Antithrombotic Effect of *Sorbus commixta* in an Arterial Thrombosis Mouse Model

Mikyung Kim¹, Jung-Min Park¹,², Young-Mi Kim³, Young-Won Chin³ and Moo-Yeol Lee¹,²

Abstract

*Sorbus commixta* Hedlund (*S. commixta*) is known to possess cardiovascular protective activity. However, its effect on thrombosis has not been reported, although thrombosis plays a primary role in diverse circulatory diseases. Thus, this study was performed to assess the antithrombotic activity of dried *S. commixta* fruit extract. Mice were fed with a diet containing 8.3% extract for 4 weeks, which corresponded to the consumption of approximately 1000 mg extract/kg/day. The antithrombotic effect was tested in a FeCl₃-induced arterial thrombosis model. Additionally, the effects on platelet aggregation and plasma coagulation were examined *in vitro* using washed platelets (WP) and isolated blood plasma, respectively. Thrombotic vascular occlusion time was significantly prolonged by the 4-week administration of the extract. Treatment of WP with the extract resulted in a decrease in thrombin-induced aggregation, which was concentration-dependent over 10 to 100 μg/mL and was not attributed to the direct inhibition of thrombin. The extract did not affect blood plasma coagulation, which was assessed by measuring prothrombin time and activated partial thromboplastin time. *S. commixta* is capable of preventing arterial thrombosis and such an effect may be attributed to the inhibition of platelet aggregation.

Keywords

*Sorbus commixta*, thrombosis, platelet aggregation, plasma coagulation, bioactivity

Received: November 2nd, 2021; Accepted: January 31st, 2022.

Introduction

*Sorbus commixta* Hedlund (*S. commixta*), also known as mountain ash or rowan, is a deciduous tree species in the family Rosaceae and is native to far eastern Asia. Although the scientific basis for its biomedical applications has never been studied extensively, the fruits and bark of *S. commixta* have a long history of use as a traditional medicine or medicinal food for the treatment of asthma, bronchial disorders, rheumatoid arthritis, gastritis, edema, and stroke in Asian countries.¹⁻¹ Recent experimental studies have illustrated some of the diverse biological functions of *S. commixta*, such as its anti-inflammatory,⁴ antioxidant,⁵ and hepatoprotective⁶ activities. In particular, the extract of *S. commixta* was reported to possess vasorelaxant⁷ and anti-atherosclerotic effects¹ with regard to cardiovascular activity. However, the active constituents and molecular mechanisms responsible for these properties are unknown.

The current study investigated the effect of *S. commixta* on thrombosis based on traditional literature and empirical data, as well as recent studies implicating its cardiovascular protective activities.¹,⁷,⁸ Thrombosis is the formation of a blood clot, also known as a thrombus, inside a blood vessel. A thrombus is an abnormal aggregation of blood factors, primarily platelets and fibrin, with entrapment of cellular elements leading to the obstruction of blood flow. Hence, it is one of the major causes of stroke resulting from the shortage of blood supply to the brain. In the present study, an extract was prepared from the fruit of *S. commixta* and tested for its antithrombotic activity in an animal model. Additionally, its impact on platelet aggregation may be attributed to the inhibition of platelet aggregation.

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aggregation and coagulation of clotting factors was examined in vitro.

Results

Antithrombotic Properties of S. commixta Fruit Extract

A hot water extract of S. commixta fruit was prepared and examined for its antithrombotic effect in a ferric chloride (FeCl₃)-induced arterial thrombosis model. The prepared extract was orally administered to mice by feeding a diet containing 0.83% extract for 4 weeks, corresponding to the administration of approximately 1000 mg extract/kg/day based on the measured consumption of ~120 g feed/kg/day. Vascular occlusion was induced by applying FeCl₃-soaked filter paper to the surface of the carotid artery. The arterial occlusion time was significantly prolonged from 11.6 ± 1.0 to 18.4 ± 5.5 min after treatment with the extract (Figure 1A). An intraperitoneal injection of 30 mg/kg of the antiplatelet drug, clopidogrel, used as a positive control, effectively inhibited thrombosis. The tail bleeding time was examined to test the effect on physiological hemostasis. Unlike the effect on thrombosis, the extract exhibited a minimal influence on tail bleeding time (Figure 1B). These results indicate that the S. commixta extract is capable of preventing thrombosis, with minimal impact on normal hemostasis.

Inhibitory Effect of S. commixta Extract on Thrombin-induced Platelet Aggregation

The effect of the extract on platelet aggregation was examined to test whether the antithrombotic activity was associated with platelets. Washed platelets (WP) were prepared from whole blood of rats and incubated with the extract for 5 min. Aggregation was induced by 0.14 U/mL thrombin, which was the minimal concentration required to induce submaximal aggregation. Consistent with the in vivo thrombosis results, the extract inhibited platelet aggregation, which was concentration-dependent in the range of 10 to 100 μg/mL (Figure 2A). When the treatment duration was increased to 30 min, the aggregation inhibitory effect was sustained, without significant loss of efficacy (Figure 2B). The extract did not affect the aggregation induced by collagen (Figure 2C), whereas ADP-induced aggregation was slightly enhanced by the extract (Figure 2D). Thus, the anti-aggregatory effect of the extract appeared to be specific against aggregatory stimuli including thrombin.

Effect of S. commixta Extract on Plasma Coagulation

In addition to platelet aggregation, plasma coagulation was tested as another major player in thrombosis. Blood plasma was pretreated with 100 μg/mL extract for 5 min, and its prothrombin time (PT) and activated partial thromboplastin time (aPTT) were measured. PT and aPTT represent the activities of the extrinsic and intrinsic pathways in the coagulation cascade, respectively. The extract did not directly affect either PT or aPTT (Figure 3A). For a positive control, plasma was treated with 0.4 IU/mL heparin for 5 min, which significantly prolonged PT and aPTT.

Given that the extract was effective against thrombin-induced aggregation, its effect on thrombin activity was examined to test whether the extract inhibited the proteolytic activity of thrombin, thereby, attenuating thrombin-induced aggregation. The extract was exposed to the reaction solution containing 1.0 U/mL thrombin for 5 min, and the thrombin activity was assessed using S-2238, a chromogenic substrate for thrombin. The presence of 100 μg/mL extract did not affect the thrombin activity (Figure 3B), suggesting that its antithrombotic activity was due to its effect on platelets rather than direct inhibition of thrombin. A serine protease inhibitor phenylmethylsulfonyl fluoride (PMSF), used as a positive control, successfully inhibited thrombin activity at 10 mM.

Discussion

This study investigated the antithrombotic effect of S. commixta, which is the basis for its cardiovascular protective activity. The dietary intake of S. commixta fruit extract led to the attenuation of injury-induced arterial thrombosis in an animal model. The extract inhibited platelet aggregation by thrombin, an endogenous platelet agonist, while no direct effect was observed on plasma coagulation. A few previous experimental studies have reported the anti-atherosclerotic and vasorelaxant effects of S. commixta. In accordance with these studies, the current investigation suggests that S. commixta has a potential preventive effect against cardiovascular events.

Platelet aggregation and plasma coagulation are 2 major events resulting in thrombosis. Their dysregulation ultimately leads to the excessive or unregulated formation of thrombi. An extract of S. commixta attenuated platelet aggregation, but exhibited minimal effects on plasma coagulation. Accordingly, its antithrombotic activity could be due to its effects on platelets, although mechanisms other than its anti-aggregatory activity cannot be excluded. Given that the extract was only effective against thrombin despite various agonists being tested, its working mechanism is likely to be specific to the signaling pathways activated by thrombin. Further studies are necessary to clarify the molecular mechanisms underlying the antiplatelet activity of the extract.

As with many experimental studies, it is difficult to justify the clinical relevance of the dose and the concentration tested in this study only with the current results. S. commixta fruit has long been consumed as a food and for medicinal purposes. It is usually eaten as preserved or processed food rather than raw fruit or is taken as a decoction for medical use. The dose of S. commixta fruit extract fed to mice was approximately 1000 mg/kg/day in this study. According to allometric scaling for dose conversion from mice to humans, this dose corresponds to the human equivalent dose of ~80 mg/kg/day. The extraction yield was approximately 16.0% (Materials and
acid, and other phenolics, such as lignans. Some of these are derivatives of hydroxycinnamic acid and hydroxybenzoic acid. Indeed, S. commixta was reported to contain flavonoids such as flavone and flavane derivatives, phenolic acids including the derivatives of hydroxycinnamic acid and hydroxybenzoic acid, and other phenolics, such as lignans. Some of these compounds were found to exhibit various biological activities, including antioxidant (catechin-7-O-β-D-xylopyranoside, catechin-7-O-β-D-apiofuranoside, neochlorogenic acid, cryptochlorogenic acid, and chlorogenic acid), anti-diabetic (lupeol and lupenone), anti-inflammatory (β-sitosteryl-3-O-β-glucopyranoside and (9S,12R,13R)-(E)-9,12,13-trihydroxy-10-octadecaenoic acid), anti-viral (sakuranetin), and neuroprotective (sorcomic acid) effects. Among these compounds, chlorogenic acid and lupeol are reported to show antiplatelet activity. Chlorogenic acid inhibits platelet aggregation by ADP and collagen. Lupeol is effective against collagen, but not against either ADP or arachidonic acid. Given that the S. commixta fruit extract inhibited aggregation by thrombin, but not by either collagen or ADP (Figure 2), the antiplatelet effects of the S. commixta fruit extract are likely to be different from those of chlorogenic acid or lupeol. Therefore, chlorogenic acid or lupeol may not be the active compounds in our study. Furthermore, the effective dose of chlorogenic acid against arterial thrombosis was 200 mg/kg via intraperitoneal injection. According to our analysis, the chlorogenic acid content was estimated to be 0.19% of the extract (Materials and Methods). This indicates that the dose of 1000 mg extract/kg/day must be equivalent to 1.9 mg chlorogenic acid/kg/day, which is considerably lower than its effective dose. Therefore, the inhibition of thrombus formation by our extract must be attributed to other active ingredients, warranting further study to identify them.

The conclusions we drew from our findings are that the dietary intake of S. commixta is likely to prevent cardiovascular events through its antithrombotic effects, although the clinical validity of its antithrombotic activity remains to be assessed. The inhibition of platelet aggregation might contribute to such antithrombotic effects, although further studies are needed to identify the active constituents and elucidate the underlying mechanisms.

Methods). Hence, the dose in this study is estimated to be approximately 500 mg dried fruit/kg/day for human consumption. Medicinal herbs are usually administered in the form of decoctions obtained from crude extracts in traditional Korean medicines. Decoctions are commonly taken 3 times a day, each dose of 80 to 100 mL, which generally represents 6 to 30 g extract/day (=100-500 mg/kg/day). The human equivalent dose of ~80 mg/kg/day in this study is not higher than the general dose of decoction. Despite the possibility of an error in these estimations, even this rough calculation shows that the dose in this study may not be too unrealistic to ignore scientific merit of the findings of this study. Indeed, the validity of our findings is verified, given that the tested dose was chosen solely to examine the effects of the extract, suggesting that the extract is likely to be effective at lower doses. The effective concentration in the in vitro platelet experiments was several tens of µg/mL; however, the in vivo validity of these results is uncertain because the active constituents of the extract and, therefore, their pharmacokinetic information are unknown. Therefore, it would be worthwhile identifying the active ingredients in this extract that exert the antithrombotic effect. Such information would provide insight into the pharmacokinetic properties and the effective dosage of the S. commixta extract, as well as the mechanisms underlying its antithrombotic activity.

The chemical composition of the genus Sorbus has been studied extensively and a number of compounds have been identified. The most thoroughly investigated substances are phenolic compounds such as flavonoids and phenolic acids. Indeed, S. commixta was reported to contain flavonoids such as flavone and flavane derivatives, phenolic acids including the derivatives of hydroxycinnamic acid and hydroxybenzoic acid, and other phenolics, such as lignans. Some of these compounds were found to exhibit various biological activities, such as...
Materials and Methods

Reagents
Thrombin, ferric chloride, chlorogenic acid, PMSF, and heparin were purchased from Sigma-Aldrich. Collagen and S-2238 were acquired from Chrono-log and Chromogenix, respectively. Hematologic reagents for PT and aPTT measurements were from Fisher Diagnostics. Pentobarbital sodium was obtained from Hanlim Pharm. All other chemicals used were of the highest purity available and were purchased from standard suppliers.

Preparation of S. commixta Fruit Extract
The S. commixta fruit extract was prepared as described previously.16 Briefly, S. commixta fruit was collected from the Arboretum of the Korea Forest Service in October 2012 (Lot No. 20120316) and authenticated by Dr Chun-Soo Na (Lifetree Biotech). The air-dried fruit was extracted in 90 to 100 °C water (1:10, w/v) for 4 h and filtered. The filtered extracts were concentrated to over 15% solid content using a vacuum evaporator. Next, the extracts were freeze-dried, resulting in a final yield of approximately 16.0%. The final extract was a dark brown powder, which was kept at 4 °C until use. For the experiments, the powder was dissolved in distilled water and re-filtered through a 0.2 μm membrane (Millipore). Based on a previous report,17 the chlorogenic acid content in the extract was analyzed as a reference material.

UPLC-UV-MS Analysis of Chlorogenic Acid in the S. commixta Fruit Extract
Chlorogenic acid was analyzed in the extract as a reference material.17 Chromatographic separation of the chlorogenic acid was performed using an ultrahigh-performance liquid chromatography (UPLC) system coupled with an ultraviolet (UV) detector and mass spectrometer (MS). The chromatograms were analyzed to determine the chlorogenic acid content in the extract.
acid was performed on an ACQUITY ultra performance liquid chromatography (UPLC) system, equipped with a BEH C18 column (2.1×150 mm, 1.7 μm) and a diode array detector (DAD; Waters). The sample injection volume was 2 μL. The mobile phase consisted of 0.2% acetic acid in water (A) and acetonitrile (B) and was delivered at a flow rate of 0.3 mL/min according to the following gradient elution program: 5→10% (B, v/v) in 10 min, 10→70% (B) in 20 min, 70→100% (B) in 10 min, 100% (B) isocratic for 3 min, 100→5% (B) in 0.5 min, and 5% (B) isocratic for 4.5 min as post-run for reconditioning. The column temperature was maintained at 45 °C.

The analysis was conducted using a Xevo G2-XS quadrupole time-of-flight mass spectrometer, equipped with electrospray ionization (Q-TOF-ESI MS; Waters) in the negative ion mode. Capillary and cone voltages were set at 3.0 kV and 40 V, respectively. Gas flow rates for cone and desolvation gas (nitrogen) were 30 and 500 L/h, respectively. Source temperature and desolvation temperature were set to 110 and 450 °C, respectively. Mass spectral data were recorded in the range of 50 to 1500 m/z.

Quantitative Analysis of Chlorogenic Acid by UPLC-ESI-MS

Chlorogenic acid and the stock solution of the extract were prepared by dissolving the accurately weighed substances in a methanol–water mixture (50:50, v/v). The stock solution of chlorogenic acid was diluted to appropriate concentrations in the ranges of 2.5 to 20 μg/mL (2.5, 5, 10, 15, 20 μg/mL) to establish a calibration curve, which was constructed by plotting the peak areas versus chlorogenic acid concentrations. The equation of the chlorogenic acid calibration curve was \( y = 725.66x + 366.36 \) \( (R^2 = 0.9920) \). The chlorogenic acid content was estimated to be 0.19% of the extract.

Experimental Animals

Male ICR mice and Sprague–Dawley rats (5-6 weeks old) were purchased from Daehan Biolink and acclimated for 1 week before experiments. The laboratory animal facility was maintained at constant temperature and humidity under a 12 h light/dark cycle. Food and water were provided ad libitum.

FeCl3-induced Arterial Thrombosis Model

In vivo antithrombotic effects were evaluated in an arterial thrombosis model, as described previously, with slight modifications. A diet containing 0.83% extract was custom-ordered from Daehan Biolink. Mice were provided with the extract-containing diet for 4 weeks. Diet consumption by the mice was about 120 g/kg body weight/day. Thus, the mice consumed approximately 1000 mg extract/kg body weight/day. After treatment, anesthesia was induced with an intraperitoneal injection of 50 mg/kg sodium pentobarbital. To monitor blood flow, approximately 10 mm of the right carotid artery was exposed and an MA0.7PSB ultrasonic flow probe connected to a TS420 perivascular flowmeter module (Transonic Systems) was placed around the arterial segment. Filter paper (1×1 mm) saturated with 50% FeCl3 was applied to the external surface of the arterial segment proximal to the flow probe. Then, the time needed for occlusion to occur was measured for up to 60 min. For calculation, occlusion time was assigned a value of 60 min for vessels that did not occlude within 60 min. As a positive control, clopidogrel was injected.
intraperitoneally at a dose of 30 mg/kg, 2 h prior to thrombosis induction.

**Tail Bleeding Time Measurement**

Tail bleeding time was examined in a tail transection model, as described previously. Mice were anesthetized with sodium pentobarbital and their tails were transected at a site 3 mm proximal to the tip. The remaining tail was immersed in normal saline at 37 °C, and bleeding was followed visually. The time to attain a stable cessation of bleeding was determined when recurrent bleeding did not occur within 30 s after cessation.

**Preparation of WP and Platelet Aggregation Measurement**

WP were prepared as described previously. Blood was collected from the abdominal aorta of rats anesthetized with diethylether. Acid-citrate-dextrose (ACD; 85 mM trisodium citrate, 66.6 mM citric acid, and 111 mM glucose) was used as an anticoagulant (ACD:blood = 1:6). After centrifugation at 250 \( \times \) g for 15 min, platelet-rich plasma was obtained from the supernatant and further centrifuged at 500 \( \times \) g for 10 min. The resultant platelet sediment was then washed once with washing buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl\(_2\), 0.8 mM Na\(_2\)HPO\(_4\), 10 mM HEPES, 0.55 mM glucose, 22 mM trisodium citrate, and 0.35% bovine serum albumin; pH 6.5) by suspension and centrifugation. WP were prepared by dispersing the platelet sediment in suspension buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl\(_2\), 0.8 mM Na\(_2\)HPO\(_4\), 10 mM HEPES, 5.6 mM glucose, 0.3% bovine serum albumin, and 1 mM CaCl\(_2\); pH 7.4), after which the concentration was adjusted to \( 2 \times 10^8 \) platelets/mL.

Platelet aggregation was examined using a 490-4D optical aggregometer (Chrono-log) and AGGRO/LINK software (Ver. 5.2.3). WP were treated with \( S. \) commixta fruit extract for 5 min, and aggregation was elicited by 0.12 to 0.14 U/mL thrombin (Ver. 5.2.3). WP were treated with NaCl, 2.8 mM KCl, 0.8 mM MgCl\(_2\), 0.8 mM Na\(_2\)HPO\(_4\), 10 mM HEPES, 0.55 mM glucose, 22 mM trisodium citrate, and 0.3% bovine serum albumin; pH 6.5) by suspension and centrifugation. WP were prepared by dispersing the platelet sediment in suspension buffer (138 mM NaCl, 2.8 mM KCl, 0.8 mM MgCl\(_2\), 0.8 mM Na\(_2\)HPO\(_4\), 10 mM HEPES, 5.6 mM glucose, 0.3% bovine serum albumin, and 1 mM CaCl\(_2\); pH 7.4), after which the concentration was adjusted to \( 2 \times 10^8 \) platelets/mL.

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**Plasma Coagulation Test**

Plasma coagulability was assessed by PT and aPTT, as described previously. Blood plasma was incubated with 100 \( \mu \)g/mL \( S. \) commixta fruit extract for 5 min, and PT and aPTT were measured with a Coagulator2 coagulation analyzer (Behnk Elektronik) using either thromboplastin-D or CaCl\(_2\) and APTT-XL reagents (Fisher Diagnostics), respectively, according to the manufacturer’s instructions. Blood plasma treated with 0.4 IU/mL heparin for 5 min was used as a positive control.

**Thrombin Activity Assay**

Thrombin activity was determined using the chromogenic thrombin substrate, S-2238, as described previously. Briefly, 10 \( \mu \)L extract was added to 90 \( \mu \)L working reagent (Tris buffer 50 mM Tris-HCl, 100 mM NaCl, and 0.1% bovine serum albumin, pH 7.4) containing 1.0 U/mL thrombin and 0.2 mM S-2238) in individual wells of a 96-well plate. The increase in absorbance at 405 nm was measured for 10 min using a SpectraMax M3 microplate reader (Molecular Devices). For a positive control, the reaction solution was treated with 10 mM PMSF instead of the extract.

**Statistical Analyses**

Means and standard errors (SE) of means were calculated for all experimental groups. Data were subjected to 1-way analysis of variance, followed by Dunn’s test to identify significant differences relative to the controls. Statistical analyses were performed using the SigmaPlot software ver. 13.0 (Systat Software). \( P<.05 \) was considered statistically significant.

**Acknowledgments**

The authors would like to thank Dr Chun-Soo Na from Lifetree Biotech (Suwon, Korea) for his support in the collection and authentication of \( S. \) commixta.

**Author Contributions**

Minkyang Kim: conceptualization, investigation, methodology, writing—original draft. Jung-Min Park: funding acquisition, investigation, methodology. Young-Mi Kim: investigation, methodology. Young-Won Chir: investigation, methodology, resources, writing—original draft. Moo-Yeol Lee: conceptualization, funding acquisition, investigation; project administration, supervision, writing—original draft, writing—review & editing.

**Declaration of Conflicting Interests**

The authors declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

**Ethical Approval**

All animal experiments were conducted in accordance with protocols approved by the Institutional Animal Care and Use Committee at Dongguk University (IACUC2013015).

**Funding**

The authors disclosed receipt of the following financial support for the research, authorship, and/or publication of this article: This research was funded by the National Research Foundation of Korea (NRF-2018R1A5A2023127), the Dongguk University Research Fund of 2020 (S-2020-G0001-00095) and 2021, and the BK21 FOUR program funded by the Ministry of Education.

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