Extract of *Ulmus macrocarpa* Hance prevents thrombus formation through antiplatelet activity

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Received March 4, 2013; Accepted June 20, 2013

DOI: 10.3892/mmr.2013.1581

**Abstract.** *Ulmus macrocarpa* Hance (Ulmaceae) has been used as a traditional oriental medicine for the treatment of edema, mastitis, gastric cancer and inflammation. The aim of this study was to investigate the effects of *Ulmus macrocarpa* extract (UME) on thrombus formation *in vivo*, platelet activation *ex vivo* and fibrinolytic activity *in vitro*. To identify the antithrombotic activity of UME *in vivo*, we used an arterial thrombosis model. UME delayed the occlusion time by 13.4 and 13.9 min at doses of 300 and 600 mg/kg, respectively. UME significantly inhibited *ex vivo* platelet aggregation induced by collagen and adenosine 5'-diphosphate (ADP), respectively, but did not affect the coagulation times following activated partial thromboplastin and prothrombin activation. Therefore, to investigate the antiplatelet effect of UME, the effect of UME on collagen and ADP-induced platelet aggregation *in vitro* was examined. UME exhibited antiplatelet aggregation activity, induced by ADP and collagen. Furthermore, the fibrinolytic activity of UME was investigated. The results showed that UME significantly increased fibrinolysis at 1,000 mg/ml. In conclusion, the results suggested that UME may significantly inhibit artery thrombus formation *in vivo*, potentially due to antiplatelet activity, and also exhibits potential as a clot-dissolving agent for thrombolytic therapy.

**Introduction**

Thrombus is the main cause of atherothrombotic disease, which itself is the primary cause of increased morbidity worldwide. Therefore, inhibiting platelet function is a promising approach for preventing thrombosis (1,2). Injury to the blood vessel wall triggers rapid platelet activation and platelet plug formation, followed by blood coagulation and the formation of fibrin-containing thrombins that occlude the site of injury. These events limit vital blood loss at the site of injured tissue, but may also block narrow or diseased vessels, leading to ischemia and/or tearing of vital organs (3,4). Furthermore, platelet aggregation and subsequent thrombus formation in coronary and cerebral arteries may cause myocardial infarction and stroke, respectively. Platelets are necessary in thrombus formation in injured blood vessels (5). Thus, excessive platelet aggregation produces a pathological thrombus and plays a significant role in the initiation and pathogenesis of atherothrombotic diseases.

A thrombus is composed of fibrin, which is produced from its precursor, fibrinogen, by thrombin. An inappropriate fibrin clot must be eliminated rapidly by fibrinolysis in order to maintain homeostasis (6). Dissolution of a fibrin clot is dependent on the action of plasmin, a serine protease that is activated by a tissue plasminogen activator. In clinical therapy, thrombolytic agents (fibrinolytic enzymes), such as tissue plasminogen activator and urokinase, convert inactive plasminogen to active plasmin, allowing fibrinolysis to occur (7).

Antiplatelet agents, including aspirin, thienopyridines and platelet glycoprotein (GP)IIb/IIIa receptor inhibitors, have been extensively researched and developed as potential therapies for the treatment and prevention of cardiovascular disease. However, these reagents have several clinical disadvantages including gastrointestinal side-effects and hemorrhage (8-11).

The medicinal plant *Ulmus macrocarpa* Hance (Ulmaceae) is a deciduous tree, widely distributed in Korea (12). The stem and root bark of *Ulmus macrocarpa* (Hance) have been used as an oriental traditional medicine for the treatment of edema, mastitis, gastric cancer, and inflammation. It has been reported that *Ulmus macrocarpa* Hance, of the Ulmaceae family, exhibits marked anti-oxidative activity on lipid peroxidation and an inhibitory effect on endogenous nitric oxide (NO)-induced apoptotic cell death (13). However, the antiplatelet and anticoagulant effects of *Ulmus macrocarpa* have not been reported previously. In this study, the inhibitory activities of *Ulmus*...
Ulmus macrocarpa extract (UME) on platelet aggregation in vitro and ex vivo were examined. The fibrinolytic effects of UME were also investigated. The antithrombotic activity of UME in a ferric chloride (FeCl₃)‑induced arterial thrombosis rat model was also investigated.

Materials and methods

Materials and animals. Collagen and adenosine 5'‑diphosphate (ADP) were purchased from Chrono‑Log Co. (Havertown, PA, USA). Aspirin, fibrinogen, thrombin, plasmin and DMSO were purchased from Sigma‑Aldrich (St. Louis, MO, USA). Thromboplastin and calcium chloride were purchased from Instrumentation Laboratory Co. (Milan, Italy). Other chemicals were of analytical grade. The standard marker compounds, (+)‑catechin and (‑)‑epicatechin were purchased from Sigma‑Aldrich (purity ≥98%). HPLC‑grade reagents, methanol and water were obtained from J.T. Baker (Phillipsburg, NJ, USA).

Male Sprague‑Dawely rats weighing between 240‑250 g were obtained from Orient Co. (Seoul, Korea) and maintained in a standard laboratory animal facility with free access to feed and water and were allowed to acclimatize for at least two weeks prior to the start of the study. The animal studies were carried out in accordance with the Korea Institute of Oriental Medicine Care Committee Guidelines.

UME preparation. Ulmus macrocarpa was obtained as a dried herb from JungDo Co. (Seoul, Korea) and was authenticated, based on its microscopic and macroscopic characteristics, by the Classification and Identification Committee of the Korea Institute of Oriental Medicine. The 70% ethanol extract from Ulmus macrocarpa was made according to the following procedure. The roots of the plant were boiled twice at 70‑80°C each time for 2 h. The filtrates were collected, concentrated and evaporated to dryness at 40‑50°C under vacuum. The dosage of the extract was indicated in the powdered form. The powder was dissolved in 20 or 50% DMSO (v/v, in PBS) for the experiments.

HPLC analysis. HPLC analysis was performed on a Waters 2695 system (Waters Co. Milford, MA, USA), consisting of a solvent delivery unit, an online degasser, an autosampler and a photodiode array detector. A Luna C18 column (250 x4.6 mm; particle size 5 µm, Phenomenex, Torrance, CA, USA) was used for analysis and the mobile phase was water (A) and MeOH (B). The gradient flow was as follows: (A)/(B) = 10/90 (0 min) → (A)/(B) = 40/60 (30 min) → (A)/(B) = 0/100 (32 min; hold for 5 min) → (A)/(B) = 10/90 (38 min; hold for 12 min). The flow rate was 1.0 ml/min, the injection volume was 10 µl and the detection wavelength was 280 nm.

A standard stock solution mixture of (+)‑catechin and (‑)‑epicatechin were prepared in 70% EtOH at a concentration of 1,000 µg/ml, respectively, and stored at 4°C. UME powder (500 mg) was transferred to a 100 ml volumetric flask and dissolved up to the 100 ml mark with methanol. The mixture was subsequently sonicated for 30 min at room temperature and filtered through a 0.2 µm membrane filter. Calibration of the standard mixture was prepared by serial dilution of the standard stock solution mixture to yield concentrations of 10‑1,000 µg/ml for (+)‑catechin and (‑)‑epicatechin. These standard component solutions at five different concentrations were injected in triplicate. Calibration curves were generated by plotting the peak areas vs. the concentrations of the two components.

Arterial thrombus formation in vivo. Arterial thrombus formation in vivo was investigated as previously described (14). UME was orally administered on a daily basis at doses of 300 and 600 mg/kg for 3 days. The rats were fasted overnight, and then orally administered the extract or the 1% carboxymethylcellulose (CMC) solution as a vehicle. The rats were i.p. anaesthetized with 60 mg/kg pentobarbital sodium salt and placed on a heat source. A segment of the right carotid artery was isolated and dissected free of the vagus nerve and surrounding tissues. Aortic blood flow was measured with a Doppler velocimeter (ADInstruments, Colorado Springs, CO, USA). Arterial thrombus formation was induced by wrapping a 2 mm² Whatmann no. 1 filter paper saturated with 50% FeCl₃ around the carotid artery near the probe for 10 min. The time required for occlusion was measured for up to 60 min. An occlusion time of 60 min was assigned for vessels that did not occlude within 60 min.

Platelet aggregation and coagulation times ex vivo. Ex vivo platelet aggregation was investigated as previously described (14). The rats (n=7) were orally administered UME at a dose of 300 mg/kg for 3 days. Platelet‑rich plasma (PRP) was obtained by centrifuging the blood sample at 180 x g for 10 min, and platelet poor plasma (PPP) was obtained by centrifuging the PRP at 2,100 x g for 10 min continuously. PRP was adjusted to 4x10⁸ platelets/ml with PPP. Platelet aggregation was measured with an aggregometer (Chrono‑Log Co.), and collagen (5 µg/ml) and ADP (5 µM) were used as aggregation stimulators. The plasma‑activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured with an Automated Coagulation Laboratory 7000 Instrument (Instrumentation Laboratory) as previously described (14). PPP was incubated at 37°C for 7 min, and subsequently 100 µl of incubated plasma was mixed with 50 µl of cephalin in the process plate. Coagulation was initiated by the addition of CaCl₂ plus 100 µl thromboplastin or 100 µl polybrene for the APTT and PT assays, respectively.

Platelet preparation and aggregation in vitro. PRP was obtained by centrifuging the blood samples at 180 x g for 10 min, and PPP was obtained by centrifuging the PRP at 2,100 x g for 10 min. PRP was adjusted to 4x10⁸ platelets/ml with PPP. The number of platelets was counted using a Coulter Counter (Coulter Electronics, Inc., Hialeah, FL, USA).

Evaluating platelet aggregation was undertaken by optical platelet aggregometry in an aggregometer (Chrono‑Log Co.). Aggregation was recorded as the percentage change in light transmission; the baseline value was set using PRP and the maximal transmission using PPP. They were incubated at 37°C for 4 min in the aggregometer. Following incubation, platelet aggregation was induced by the addition of collagen (5 µg/ml) or ADP (5 µM). The inhibition of platelet aggregation for each sample was expressed as the percentage decrease...
in the maximal transmittance compared with the stimulated control.

**Fibrinolytic activity assay in vitro.** Fibrinolytic activity was evaluated according to the method of Astrup and Mullertz with minor modifications (15). The fibrin plate was prepared by mixing 0.6% human fibrinogen solution (Sigma-Aldrich) in 1X PBS (pH 7.4) with 50 NIH units of human thrombin. In order to speed up the clotting process, the plates were incubated at 37°C for 30 min. Plates were prepared fresh each time. UME (250, 500 and 1,000 mg/ml), standard solutions (plasmin) and control solutions were placed separately onto membrane discs in the fibrin plate. We used 20% DMSO in PBS as a control. The plates were incubated at 37°C for 15 h. The diameters of the transparent rings were measured with calipers (Mitutoyo Co., Tokyo, Japan), and subsequently the fibrinolysis area (mm²) was calculated. The mean diameter of the hydrolyzed clear zone was measured and volume of lysis caused by each sample was calculated.

**Statistical analysis.** Experimental results were expressed as the means ± SD. Differences between the groups were used for multiple comparisons followed by an unpaired Student's t-test. P<0.05 was considered to indicate a statistically significant difference.

**Results**

**HPLC analysis.** We employed HPLC to confirm and quantify the standard contents of UME, such as catechin and epicatechin, for standardization and quality control. The representative chromatogram at 280 nm reveals that UME contained two marker compounds, catechin and epicatechin whose peaks were retained at 18.55 and at 24.83 min, respectively (Fig. 1). Correlation coefficients were better than 0.999 for all standard components. The limits of detection (LOD) and quantification (LOQ) for both components were 1.5 and 5.0 µg/ml, respectively. The catechin and epicatechin levels in UME were quantified as 1.397 and 0.069%, respectively.

**Effect of UME on arterial thrombus formation in vivo.** The effect of UME on arterial thrombus formation in vivo was evaluated by using the FeCl₃-induced rat carotid artery injury model. Following the application of 50% FeCl₃, injured vessels of the control group occluded within 21.0 ± 5.7 min (n=7). Oral UME treatment for 3 days significantly extended the occlusion times to 13.4 ± 8.0 (n = 7) and 13.9 ± 13.6 min (n = 7) for doses of 300 and 600 mg/kg, respectively (Fig. 2).

**Effect of UME on platelet aggregation and coagulation times ex vivo.** UME significantly inhibited ADP- and collagen-induced platelet aggregations ex vivo (Fig. 3A). Collagen- and ADP-stimulated aggregations were inhibited by 18.0±10.0 and 8.0±9.4% at 300µg/kg, and 24.7±7.3 and 9.3±3.3% at 600µg/kg, respectively (n=7). The APTT and PT of the control group were 17.5±1.7 and 14.7±10.0 sec, respectively. UME treatment did not significantly alter APTT and PT, which were 17.3±0.9 and 15.1±0.7 sec at 600 mg/kg, respectively. These data indicate that UME significantly inhibits platelet aggregation but does not affect the coagulation system. Aspirin (20 mg/kg), was used as a positive control and completely blocked collagen-induced aggregation.

**Effect of UME on platelet aggregation in vitro.** UME significantly inhibited collagen-(5 µg/ml), and ADP (5 µM)-induced platelet aggregations in vitro in a concentration-dependent manner, with IC₅₀ values of 915.7±4.6 and 833.3±2.5 µg/ml, respectively (Fig. 4). This result supports the anti-platelet effect on platelet aggregation ex vivo.

**Effect of UME on fibrinolytic activity in vitro.** In the fibrin plate, fibrinolysis was increased by treatment with UME in a dose-dependent manner (Fig. 5). UME induced fibrinolysis at 1,000 µg/ml, compared with the control group (2.2±0.4 vs. 0.0±0.0 mm²), respectively.
Discussion

Blood flow disturbances at sites of atherosclerotic plaque rupture promote platelet activation and arterial thrombus formation (16,17). In the present study, UME significantly inhibited thrombus formation in rat carotid arteries in vivo and platelet aggregation ex vivo, but did not affect coagulation times such as PT and APTT. Therefore, UME has the potential to prevent thrombotic or cardiovascular diseases via antiplatelet, rather than anticoagulation activity.

Thus, we investigated the activities of UME as an antiplatelet and thrombolytic agent. The results indicated that Ulmus macrocarpa may have the potential to prevent thrombotic and cardiovascular diseases, which may be due to its antiplatelet and fibrinolytic effects. Inappropriate thrombus formation is associated with a failure in homeostasis, and the major elements involved are coagulation factors, platelets and blood vessels (18).

We examined the in vivo antithrombotic effect of UME in a FeCl₃-mediated artery thrombosis in a rat model, composed of fibrin, activated platelets and entrapped erythrocytes. This type of thrombus is found in the coronary arteries following sudden mortality and acute myocardial infarction (19,20). In this model, FeCl₃ induces oxidative injury and exposes the subendothelial matrix. Platelets interact with the matrix via GPIb-V-IX and α₂bβ₃ on the platelet membrane, and collagen and vWF in the matrix. Glycoprotein VI binding to collagen is required for platelet activation, and activated platelets undergo calcium mobilization and the release of ADP and TXA₂ to accelerate platelet recruitment and activation for thrombus formation (21). Thus, platelet aggregation contributes to thrombus formation. UME significantly increased the occlusion time in a dose-dependent manner (Fig. 2), which indirectly indicated that UME may inhibit thrombus formation in vivo.

The exact mechanism by which thrombus formation was trig-
gered in this model is unclear, but it has been demonstrated that the morphology of the thrombi is similar to those found in humans (22). Therefore, we examined whether UME affected platelet activity and plasma coagulation using \textit{ex vivo} platelet aggregation and coagulation assays. UME significantly and dose-dependently inhibited collagen- and ADP-induced platelet aggregation but did not affect plasma coagulation times (Fig. 3). Thus, the antithrombotic effect of UME in the artery thrombosis model may result from its antiplatelet rather than its anticoagulation activity (23-25).

UME was found to exhibit antiplatelet aggregation activity, induced by ADP and collagen (Fig. 4). This supports the theory that UME has an anti-platelet effect on platelet aggregation \textit{ex vivo}. Furthermore, we investigated the fibrinolytic activity of UME using plasmin as a positive control. Plasmin was converted from plasminogen, whereupon plasmin degraded the fibrin network in clots (26). In this study, UME dose-dependently increased fibrinolysis in the fibrinolytic assay (Fig. 5).

In conclusion, we demonstrated that UME significantly inhibits artery thrombus formation \textit{in vivo}, which may be due to its antiplatelet activity, and also demonstrated potential as a clot-dissolving agent for thrombolytic therapy. The beneficial effect of UME on the cardiovascular system may be due to its modulation of platelet activation.

Acknowledgements

This study was supported by the ‘Discovery of Herbal Medicine for the Prevention of Prehypertension’ project (K13202) from the Ministry of Education, Science and Technology of Korea.

References