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

WON-KYUNG YANG[1], JUNG-JIN LEE[2], YOON-YOUNG SUNG[1], DONG-SEON KIM[1], CHANG-SEON MYUNG[3] and HO KYOUNG KIM[1,4]

[1]Basic Herbal Medicine Research Group, Korea Institute of Oriental Medicine;
[2]Korea Medicine (KM)-Based Herbal Drug Research Group, Korea Institute of Oriental Medicine;
[3]Department of Pharmacology, Chungnam National University, College of Pharmacy; [4]Herbal Material Management Team, Korea Institute of Oriental Medicine, Daejeon 305-811, Republic of Korea

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*

Correspondence to: Dr Ho Kyoung Kim, Herbal Material Management Team, Korea Institute of Oriental Medicine, 1672 Yuseong-daero, Yuseong-gu, Daejeon 305-811, Republic of Korea
E-mail: hkkim@kiom.re.kr

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 ($FeCl_3$)-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 Whatmanno no. 1 filter paper saturated with 50% $FeCl_3$ 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 $4x10^8$ 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 $CaCl_2$ 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 $4x10^8$ 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 (mm^2) 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 $FeCl_3$-induced rat carotid artery injury model. Following the application of 50% $FeCl_3$, 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 300mg/kg, and 24.7±7.3 and 9.3±3.3% at 600mg/ kg, respectively (n=7). The APTT and PT of the control group were 17.5±1.7 and 14.7±10.0 sec, respectively (Fig.3B). UME treatment did not significantly alter APTT and PT, which were 17.8±0.8 and 15.2±0.9 sec at 300mg/kg, and 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
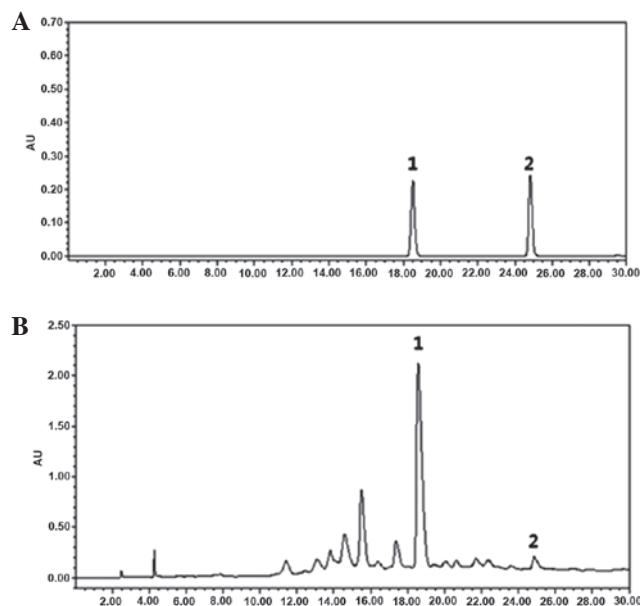

Figure 1. HPLC chromatograms of a standard mixture and the extract of the bark of *Ulmus macrocarpa* (UME). Chromatograms of (A) standard mixture and (B) UME are recorded at 280 nm. Catechin (1) and epicatechin (2) peaks are indicated.
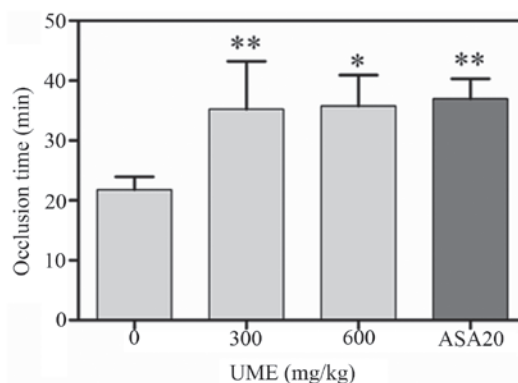

Figure 2. Effect of *Ulmus macrocarpa* extract (UME) on arterial thrombus formation *in vivo*. Following oral administration, the carotid artery was subjected to chemical injury with 50% $FeCl_3$. Blood flow was measured with a doppler velocimeter. Data are expressed as the means ± SD. *$P<0.05$ and **$P<0.01$.

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 IC_{50} 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 mm^2), respectively.
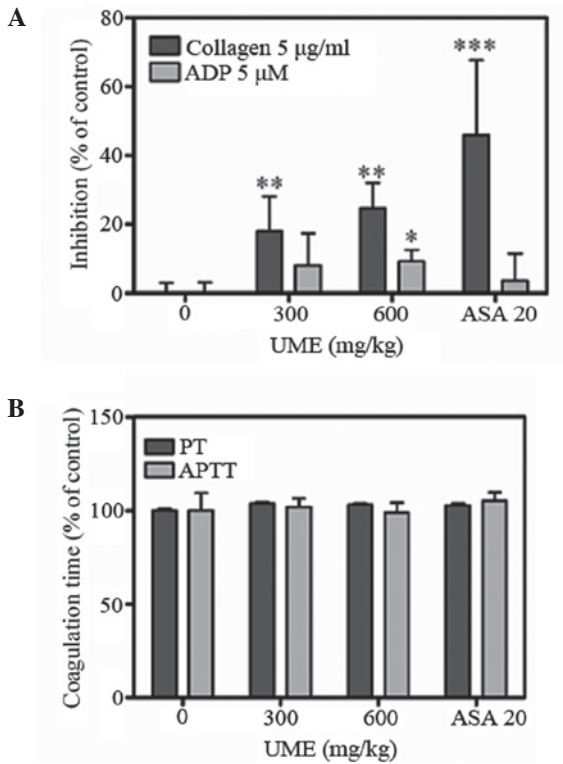
A

B

Figure 3. Effect of *Ulmus macrocarpa* extract (UME) on platelet aggregation and coagulation times *ex vivo*. (A) Platelet aggregation was induced by collagen (5 μg/ml) or adenosine 5'-diphosphate (ADP) (5 μM). In the coagulation assay, platelet poor plasma (PPP) was obtained by centrifuging platelet-rich plasma (PRP), and (B) activated partial thromboplastin time (APTT) and prothrombin time (PT) were measured as described in Materials and methods. Data are expressed as the means ± SD. *P<0.05, **P<0.01 and ***P<0.001.

A

B

Figure 5. Effects of *Ulmus macrocarpa* extract (UME) on fibrinolytic activity. (A) Measurement of fibrinolytic area on UME fibrin plate. (B) Images of UME fibrin plate. Data are expressed as the means ± SD, from three independent experiments. ***P<0.001.
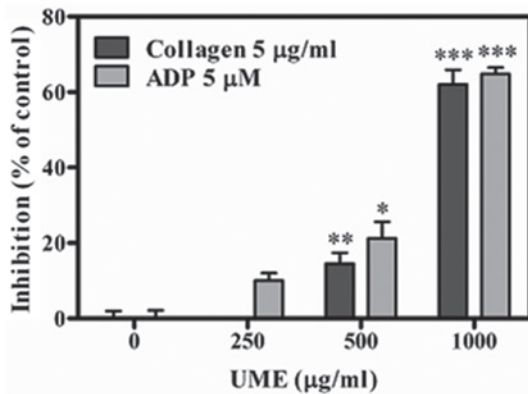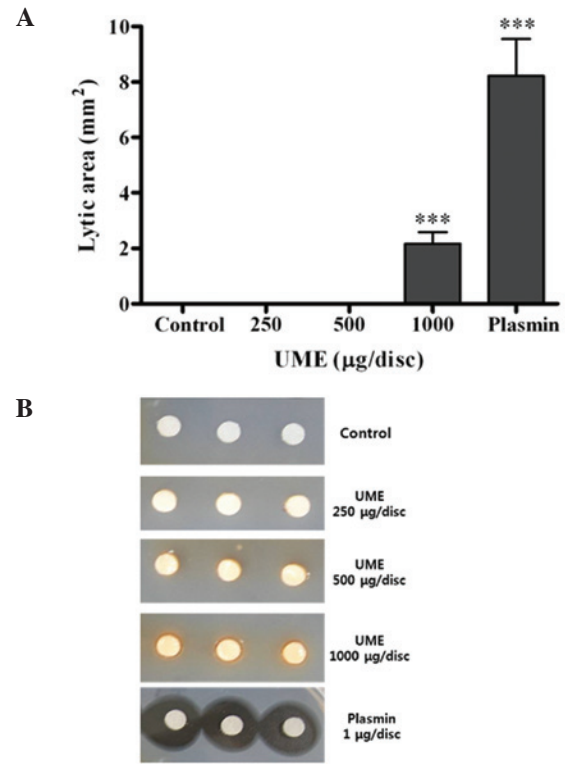

Figure 4. Effect of *Ulmus macrocarpa* extract (UME) on platelet aggregation *in vitro*. The platelet suspension was incubated at 37°C in an aggregometer and stirred at 1,000 rpm. Following preincubation with UME for 3 min, platelet aggregation was induced by the addition of collagen (5 μg/ml), and adenosine 5'-diphosphate (ADP) (5 μM). The aggregation percentages are expressed as the percentage of the maximum aggregation by the respective inducers. Data are expressed as the means ± SD from three independent experiments. *P<0.05, **P<0.01 and ***P<0.001.

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 FeCl$_3$-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, FeCl$_3$ induces oxidative injury and exposes the subendothelial matrix. Platelets interact with the matrix via GPIb-V-IX and $\alpha2b\beta3$ 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 TXA$_2$ 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 *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 *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 *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

References

1. Pepine CJ: Residual risk for secondary ischemic events in patients with atherothrombotic disease: opportunity for future improvements in patient care. Ann Med 42: 19-35, 2010.
2. Varon D and Spectre G: Antiplatelet agents. Hematology Am Soc Hematol Educ Program: 267-272, 2009.
3. Aronow WS: Management of peripheral arterial disease of the lower extremities in elderly patients. J Gerontol A Biol Sci Med Sci 59: 172-177, 2004.
4. McNicol A and Israels SJ: Platelets and anti-platelet therapy. J Pharmacol Sci 93: 381-396, 2003.
5. Stoyioglou A and Jaff MR: Medical treatment of peripheral arterial disease: a comprehensive review. J Vasc Interv Radiol 15: 1197-1207, 2004.
6. Medved L and Nieuwenhuizen W: Molecular mechanisms of initiation of fibrinolysis by fibrin. Thromb Haemost 89: 409-419, 2003.
7. Collen D and Lijnen HR: Thrombolytic agents. Thromb Haemost 93: 627-630, 2005.
8. Rezkalla SH and Benz M: Antiplatelet therapy from clinical trials to clinical practice. Clin Med Res 1: 101-104, 2003.
9. Gum PA, Kottke-Marchant K, Welsh PA, White J and Topol EJ: A prospective, blinded determination of the natural history of aspirin resistance among stable patients with cardiovascular disease. J Am Coll Cardiol 41: 961-965, 2003.
10. Bhatt DL and Topol EJ: Scientific and therapeutic advances in antiplatelet therapy. Nat Rev Drug Discov 2: 15-28, 2003.
11. Eisert WG: How to get from antiplatelet to antithrombotic treatment. Am J Ther 8: 443-449, 2001.
12. Kim KW, Park JS, Kim KS, *et al*: Inhibition of *Ulmus davidiana* Planch (Ulmaceae) on bone resorption mediated by processing of cathepsin K in cultured mouse osteoclasts. Phytother Res 22: 511-517, 2008.
13. Jun CD, Pae HO, Kim YC, *et al*: Inhibition of nitric oxide synthesis by butanol fraction of the methanol extract of *Ulmus davidiana* in murine macrophages. J Ethnopharmacol 62: 129-135, 1998.
14. Jin YR, Ryu CK, Moon CK, Cho MR and Yun YP: Inhibitory effects of J78, a newly synthesized 1,4-naphthoquinone derivative, on experimental thrombosis and platelet aggregation. Pharmacology 70: 195-200, 2004.
15. Astrup T and Mullertz S: The fibrin plate method for estimating fibrinolytic activity. Arch Biochem Biophys 40: 346-351, 1952.
16. Chou J, Mackman N, Merrill-Skoloff G, Pedersen B, Furie BC and Furie B: Hematopoietic cell-derived microparticle tissue factor contributes to fibrin formation during thrombus propagation. Blood 104: 3190-3197, 2004.
17. Steinhubl SR and Moliterno DJ: The role of the platelet in the pathogenesis of atherothrombosis. Am J Cardiovasc Drugs 5: 399-408, 2005.
18. Azevedo AP, Farias JC, Costa GC, *et al*: Anti-thrombotic effect of chronic oral treatment with *Orbignya phalerata* Mart. J Ethnopharmacol 111: 155-159, 2007.
19. Kurz KD, Main BW and Sandusky GE: Rat model of arterial thrombosis induced by ferric chloride. Thromb Res 60: 269-280, 1990.
20. Lockyer S and Kambayashi J: Demonstration of flow and platelet dependency in a ferric chloride-induced model of thrombosis. J Cardiovasc Pharmacol 33: 718-725, 1999.
21. Furie B and Furie BC: Thrombus formation in vivo. J Clin Invest 115: 3355-3362, 2005.
22. Farrehi PM, Ozaki CK, Carmeliet P and Fay WP: Regulation of arterial thrombolysis by plasminogen activator inhibitor-1 in mice. Circulation 97: 1002-1008, 1998.
23. Cui X, Sakaguchi T, Shirai Y and Hatakeyama K: Orally administered *Panax ginseng* extract decreases platelet adhesiveness in 66% hepatectomized rats. Am J Chin Med 27: 251-256, 1999.
24. Park HJ, Rhee MH, Park KM, Nam KY and Park KH: Effect of non-saponin fraction from *Panax ginseng* on cGMP and thromboxane A2 in human platelet aggregation. J Ethnopharmacol 49: 157-162, 1995.
25. Yun YP, Do JH, Ko SR, *et al*: Effects of Korean red ginseng and its mixed prescription on the high molecular weight dextran-induced blood stasis in rats and human platelet aggregation. J Ethnopharmacol 77: 259-264, 2001.
26. Baruah DB, Dash RN, Chaudhari MR and Kadam SS: Plasminogen activators: a comparison. Vascul Pharmacol 44: 1-9, 2006.