food for the silkworm, are used to treat hypertension and arthritis. In addition, the fruit is a diuretic and tonic agent^{13, 14)}. Previous studies have focused on isolating active compounds from *Morus alba* and have reported that its constituents are primarily polyphenolic in nature and include isoprenyl flavonoids, bezofuranes, and adducts of the Diel-Alder

type. These constituents demonstrated pharmacological properties such as antioxidant actions, the suppression of lectin-like oxidized low density lipoprotein receptor-1, cytotoxicity against cancer cells, and inhibition of the invasion and migration of cancer cells¹⁵⁻¹⁸⁾.

Morusin is an important prenylated flavonoid recently isolated from *Morus australis* (Japanese mulberry)¹⁹⁾. Morusin inhibits arachidonic acid-, platelet-activating factor (PAF)-, and collagen-induced platelet aggregation²⁰⁾; suppresses superoxide anion formation²¹⁾; and shows antibacterial activity¹⁶⁾. Morusinol is among the other *Morus* flavones and was first extracted from mulberry root bark²²⁾; however, the biological activity of morusinol has not yet been reported. Therefore, the present study investigated the effects of morusinol on platelet aggregation and TXB₂ formation *in vitro*, as well as rat thrombosis *in vivo*.

Material and Methods

Materials

Indomethacin, bovine serum albumin (BSA), ethylene glycol-bis (β -aminoethyl ether)-N,N,N',N'-tetraacetic acid (EGTA), carboxymethyl cellulose (CMC), calcium chloride, and dimethyl sulfoxide (DMSO) were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Collagen, arachidonic acid, and thrombin were purchased from Chrono-Log Co. (Havertown, PA, USA). TXB₂ formation kit was purchased from Cayman Chemical Co. (Avenue, NY, USA). Other chemicals used in this study were of analytical grade.

Extraction and Isolation of Morusinol

The dry material of Mori Cortex Radicis (the root epidermis of *Morus alba*) (10 kg) was extracted three times with methanol (MeOH) at room temperature. Removal of the solvent *in vacuo* yielded the MeOH extract (1.5 kg). The MeOH extract was suspended in distilled water and then successively partitioned with *n*-hexane and ethyl acetate (EtOAc) (610 g). A portion of the EtOAc-soluble layer (75 g) was subjected to column chromatography using silica gel column chromatography (70-230 mesh; $\Phi=10$ cm, $L=28$ cm) and eluted with a stepwise gradient of chloroform (CHCl₃)/MeOH (1 : 0, 80 : 1, 50 : 1, 20 : 1, 15 : 1, 10 : 1, 5 : 1, 3 : 1, 1 : 1, v/v) to yield 12 fractions (SS2-1 to 12). Fraction SS2-7 (3 g) was subjected to column chromatography on an RP-18 CC silica gel column ($\Phi=3$ cm, $L=20$ cm) and eluted with methyl cyanide-H₂O (MeCN-H₂O) (stepwise, 30, 40, 50, 60, 70, 80, 90, 100% MeCN in H₂O, v/v) to give nine fractions (SS2-7-1 to 9). Morusinol (152

mg) was isolated from fraction SS2-7-6 (450.1 mg) by column chromatography ($\Phi=25$ mm, $L=20$ cm) on an MCI gel CHP-20 column and eluted using MeOH-H₂O (stepwise, 40, 50, 60, 70, 80, 90% MeOH in H₂O, v/v).

Identification of Morusinol Using Nuclear Magnetic Resonance and Electrospray Ionization Mass Spectroscopy

The ¹H- and ¹³C-NMR spectra for morusinol were recorded on a Varian 500 nuclear magnetic resonance (NMR) spectrometer using deuterated DMSO (DMSO-*d*₆) as the solvent. The electrospray ionization mass spectroscopy (ESI-MS) spectrum was obtained on a Waters Q-TOF micro mass spectrometer. The ¹H- and ¹³C-NMR spectra for morusinol were as follows: ¹H-NMR (500 MHz, DMSO-*d*₆) δ 13.3 (1H, s, OH-5), 7.16 (1H, d, $J=8.0$ Hz, H-6'), 6.50 (1H, d, $J=10.0$ Hz, H-14), 6.43 (1H, d, $J=2.0$ Hz, H-3'), 6.34 (1H, dd, $J=8.0, 2.0$ Hz, H-5'), 6.21 (1H, s, H-6), 5.68 (1H, d, $J=10.0$ Hz, H-15), 2.33 (2H, m, H-9), 1.44 (2H, m, H-10), 1.41 (6H, s, CH₃-17, CH₃-18), 0.96 (6H, s, CH₃-12, CH₃-13); ¹³C-NMR (125 MHz, DMSO-*d*₆) δ 182.0 (C-4), 161.8 (C-7), 161.0 (C-5), 160.4 (C-4'), 158.4 (C-2'), 156.4 (C-2), 151.7 (C-8a), 131.1 (C-6'), 127.6 (C-15), 121.2 (C-3), 114.1 (C-14), 110.8 (C-1'), 106.8 (C-5'), 104.2 (C-4a), 100.4 (C-8), 98.7 (C-6), 78.0 (C-16), 68.7 (C-11), 41.9 (C-10), 28.8 (C-12), 28.8 (C-13), 27.7 (C-17), 27.7 (C-18), 20.1 (C-9). The ESI-MS spectrum was as follows: ESI-MS m/z 437 [M - H]⁻, as reported previously (Konno *et al.*, 1977). The chemical structure of morusinol is presented in Fig. 1.

Animals

Male Sprague-Dawley rats (250-300 g) and New Zealand white rabbits (2.5-3 kg) were purchased from Sam-Tako Animal Co. (Osan, Korea) and acclimated for one week at a temperature of $24 \pm 1^\circ\text{C}$ and a humidity level of $55 \pm 5\%$. The animals had free access to drinking water and a commercial pellet diet obtained from Samyang Co. (Wonju, Korea). The animal studies were carried out in accordance with the Guide for the Care and Use of Laboratory Animals, Chungbuk National University, Korea.

Washed Rabbit Platelet Preparation and Platelet Aggregation *in vitro*

Blood was withdrawn from the ear artery of male New Zealand white rabbits and collected directly into anticoagulant citrate dextrose (ACD) solution that contained 0.8% citric acid, 2.2% trisodium citrate, and 2% dextrose (w/v). This procedure yielded a 1 :

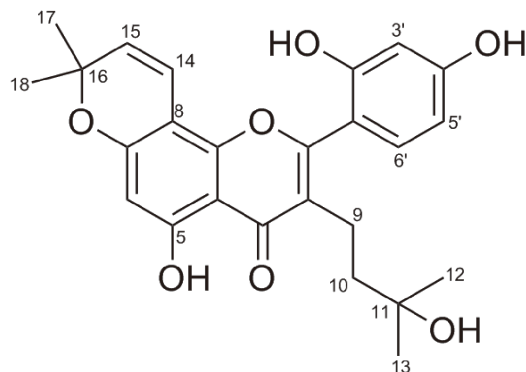


Fig. 1. Chemical structure of morusinol.

0.15 (v/v) blood/ACD mixture. Washed platelets were prepared as previously described¹². Briefly, platelet-rich plasma (PRP) was obtained by centrifugation of rabbit blood at $230 \times g$ for 10 min. Platelets were sedimented by centrifugation of the PRP at $800 \times g$ for 15 min and washed with HEPES buffer (137 mM NaCl, 2.7 mM KCl, 1 mM MgCl₂, 5.6 mM glucose, 3.8 mM HEPES, pH 6.5) containing 0.35% BSA and 0.4 mM EGTA. The washed platelets were resuspended in HEPES buffer (pH 7.4) and the cell dilution was adjusted to 4×10^8 cells/mL. Platelet aggregation was measured with an aggregometer (Chrono-Log Co., Havertown, PA, USA) according to the turbidimetry method of Born^{22, 23}, as previously described¹². Briefly, the washed platelet suspension was incubated at 37°C in the aggregometer with stirring at 1,000 rpm and then various concentrations (5-30 μM) of morusinol were added, respectively. After 3 min preincubation, platelet aggregation was induced by the addition of agonists such as collagen (10 $\mu\text{g/mL}$), arachidonic acid (100 μM), or thrombin (0.05 U/mL). The extent of platelet aggregation was expressed as a percentage of the control value stimulated by an agonist alone.

Thromboxane B₂ Formation

TXB₂ is released from platelet granules through the agonist-activated pathway in platelets¹⁰. TXB₂ formation was assayed with a TXB₂ formation kit according to the manufacturer's instructions. Briefly, washed rabbit platelets (4×10^8 cells/mL) were preincubated with morusinol (5-30 μM) or aspirin (100 and 200 μM , as the positive control) at 37°C for 3 min in the aggregometer, as described above. After a 3 min preincubation, platelet aggregation was induced by the addition of collagen (10 $\mu\text{g/mL}$), arachidonic acid (100 μM), or thrombin (0.05 U/mL). The reaction was stopped by the addition of 5 mM indomethacin and 2 mM EGTA. The resultant platelet suspen-

sions were centrifuged at 12,000 g for 2 min, and TXB₂ formation was measured by enzyme-linked immunoassay (ELISA). The TXA₂ content in each sample was determined by the TXB₂ level, given that TXB₂ is the stable, inactive metabolite of TXA₂. Aspirin (100 and 200 μ M), an antiplatelet agent, was used as the positive control.

Arterial Thrombus Formation *in vivo*

Arterial thrombus formation *in vivo* was investigated as previously described¹². In brief, oral morusinol (20 mg/kg) or aspirin (20 mg/kg) was administered to male Sprague-Dawley rats ($n=10$) for 3 days. Rats were then anesthetized with sodium pentobarbital (60 mg/kg, i.p.) and maintained at 37°C on a servo-controlled, heated rodent operating table. A segment of the right carotid artery was isolated and dissected away from the vagus nerve and surrounding tissues. Aortic blood flow rate was monitored with a blood flowmeter (ADInstruments, Colorado Springs, CO, USA). Arterial thrombus formation was induced by wrapping a 2 mm² piece of Whatman no. 1 filter paper saturated with 50% ferric chloride (FeCl₃, in distilled water) around the carotid artery near the flowmeter probe for 10 min. The time required for thrombotic arterial occlusion to occur was measured for up to 60 min, and occlusion time was assigned a value of 60 min for vessels that did not occlude within this time frame.

Statistical Analysis

All experimental results are expressed as the mean \pm S.E.M., with the exception of blood vessels that did not occlude within 60 min. As noted above, occlusion time was assigned a value of 60 min under these circumstances. One-way analysis of variance (ANOVA) was used for multiple comparisons followed by Dunnett's test. Statistical significance was set at $p < 0.05$.

Results

Effect of Morusinol on Platelet Aggregation *in vitro*

Morusinol significantly inhibited collagen- and arachidonic acid-induced washed rabbit platelet aggregation *in vitro* in a concentration-dependent manner, with IC₅₀ values of 13.4 ± 5.4 and 19.8 ± 3.5 (μ g/mL), respectively (Fig. 2); however, the inhibitory effect of morusinol was not observed for thrombin-induced platelet aggregation. These data indicate that the effect of morusinol is specific for collagen and arachidonic acid.

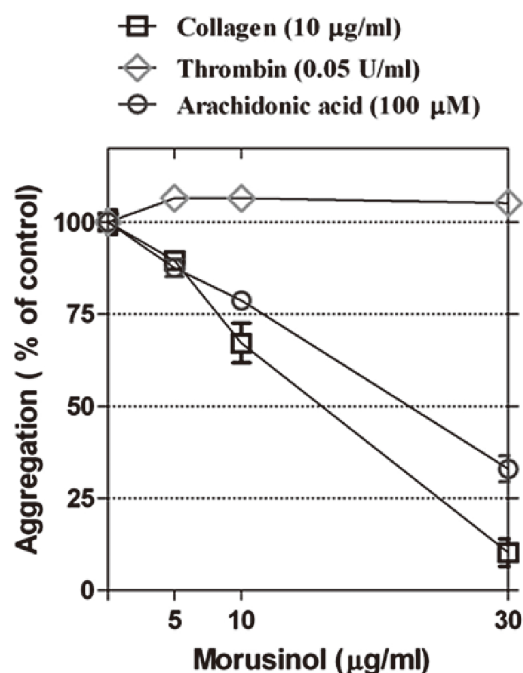


Fig. 2. Effect of morusinol on washed rabbit platelet aggregation *in vitro*. After preincubation with morusinol for 3 min, platelet aggregation was induced by the addition of collagen (10 μ g/mL), arachidonic acid (100 μ M), or thrombin (0.05 U/mL). The aggregation percentages are expressed as the percentage of maximum aggregation by the respective inducers. Data are expressed as the mean \pm S.E.M. ($n=4$).

Effect of Morusinol on Thromboxane B₂ Formation

To further examine the underlying antiplatelet activity of morusinol, washed rabbit platelets were stimulated with collagen or arachidonic acid in the presence or absence of morusinol (Fig. 3). Collagen-induced TXB₂ formation was significantly decreased by 32.1, 42.0, and 99.0% with 5, 10, and 30 μ g/mL morusinol, respectively, compared with the control value stimulated by an agonist alone. Furthermore, arachidonic acid-induced TXB₂ formation was decreased by 8.0, 24.1, and 29.2% with 5, 10, and 30 μ g/mL morusinol, respectively.

Effect of Morusinol on Arterial Thrombus Formation *in vivo*

The effect of morusinol on arterial thrombus formation *in vivo* was evaluated by using the FeCl₃-induced rat carotid artery injury model described in Materials and Methods. After 50% FeCl₃ application, injured vessels of the control group (1% CMC) ($n=7$) were occluded within 22.5 ± 2.5 min. Oral morusinol (20 mg/kg) or aspirin treatment (20 mg/kg) for 3 days

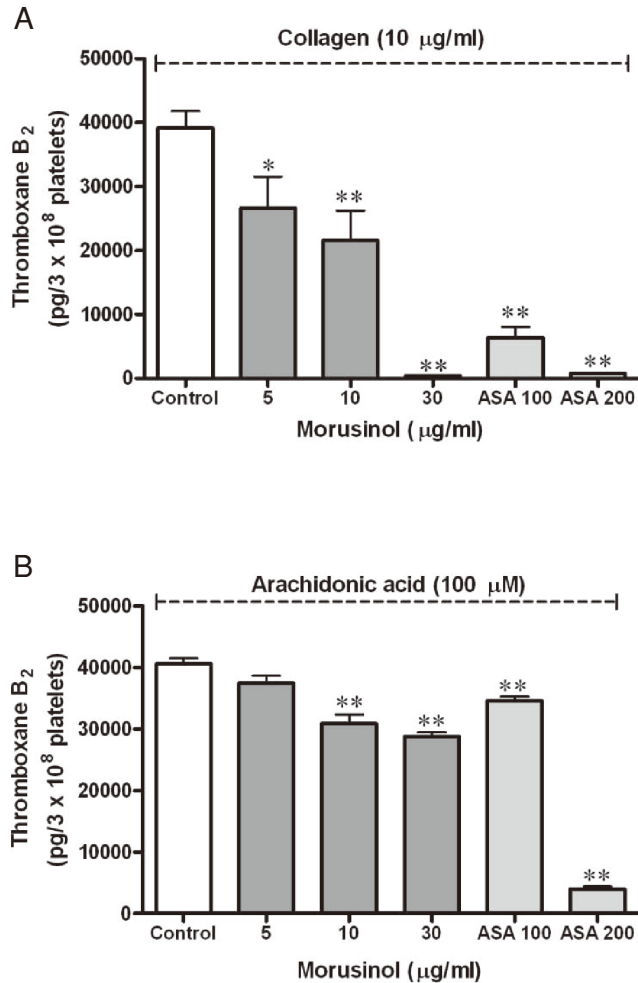


Fig. 3. Effect of morusinol on TXB₂ formation. TXB₂ formation in platelets was assayed with a TXB₂ formation kit according to the manufacturer's instructions. Platelet aggregation was then induced by the addition of collagen (A, 10 µg/ml) or arachidonic acid (B, 100 µM). Aspirin (ASA, 100 and 200 µM), an antiplatelet agent, was used as the positive control. TXA₂ formation was reflected by the amount of TXB₂ produced. Data are expressed as the mean ± S.E.M. ($n=3$). * $p < 0.05$ and ** $p < 0.01$ vs. stimulus control.

significantly prolonged occlusion time to 42.8 ± 4.8 min ($n=7$) or 29.3 ± 1.5 min ($n=7$) (**Fig. 4**). These data suggest that the inhibitory effect of morusinol on arterial thrombus formation appears to be stronger than the positive control, aspirin at a dosage of 20 mg/kg.

Discussion

Blood flow disturbances at sites of atherosclerotic plaque rupture promote platelet activation and arterial thrombus formation. This work evaluated the inhibi-

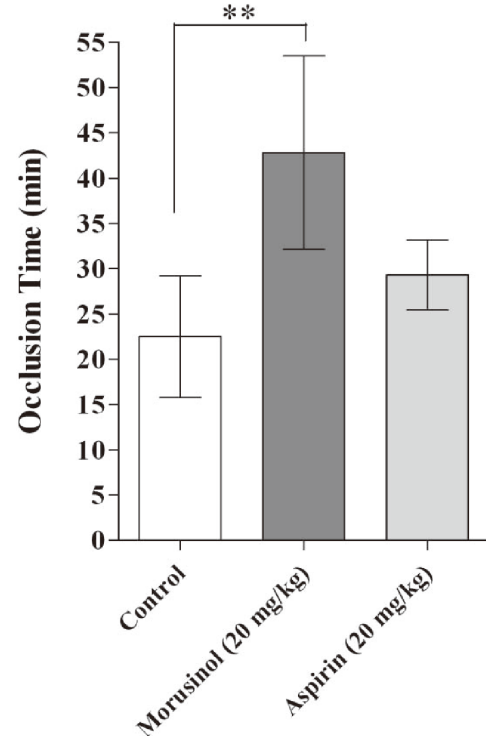


Fig. 4. Effect of morusinol on arterial thrombus formation *in vivo*. Rat carotid arteries were subjected to chemical injury by placing a 2 mm² piece of Whatman no. 1 filter paper saturated with 50% FeCl₃ around the carotid artery for 10 min. Blood flow was then measured with a blood flowmeter. Morusinol (20 mg/kg) or aspirin (20 mg/kg) were orally administered for three days, and the results are expressed as time to occlusion ($n=7$). ** $p < 0.01$ vs. control.

tory actions of morusinol on platelet aggregation and TXB₂ formation in washed platelets, as well as the antithrombotic effect of morusinol in a FeCl₃-induced rat arterial thrombosis model. In this model, the thrombotic plug is composed of fibrin, activated platelets, and entrapped erythrocytes. This type of thrombus is found in coronary arteries after myocardial sudden death and acute myocardial infarction and is a causative factor^{24, 25}.

Ferric chloride (FeCl₃) brings about oxidative endothelial injury and exposes the subendothelial extracellular matrix. Platelets then interact with vWF and collagen in the matrix via their respective platelet membrane receptors, the glycoprotein (GP)Ib-V-IX complex and integrin α IIb β 3, resulting in platelet adhesion. Glycoprotein VI is an additional platelet membrane receptor for collagen, and GPVI binding to collagen leads to platelet activation. Activated platelets undergo calcium mobilization and the release of

ADP and TXA₂ to accelerate further platelet recruitment and aggregation for thrombus formation²⁶.

Morusinol significantly increased the time to occlusion in this study, which indirectly indicates that morusinol can inhibit thrombus formation *in vivo*. Previous work investigated the antiplatelet effects of the active constituents isolated from *Morus australis* (e.g., morusin and kuwanon C) by evaluating the impact of morusin and kuwanon C on collagen-, arachidonic acid-, and thrombin-induced aggregation of washed rabbit platelets. Morusin inhibited the aggregation of washed rabbit platelets, but the degree of inhibition depended on the specific aggregation inducer employed²⁰. For example, collagen- and arachidonic acid-induced aggregation were completely inhibited by both morusin and kuwanon C, while thrombin-induced aggregation was only slightly affected by morusin and markedly inhibited by kuwanon C²⁰. In the current study, morusinol, like morusin, inhibited platelet aggregation induced by collagen and arachidonic acid, but not by thrombin; therefore, morusinol and morusin may not be involved in the thrombin-induced platelet aggregation pathway. As a possible mechanism, morusinol may have an antiplatelet effect through collagen- and arachidonic acid-induced intracellular calcium mobilization and arachidonic acid metabolites, including TXA₂, prostaglandin and eicosanoid; however, the present study has a limitation because this potential mechanism has not been proved in this study.

As noted above, morusinol potently inhibited platelet aggregation induced by collagen (**Fig. 2**), indicating that morusinol may interfere with the tyrosine-mediated signaling pathway. Morusinol also significantly inhibited TXB₂ formation (**Fig. 3**) and arachidonic acid-mediated platelet aggregation (**Fig. 2**) in a concentration-dependent manner. Arachidonic acid directly activates the membrane cyclooxygenase (COX)-TXA₂ synthase pathway to stimulate its own conversion into TXA₂, which then activates phospholipase C. Thus, arachidonic acid mediates platelet aggregation via the same pathway as U46619, a synthetic TXA₂ agonist²⁷ that acts directly on the TXA₂ receptor to induce G protein-coupled phospholipase C β activation, resulting in an increase of protein kinase C activation^{2, 28}. Hence, morusinol may inhibit arachidonic acid-mediated TXA₂ formation and therefore TXA₂-mediated platelet aggregation. In a future study, the influence of morusinol on the generation of TXA₂ and prostaglandin D₂ (PGD₂) will be investigated, both of which are produced from arachidonic acid through the COX-1 pathway in platelets²⁹.

Conclusion

In conclusion, the present study demonstrated that morusinol can significantly inhibit arterial thrombus formation *in vivo*, which may be due to its antiplatelet activity. Thus, morusinol may have great potential for the treatment of cardiovascular injury via the modulation of platelet activation.

Acknowledgments

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