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Anti-thrombotic effect of rutin isolated from *Dendropanax morbifera* Leveille

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***Dendropanax morbifera* H. Lev. is well known in Korean traditional medicine for improvement of blood circulation. In this study, rutin, a bioflavonoid having anti-thrombotic and anticoagulant activities was isolated from a traditional medicinal plant, *D. morbifera* H. Lev. The chemical characteristics of rutin was studied to be quercetin 3-*O*-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside using high performance liquid chromatography mass spectrometry (HPLC-MS), proton nuclear magnetic resonance (^{1}H NMR) and carbon-13 nuclear magnetic resonance (^{13}C NMR). Turbidity and fibrin clotting studies revealed that rutin reduces fibrin clot in concentration dependent manner. Rutin was found to prolong activated partial thromboplastin time (aPTT), prothrombin time (PT) and closure time (CT). Furthermore, it decreased the activity of pro-coagulant protein, thrombin. *In vivo* study showed that rutin exerted a significant protective effect against collagen and epinephrine (or thrombin) induced acute thromboembolism in mice. These results suggest that rutin has a potent to be an anti-thrombotic agent for cardiovascular diseases.**

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[**Key words:** *Dendropanax morbifera* Leveille; Rutin; Antithrombotic; Thromboembolism; Thrombosis]

The pathophysiological process in these circulatory disorders such as myocardial infarction, stroke, deep-vein thrombosis (DVT), and pulmonary embolism (PE) is the formation of the abnormal blood clot inside the blood vessels. Thrombosis occurs as a result of an activation of thrombin which converts fibrinogen into fibrin, finally resulting in insoluble fibrin clot (or polymer). Fibrin is the major structural component of blood clots, forming a cross-linked network of fibers and plays an important role in the blood coagulation process. Fibrinogen, a precursor of the major protein of clotted blood-fibrin, is a plasma glycoprotein that consists of a pair of three polypeptide chains designated Aα, Bβ and γ (1). Platelets and coagulation factors are the key players in the process of thrombus formation. Thrombus is formed by platelet activation and adhesion is a necessary in the process of hemostasis. Exposures of collagen at vessel on integrin αIIbβ3 and fibrinogen receptor of the platelet surface conducing to platelet adhesion (2). Also, the coagulation cascade is the sequential process of interaction and activation of coagulation factors, resulting in generation of fibrin, the major protein component of thrombus (3). But, excessive thrombus formation could block blood flow resulting in acute circulatory disorders. Currently available agents for circulatory disorders in clinical usage are plasminogen activator (PA) (indirect thrombolytic) and low-molecular-weight heparin (anti-thrombotic) (4,5). Based on the mode of action that the PAs acts upon, they are described as indirect thrombolytic agents and has been using over the last several years in thrombolytic therapy (4). Although, these indirect thrombolytic and anti-thrombotic agents are effective in circulatory disorders, an avoidable risk of bleeding and coagulation limits their uses (4,5).

Plants are abundant source of novel bioactive substances. Isolation and characterization of pharmacologically active compounds from plants continue today. In recent years, anti-thrombotic compounds and agents found in non-animal sources have received considerable attentions because of their safety. Salvianolic acid A from *Salvia miltiorrhiza*, polysaccharide from *Umbilicaria esculenta*, kaempferol, quercetin and tiliroside from *Rubus chingii* leaves possess inhibition activities of thrombus, platelet aggregation and coagulation (6–8). In particular, flavonoids, a large group of naturally occurring compounds, are receiving attention because of their wide range of biological activities (9). Rutin, a quercetin glycoside, is found in a number of plants (10). Previous studies have reported that rutin inhibit platelet aggregation in human platelets stimulated by collagen (11), decrease capillary fragility (12) and prolong aPTT (13). In Republic of Korea, *Dendropanax morbifera* Leveille has long been used as a traditional medicine and healthy food. It is recommended for traditional medicinal preparations to improve blood circulation in original text of Donguibogam (written in the 17th century, Korean). *D. morbifera* extracts have had a history of use in traditional medicine for the treatment of various diseases (14). Nonetheless there is no information on the anti-thrombotic effect of rutin against clotting and thromboembolism model has not yet been reported. Therefore, in present study, we have attempted to isolate rutin from

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D. morbifera and investigated the anti-thrombotic activities of rutin on fibrin and blood clot, coagulation and platelet activation and examined the anti-thrombotic activity on thromboembolism mouse model.

MATERIALS AND METHODS

Materials Silica gel 60 (70–230 mesh) and precoated silica gel 60 F_{254} TLC plates (0.25 mm) were purchased from E. Merck (Darmstadt, Germany). Highly-porous synthetic resin Diaion HP-20 was purchased from Mitsubishi Chemical Co., Ltd. (Tokyo, Japan). Human fibrinogen, thrombin, factor X activated, collagen VII, epinephrine and dimethyl sulfoxide (DMSO) were purchased from Sigma–Aldrich (St. Louis, MO, USA). Paranitroaniline (pNA) chromogenic substrates were obtained from Chromogenix (Milano, Italy). Other reagents used were of analytical grade from commercial sources.

Instrumental analysis UV absorption spectum was recorded on a Beckman DU-70 spectrophotometer. Positive and negative-ion electrospray ionization ESI-MS spectrometry were done on an Agilent 1100. ^{1}H NMR and ^{13}C NMR spectra were recorded on a JEOL FT-NMR spectrometer at 600 MHz and 150 MHz in MeOH-d4, respectively.

Animals Male ICR (Imprinting Control Region), the outbred strain of mice (20–30 g), approximately 4–6 week old was used in the experiments. The mices were housed four per cage. All animals were maintained under controlled environmental conditions (22 ± 2°C, 12 h light/dark cycle). Food (Certified Rodent Diet 5002, Orient Bio, Seongnam, Korea) and tap water were available ad libitum. All efforts were made to minimize animal suffering and to reduce the number of animals used. All experimental procedures were performed in accordance with the NIH Guide for the Care and Use of Laboratory Animals (15) and the Guiding Principles for the Care and Use of Animals approved by the internal Ethics Committee of Chosun University.

Plant material Fresh leaves of *D. morbifera* were collected from Wando-Island, Republic of Korea in the month of May and authenticated by Professor Myung-Kon Kim, Department of Bio-food technology, Chonbuk National University, South Korea. A voucher specimen was deposited at Chonbuk National University. The leaves were dried at room temperature and ground into fine powder.

Extraction and isolation of active compound The powdered leaves of *D. morbifera* (500 g) were extracted with 80% ethanol (3 L × 3) at room temperature to yield the crude extract (135 g) after filtration and solvent evaporation using a rotary evaporator. The extract (135 g) dissolved in H_2O (500 mL), was subjected to porous synthetic resin (Diaion HP-20) column chromatography (5 cm × 50 cm) eluted with a stepwise-gradient, in sequence, of H_2O (1 L), methanol-H_2O (1:4, 1 L), (2:3, 1 L), (3:2, 1 L), (4:1, 1 L), and MeOH (1 L) to give 24 fractions of 300 mL each. The fractions 12–14 (7.5 g, MeOH–H_2O = 3:2 eluate) were subjected to column chromatography (5 × 50 cm) on silica gel (300 g), eluted with $CHCl_3$–MeOH–H_2O (80:20:2, 2.0 L), $CHCl_3$–MeOH–H_2O (70:30:3, 2 L) and $CHCl_3$–MeOH–H_2O (65:35:10, lower phase, 2 L), 50 mL fractions being collected to give 120 fractions. The fraction 81–93 (3.7 g) was subjected to column chromatography (4 cm × 35 cm) on silica gel (200 g), eluted with $CHCl_3$–MeOH–H_2O (65:35:10, lower phase, 2 L) to give 8 sub-fractions (A-H). The sub-fraction H (1.24 g) was subjected to re-chromatography (4 cm × 40 cm) on silica gel (200 g) with the solvent system $CHCl_3$–MeOH–H_2O (65:35:10, lower phase) to give active compound (762 mg). Active compound was obtained as pale yellow powder with UV (MeOH) λ_{max} 257.5 and 357.0 nm. The chemical structure of active compound were identified according to HPLC-MS, ^{1}H NMR and ^{13}C NMR spectra. HPLC-MS (positive mode): *m/z* 610.8 $[M+H]^+$, 534.8 $[M+H\text{-glucose}]^+$, 302.8 $[M+H\text{-glucose-rhamnose}]^+$, negative mode: 609.0 $[M-H]^-$. From these results, molecular weight of main compound was estimated as 610 ($C_{27}H_{30}O_{16}$). ^{1}H NMR (600 MHz, MeOH-d_4): δ8.29 (1H, dd, J = 2.4, H-6′), 8.06 (1H, dd, J = 8.1, 1.8, H-2′), 6.60 (1H, d, J = 2.4 Hz, H-5′), 6.56 (1H, d, J = 1.2 Hz, H-8), 5.93 (1H, d, J = 7.2 Hz, H-6), 5.86 (1H, d, J = 7.8 Hz, H-1″), 5.18 (1H, d, J = 1.8 Hz, H-1‴), 1.44 (3H, d, J = 5.4 Hz, H-6‴). ^{13}C NMR chemical shifts (150 MHz, MeOH-d_4): See in Supplementary Table S1. From these results, main compound was identified as rutin (quercetin 3-*O*-α-L-rhamnopyranosyl-(1-6)-β-D-glucopyranoside) (Supplementary Fig. S1).

Turbidity assay The inhibition activity of fibrin clotting was determined by turbidity assay (16). The assay was performed in 96-well plates at room temperature (RT). Initially, the reaction mixtures were prepared by addition of fibrinogen (2.9 μM), thrombin (0.5 U/mL) and $CaCl_2$ (5 mM) in 20 mM Tris–HCl (pH 7.4) with rutin (1, 5, 10, 20 and 50 μg) in a volume of 100 μL at 37°C for 1 h. Immediately, turbidity was monitored every 1 min at 405 nm for 80 min in a microplate reader (Molecular Devices, Sunnyvale, CA, USA). After reaction, the density of clots was presented as a percentage of inhibition.

Fibrin clotting assay The inhibition activity of fibrin clotting was determined by fibrin clotting assay. Initially, the fibrin clot solution was prepared by addition of 1.5% fibrinogen (90 μL), thrombin (0.5 U/mL) and $CaCl_2$ (5 mM) in 20 mM Tris–HCl (pH 7.4) with rutin (5, 10, 25 and 50 μg) in a volume of 100 μL. Then, this solution was incubated at 37°C for 1 h. After reaction, the clots in test tube were photographed and weighted. The density of clots was presented as weight of the remaining clots.

Thrombin and factor X activated assays Thrombin and factor X activated (FXa) activity assays were performed according to the method of Gaspar et al. (17) with slight modification in a 96-well plate. For thrombin activity assay, 20 μL of human thrombin (40 mU) in 20 mM Tris–HCl (pH 7.4) was mixed with 100 μL of varying amounts of rutin and incubated for 15 min at 37°C. Thereafter, 1 mM of chromogenic substrate for thrombin, S-2238 was added to a mixture. After incubation for 1 h 37°C, absorbance was measured at 405 nm. For FXa activity assay, 50 μL of human FXa (20 mU) was pre-incubated with 100 μL of varying amounts of rutin in 20 mM Tris–HCl (pH 7.4) for 15 min at 37°C. To initiate the reaction, chromogenic substrate *N*-benzoyl-Ile-Glu-Gly-Arg-*p*-nitroanilide (1 mM) was added to a mixture and the reactions were followed for 1 h at 37°C and residual activity was measured at 405 nm at room temperature.

***In vitro* antithrombotic activities** Fibrin clots were prepared by mixing human fibrinogen (2.6 μM) and Alexa Fluor 488 fibrinogen (0.4 μM) clotted with 0.5 U/mL of thrombin with saline and/or rutin in 20 mM Tris–HCl (pH 7.4) in glass-bottomed dish. The mixtures were incubated in the dark at 37°C for 1 h. The fibrin clots were observed with fluorescent microscope (Nikon, Eclipse TE 2000-U, Tokyo, Japan) and photographed. Saline-pretreated fibrin clot was used as a control. Also, u-PA-pretreated fibrin clot was used as a positive control.

Blood clot assay was performed as described previously (18) with some modification. Before coagulation, 300 mL of blood sample collected from mice by cardiac puncture was immediately pretreated with 50 μg of rutin with positive control (u-PA, 50 IU) and was incubated for 1 h at 37°C. The clot images were obtained using a Canon IXUS100IS digital camera. The relative density was expressed as a density of the remaining blood clots. Quantitative analysis was performed by Image J 1.46b software (Wayne Rasband, NIH, MD, USA).

Collagen and epinephrine (or thrombin)-induced acute thromboembolism model Two models, collagen and epinephrine and/or thrombin induced thromboembolism were performed in male ICR mice according to the methods of DiMinno and Silver (19) and Gomi et al. (20). Five experimental groups of twenty animals per group were studied. Group 1 was treated with saline, group 2 was treated with collagen (250 μg/mL) + epinephrine (150 μg/mL) and/or human thrombin (3300 NIH U/mg), group 3 was treated with aspirin (20 mg/kg) + mixture of collagen and epinephrine or human thrombin, group 4 and 5 were respectively given 8 mg/kg and 16 mg/kg rutin + mixture of collagen and epinephrine or human thrombin. One hour before the thrombotic challenge, mice were injected intraperitoneally with aspirin and rutin. Then, mice were injected rapidly with 0.1 mL of the mixture of collagen and epinephrine into the tail vein. The mortality or paralysis (loss of function of the hind limbs for more than 15 min) of mice was recorded for 15 min and all surviving mice were sacrificed immediately after the experiment.

Recalcification time assay Recalcification time assay was performed according to the method of Ribeiro et al. (21) with slight modification in a 96-well plate. For recalcification time assay 30 μL of human platelet-poor plasma was mixed with 30 μL of rutin (5,10,20 and 50) dissolved in DMSO in 96-well plates and incubated for 10 min at 37°C. Thereafter, 30 μL of 25 mM prewarmed $CaCl_2$ was added to the mixture and absorbance was taken every 30 s at 650 nm for 30 min in a microplate reader. The clotting time was presented as reaching a 0.1 absorbance value.

Coagulation assays The effect of rutin on activated partial thromboplastin time (aPTT) and prothrombin time (PT) were evaluated on coagulometer (Thrombostat 1, Behnk Elektronik GmbH & Co, Germany) using reagents from Fisher Diagnostics (Middletown, USA) according to the manufacturer's instructions. Rutin and heparin were added to the plasma in concentrations of 5, 10, 20, 40 and 50 μg and 1 U/mL.

Platelet function assay This assay measures platelet aggregation/activation and adherence in whole blood under high shear stress flow conditions. The effect of rutin on platelet function was analyzed by platelet function analyzer (PFA-100) with collagen/epinephrine cartridge (Dade Behring, Marburg, Germany). Blood samples were drawn from healthy donors in 3.8% (w/v) sodium citrate. Aliquots of whole blood were treated with different concentrations of rutin and incubated for 5 min at RT. Blood samples were then loaded into a collagen/epinephrine cartridge and the time required for occlusion of the aperture was measured by machine and reported as closure times (CT). Untreated blood was used as a control.

Hemostasis assessment Tail bleeding time was measured by using the tail-transection test as described earlier (22). Mice were anesthetized as described above and the tested samples (u-PA (4000 IU/kg) and rutin (16 mg/kg)) were injected intravenously in the tail veins of mice. After 30 min, mice were placed on a 37°C heating pad and the distal 2.5 mm segment of the tail was transected. The tail was immediately immersed in a tube containing warmed saline (37°C). The bleeding time was recorded for 30 min. Hemorrhagic activity was examined by the method of Song et al. (23) with some modifications. Briefly, mice were anesthetized as described above and injected with rutin (16 mg/kg) subcutaneously into the dorsal skin of mice. After 2 h, the mice were sacrificed and the skin was removed to observe the hemorrhagic spot. Saline solution was used as negative control.

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