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Antithrombotic and Fibrinolytic Activities of Methanolic Extract of Aged Sorghum Vinegar

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Antithrombotic activities, namely, in vitro platelet aggregation and in vivo pulmonary thrombosis, of the methanolic extract of aged vinegar were evaluated. The ability of the extract to inhibit platelet aggregation induced by adenosine diphosphate (ADP) and thrombin was concentration-dependent. IC_{50} values for the inhibition of platelet aggregation induced by ADP and thrombin were 1.7 ± 0.3 and 8.9 ± 1.9 mg/mL, respectively. When administered orally at >100 mg/kg of body weight, the extract protected the rats against thrombotic death induced by collagen and epinephrine. Furthermore, the low molecular weight fraction of the extract showed strong fibrinolytic activity and altered coagulation parameters such as activated partial thromboplastin time (APTT), prothrombin time (PT), and throbin time (TT) in rat platelet. These results suggested that the antithrombotic ability of the vinegar extract corresponded to both antiplatelet and anticoagulation activities.

KEYWORDS: Antithrombotic activity; aged vinegar; sorghum

INTRODUCTION

Arterial thrombosis induced by platelet aggregation may cause life-threatening disorders such as unstable angina and reocclusion after angioplasty. Hence, inhibition of platelet aggregation is important in the prevention and treatment of cardiovascular diseases (*1*, *2*). Although certain pharmacological agents are effective in preventing the occurrence of the cardiovascular disorders, their safety cannot be guaranteed.

During the initial stage of thrombosis, damage in blood vessels causes the production of adhesive proteins (such as collagen and von Willebrand factor) and soluble agonists (such as ADP and thrombin) at the injury site; this event then stimulates platelet adhesion, activation, and aggregation, resulting in the formation of a platelet-rich thrombus (*3*). Activated platelets facilitate thrombin formation by providing a catalytic surface on which coagulation activation can occur. Thrombin not only is responsible for the formation of fibrin but also acts an extreme platelet activator. The growing mound of activated platelets is eventually stabilized by cross-linked fibrin and results in the formation of a platelet-rich thrombus (*4*, *5*). Therefore, the inhibition of platelet function is a promising approach for the prevention of thrombosis.

Vinegar derived from grain fermentation is a common seasoning in China. It is also used for treating diseases in traditional Chinese medicine (*6*). Studies have shown that vinegar possesses a wide spectrum of physiological effects, including alleviating exhaustion (*7*), regulating blood glucose (*8*) and blood pressure (*9*), aiding digestion (*10*), and assisting calcium absorption (*11*). The antioxidant activity of vinegar is well established and generally attributed to phenolic compounds, such as dihydroferulic acid and dihydrosinapic acid (*12*, *13*), as well as melanoidins (*14*). The latest study demonstrated that the high molecular weight of melanoidins in vinegar had strong in vitro antioxidant activity (*15*). Vinegar has also been reported to improve blood fluidity, leading to the prevention of hypertension (*16*). Thus, the role of vinegar in the prevention and treatment of cardiovascular disease has recently received considerable attention. However, little information is available about the antithrombotic activity of vinegar.

Aged vinegar is very common in northern China. In this study, we investigated the antiplatelet activity and anticoagulation effect of aged vinegar by the measurement of platelet aggregation in vitro. The antithrombotic activity of the vinegar was also investigated in vivo.

MATERIALS AND METHODS

Animals and Materials. Male New Zealand white rabbits weighing 2.3−3.0 kg and male Kun-Ming rats weighing 18−25 g were purchased from the Department of Laboratory Animal Science, Health Science Center, Peking University (Beijing, China) and fed with standard laboratory diet and water. All rats were housed in stainless steel wire-bottom

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cages in an air-conditioned room kept at a controlled ambient temperature (22 ± 1 °C) and humidity (50 ± 10%) with a 12 h light/dark cycle. The experiment was carried out according to European Community guidelines for the use of experimental animals and approved by the Peking University Committee on Animal Care and Use.

Aged vinegar was obtained from Shanxi Ziyuan Microorganism R&D Co., Ltd. (Shanxi, China) in 2007. Reagent kits for measuring activated partial thromboplastin time (APTT), prothrombin time (PT), and throbin time (TT) were purchased from Shanghai Sunbiote Co. Ltd. (Shanghai, China). Adenosine diphosphate (ADP) and heparin sodium (160 U/mg) were purchased from Beijing Solarbio Science and Technoloy Co. Ltd. (Beijing, China). Folin−Ciocalteu (FC) reagent, rutin, gallic acid, ferulic acid, vanillic acid, cinnamic aicd, caffeic acid, 3,4-dihydroxybenzoic acid, syringic acid, chlorogenic acid, *p*-coumaric acid, epigallocatechin, catechin, epicatechin, thrombin, and aspirin were obtained from Sigma Chemical Co. (St. Louis, MO). Collagen and epinephrine were purchased from Chrono-log Reagents Co. (Havertown, PA). All other chemicals and reagents were of analytical grade.

Preparation of Methanolic Vinegar Extract. Approximately 5 L of the aged vinegar was mixed with saturated sodium hydrogen carbonate solution (5 L) to remove organic acids, followed by freeze-drying. The freeze-dried vinegar was defatted with 3 volumes of dichloromethane three times. The residue was mixed with methanol at a 1:10 ratio (w/v) for 2 h at room temperature. Subsequently, the mixture was centrifuged (8000*g*, 15 min), and the resulting supernatant was filtered through a 0.45 μm membrane. The filtrate was dried under vacuum at 40 °C. The dried extract was used for further analysis.

Proximate Chemical Analysis and Phenolic Compound Analysis. Official methods (AOAC, 2000) were used to measure moisture (vacuum oven method 925.09), protein (method 997.09), carbohydrate (method 985.10), lipid (method 952.13), and ash (method 924.05) contents of the vinegar extract. Total phenolics content in the vinegar extract was measured spectrophotometrically by using the Folin−Ciocalteu method (*17*). Total flavonoid content in the vinegar extract was determined as described in ref *18*.

Phenolic compounds in the extracts were estimated by reversed-phase HPLC equipped with UV detection. All samples were filtered through a 0.45 μm pore size syringe-driven filter before injection. A 20 μL aliquot of the sample was fractionated using a Shimadzu HPLC system equipped with a diode array detector on a C_{18}-ODS analytical column (150 mm × 4.6 mm; i.d., 5 μm) (Waters Associates, Milford, MA). Phenolics were analyzed by HPLC with a linear gradient of acetonitrile (mobile phase B) at a flow rate of 0.8 mL/min. Mobile phase A consisted of purified water and 0.1% trifluoroacetic acid (TFA). Gradient elution was performed as follows: from 0 to 5 min, linear gradient from 10 to 15% solvent B; from 5 to 15 min, linear gradient from 15 to 25% solvent B; from 15 to 25 min, linear gradient from 25 to 30% solvent B; and from 25 to 30 min, linear gradient from 30 to 40% solvent B. Column temperature was set at 40 °C. The phenolic compounds were identified at 280 nm by comparing the retention times of standards and further confirmed by their internal standards. The phenolic compounds were quantified by comparing the peak area of the samples with the peak area of the external calibration curves for all of the phenolic compounds (all $r^2 > 0.99$). The detection limits of the phenolic standards ranged from 20 to 300 μg/L (S/N = 3).

Antiplatelet Activity. Antiplatelet activity of the vinegar extract was performed as described (*19*). Fresh blood from an ear artery of the male New Zealand white rabbits was collected into plastic tubes containing ACD-C (65 mM citric acid, 85 mM trisodium citrate, 2% glucose, pH 4.5) ($^1/_6$ volume of blood). Platelet-rich plasma (PRP) was harvested by centrifugation at 250*g* for 15 min at room temperature. The PRP was then centrifuged at 650*g* for 10 min at room temperature (20−25 °C). The collected platelets were washed twice with a Tyrode/HEPES solution [138.3 mM NaCl, 2.68 mM KCl, 1.0 mM $MgCl_2 \cdot 6H_2O$, 4.0 mM $NaHCO_3$, 10 mM HEPES, 0.1% glucose (w/v), and 0.35% albumin (w/v); pH 6.35]. The resultant pellet was resuspended in a volume of Tyrode/HEPES solution (pH 7.35) that produced an OD of 0.15 at 650 nm. Assays were done in 96-well microplates using a microplate reader (model 680, Bio-Rad, Hercules, CA) to monitor aggregation activity of the washed platelets. Two hundred microliters of the platelet suspension was incubated at 37 °C with stirring at 1000 rpm. Various concentrations (1, 2, or 4 mg/mL) of the vinegar extract (20 μL) were then added. After 3 min of preincubation, platelet aggregation was induced by the addition of ADP (10 μM) or thrombin (0.5 nM). Decreases in absorbance at 650 nm were recorded for 9 min at 11 s intervals.

Table 1. Chemical Composition of Vinegar Extract[a]

composition	content[b]
moisture (g/100 g of vinegar extract)	21.4 ± 1.7
protein (g/100 g of vinegar extract)	5.2 ± 0.2
soluble sugars (g/100 g of vinegar extract)	35.6 ± 2.3
lipids (g/100 g of vinegar extract)	0.2 ± 0.0
ash (g/100 g of vinegar extract)	7.1 ± 1.2
phenolics (FC) (mg/g of vinegar extract)	305.5 ± 11.8
flavonoids (mg/g of vinegar extract)	107.0 ± 8.1

[a] Approximately 5 L of vinegar was mixed with a saturated sodium hydrogen carbonate solution (5 L) to remove the organic acids. Freeze-dried vinegar was extracted with methanol at 1:10 ratio (w/v) for 2 h at room temperature. Subsequently, the extract was centrifuged (8000*g*, 15 min), and the supernatants were filtered through a 0.45 μm membrane. The filtrate was dried under vacuum at 40 °C. The dried extract was defined as vinegar extract. [b] Data are means ± standard deviations of triplicate determinations.

In Vivo Antithrombosis Assay. Antithrobosis activity of the vinegar extract was studied as described by DiMinno and Silver (*20*) using pulmonary thrombosis test on fasting Kun-Ming rats. The vinegar extract of different concentrations (50, 100, and 200 mg/kg), aspirin (10 and 20 mg/kg) as a positive control, and saline were administered orally. One hour after oral administration of the sample, a mixture of collagen (115 μg/rat) and epinephrine (1.80 μg/rat) was injected into the tail vein at a rate of about 20 μL/s to induce pulmonary thrombosis. The mortality of the rats was observed for 15 min. The efficacy of test samples was expressed as the percent decrease in mortality rate in the treated group compared to that of the control group.

Bleeding Time. The vinegar extract (100 mg/kg), aspirin (30 mg/kg), or saline as vehicle was administrated by intraperitoneal injection to anesthetized rats for 7 days. Bleeding time was assessed 5 min after the bolus injection (150 μL) by amputating 3 mm of the tail tip with a scalpel and blotting the bleeding tail onto a filter paper every 15 s until the paper was no longer stained with blood. When necessary, bleeding was manually stopped after 20 min to prevent the rat's death.

In Vitro Anticoagulation Assay. Measurements of APTT, PT, and TT were performed according to the manufacturer's specifications by using a TYXN-96 Multifunctional Blood Coagulation Instrument (Shanghai Research Institute of General Electromechanic Technology, Shanghai, China). In brief, the platelet-poor-plasma of the rabbit was incubated with the vinegar extract or heparin for 7 min at 37 °C. One hundred microliters of the incubated plasma was mixed with 50 μL of phosphatidylethanolamine in the process plate, and the coagulation was started by the addition of $CaCl_2$ (1 mM), 100 μL of thromboplastin, and 100 μL of bovine thrombin into 100 μL of incubated plasma for APTT, PT, and TT assay, respectively.

Fibrinolytic Activity. Twenty grams of the vinegar extract was dissolved in distilled water (200 mL) and dialyzed into two fractions, namely, the low molecular weight fraction (LMW, MW < 5000 Da) and the high molecular weight fraction (HMW, MW > 5000 Da). The freeze-dried LMW fraction was dissolved in deionized water to reach a concentration of 4 mg/mL and used for evaluating the fibrinolytic activity.

The fibrinolytic activity of the vinegar extract (LMW) was observed by artificial blood clot degradation (*21*). An artificial blood clot was made by spontaneous coagulation of fresh rabbit blood in a glass test tube. One hour later, the artificial blood clot was rinsed out repeatedly. The artificial blood clot was dipped in 4 mg/mL of LMW vinegar extract at room temperature. Normal saline was used as a control.

Statistical Analysis. Analysis of variance (ANOVA) was performed by using SAS (version 9.0; SAS Institute, Cary, NC). Duncan's multiple-range test was used to determine the difference of means. The experimental results were expressed as the mean ± SD. The χ^2-test was used for determining whether there were significant differences in effects between rats that received antithrombotic agents and the controls which did not.

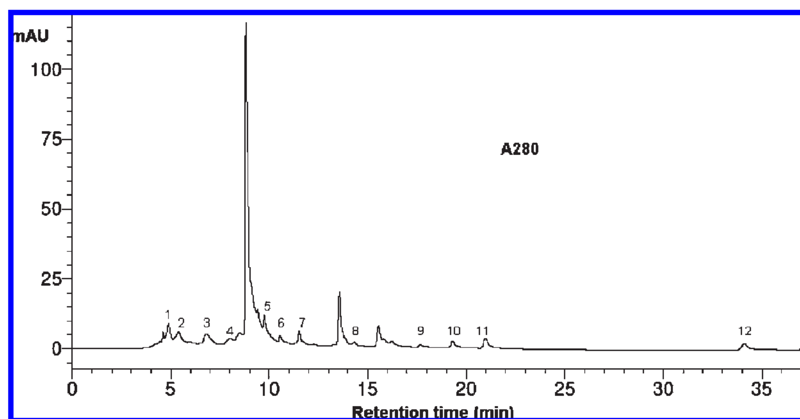

Figure 1. Chromatography of the vinegar extract at 280 nm. The chromatographic conditions are described under Materials and Methods: column, C_{18}-ODS (150 × 4.6 mm, 5 μm); detection, 280 nm; flow rate, 1.0 mL/min. Peaks: 1, gallic acid; 2, 3,4-dihydroxybenzoic acid; 3, chlorogenic acid; 4, catechin; 5, vanillic acid; 6, ferulic acid; 7, caffeic acid; 8, epicatechin; 9, cinnamic acid; 10, syringic acid; 11, vanillic acid; 12, *p*-coumaric acid.

Table 2. Phenolic Composition in Vinegar Extract[a]

composition	content (mg/g of vinegar extract)
gallic acid	75.8 ± 6.5
3,4-dihydroxybenzoic acid	29.4 ± 1.4
chlorogenic acid	42.4 ± 3.1
caffeic acid	8.1 ± 0.3
vanillic acid	14.9 ± 0.2
syringic acid	6.4 ± 1.1
p-coumaric acid	6.5 ± 0.8
ferulic acid	1.9 ± 0.1
cinamic acid	1.7 ± 0.2
epigallocatechin	1.9 ± 0.1
catechin	2.4 ± 0.1
epicatechin	0.3 ± 0.1
total phenolics (HPLC)	191.3 ± 22.8

[a] All samples were quantified in triplicate. Data are means ± standard deviations.

RESULTS

Proximate Chemical Composition and Phenolics of the Vinegar Extracts. The proximate composition of the vinegar extract is shown in **Table 1**. Total amounts of phenolics and flavonoids present in the vinegar extract were 305.5 ± 11.8 and 107.0 ± 8.1 mg/g, respectively.

Figure 1 shows a typical chromatogram for the vinegar extract at 280 nm. The phenolic compounds found in the vinegar extract are listed in **Table 2**. HPLC analysis showed that the vinegar extract was rich in gallic acid, chlorogenic acid, 3,4-dihydroxybenzoic acid, and vanillic acid. These phenolic acids accounted for 84.9% of the total phenolic compounds in the extract. Amounts of other phenolic compounds in the vinegar extract, namely, cinnamic, ferulic, syringic, *p*-coumaric, and caffeic acids, ranged from 1.7 ± 0.2 to 8.1 ± 0.3 mg/g. Amounts of flavonoid compounds in the vinegar extract, namely, epigallocatechin, catechin, and epicatechin, were 1.6 ± 0.1, 2.4 ± 0.1, and 0.3 ± 0.1 mg/g, respectively. There were some phenolics that were not determined by using HPLC.

Effect of the Vinegar Extract on Platelet Aggregation In Vitro. **Figure 2** shows that the effect of the vinegar extract on rabbit platelet aggregation in vitro was concentration-dependent. The IC_{50} values of the extract for inhibiting ADP- and thrombin-induced platelet aggregation were 1.7 ± 0.3 and 8.9 ± 1.9 mg/mL, respectively.

Effect of the Vinegar Extract on Thrombosis in Vivo. Intravenous injection of collagen and epinephrine into the tail vein of the rats led to total mortality in the control group (**Table 3**). Oral administration of the vinegar extract prior to the injection exhibited a significant ($p < 0.05$) dose-dependent preventive effect against thrombotic death. Survival rates of 100, 77.8, and 44.4% were observed in rats given 200, 100, and 50 mg/kg of the extract prior to the injection, respectively. Aspirin, which was used as a reference drug, showed a survival rate of 88.9% used at 20 mg/kg. These results are consistent with those reported by Kim and Lee (*22*).

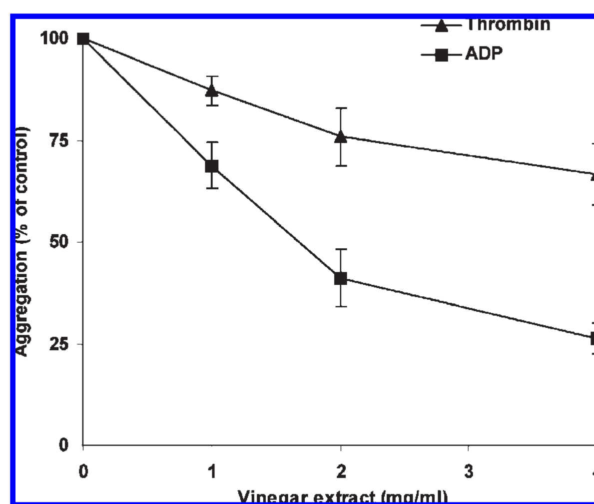

Figure 2. Effect of vinegar extract on rabbit platelet aggregation induced by thrombin (▲) and ADP (■). Data are expressed as mean ± SD ($n = 3$).

Table 3. Effect of Vinegar Methanolic Extract on Pulmonary Thrombosis in Rats[a]

sample	dose (mg/kg)	no. of dead/total[b]	inhibition[c] (%)
control	saline	10/10	0
vinegar extract	200	0/9	100**
	100	2/9	77.8**
	50	5/9	44.4*
aspirin	20	1/9	88.9**
	10	2/9	77.8**

[a] Each sample was administered orally 1 h prior to the thrombotic challenge. [b] Number of dead, the number of animals that died during the study. [c] **, $p < 0.01$ compared with control; *, $p < 0.05$ compared with control.

Effect of the Vinegar Extract on Tail Bleeding Time of Rats. Effects of the vinegar extract on tail bleeding time of rats are

Table 4. Effect of Vinegar Extract on the Tail Bleeding Time of Rats[a]

sample	dose (mg/kg)	tail bleeding time[b] (s)	n
control	saline	65.1 ± 6.9	10
vinegar extract	100	140.0 ± 16.0*	9
aspirin	30	153.4 ± 36.3*	9

[a] Samples were administered intraperitoneally once a day for 7 days. Aspirin was injected intravenously 2 min before thrombin. [b] *, $p < 0.01$ compared with control.

Table 5. Effects of Vinegar Extract on Rat Plasma Coagulation Times[a]

sample	dose (mg/kg)	APTT (s)	PT (s)	TT (s)
control	saline	32.9 ± 1.6	13.2 ± 0.7	7.6 ± 0.6
vinegar extract	50	37.2 ± 1.5*	16.6 ± 1.1*	11.9 ± 0.5*
	100	41.6 ± 2.1*	20.9 ± 1.6*	14.6 ± 0.9*
	200	46.3 ± 1.8*	24.3 ± 1.1*	16.3 ± 0.9*
heparin	50	>100*	32.3 ± 1.9*	>100*

[a] The results are expressed as mean ± SD ($n = 6$). *,$p < 0.01$ compared with control.

shown in **Table 4**. The vinegar extract (100 mg/kg) significantly prolonged ($p < 0.05$) the bleeding time (> 74 s) compared to that of saline injection (65.1 ± 6.9 s). The tail bleeding time of rats administered aspirin (30 mg/kg) was similar to that of rats given the vinegar extract.

Anticoagulation Activity of the Vinegar Extract in Vitro. The APTT, PT, and TT of the control were 32.9 ± 1.6, 13.2 ± 0.7, and 7.6 ± 0.6 s, respectively ($n = 6$) (**Table 5**). The APTT, PT, and TT of the heparin group were > 100, 32.3 ± 1.9, and > 100 s, respectively ($n = 6$). The vinegar extract significantly prolonged the APTT, PT, and TT ($p < 0.01$). The APTT, PT, and TT were prolonged by the vinegar extract in a dose-dependent manner from 37.2 ± 1.5 to 46.3 ± 1.8 s, from 16.6 ± 1.1 to 24.3 ± 1.1 s, and from 11.9 ± 0.5 to 16.3 ± 0.9 s when the amount of the extract increased from 50 to 200 mg/kg, respectively.

Fibrinolytic Effect of the LMW of Vinegar Extract. To investigate the fibrinolytic effect of the vinegar extract, the vinegar extract was dialyzed into two fractions, namely, the HMW (MW > 5000 Da) and the LMW (MW < 5000 Da). The HMW (MW > 5000 Da) of the vinegar extract was puce and was not suitable for direct observation of blood clot degradation, whereas the LMW (MW < 5000 Da) of the vinegar extract was white and was used to observe the blood clot degradation (**Figure 3**). In the test tube of normal saline, the blood clot was observed to have a slight degradation until 90 min. In the test tube of the vinegar extract, blood clot degradation was observed with the spread of red blood cells by multiple fibrins net in the solution after 90 min. These phenomena suggest that the LMW of the vinegar extract has a direct fibrinolytic effect on the blood clot.

DISCUSSION

Much research haa been carried out worldwide to develop antithrombotic agents with improved efficacy for preventing or treating arterial or venous thrombosis (*1*, *2*). Vinegar has been used both as a seasoning and as a medicine in China. Its ability to improve blood fluidity has already been proven (*16*). It is demonstrated for the first time that the vinegar extract exhibits a potent antithrombotic activity and fibrinolytic activity.

The interactions between platelets and various adhesive proteins (such as collagen and von Willebrand factor) and soluble agonists (such as thrombin, thromboxane A_2, ADP, platelet-activating factor (PAF), serotonin, and epinephrine) provide potential targets for developing antiplatelet agents (*23*, *24*).

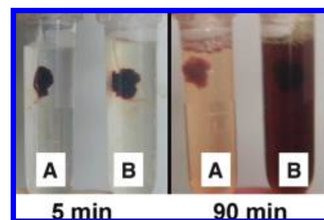

Figure 3. Direct fibrinolytic effect observed in the vinegar extract solution. Tubes containing the artificial blood clots were filled with either normal saline (tube A) or LMW (MW < 5000 Da) of vinegar extract (tube B) in each panel. In tube B, the blood clot degradations were observed by spreading of the red blood cells in the solution after 5 min and after 90 min.

Thrombin is an extreme platelet activator that strongly promotes release and aggregation of platelets. It is also responsible for the transformation of fibrinogen into fibrin, which results in the final formation of thrombus (*25*). ADP, on the other hand, plays an important role in platelet aggregation by inducing the interaction of membrane receptor glycoprotein IIb−IIIa with fibrinogen (*25*). During platelet aggregation, the vinegar extract significantly inhibited ADP- and thrombin-induced platelet aggregation. Moreover, oral administration of the vinegar extract prior to the injection of collagen and epinephrine effectively prevented the thrombotic death of rats. All of the results suggest that the antithrombotic activities of the vinegar extract were related to its ability to inhibit platelet aggregation. To investigate the interactions of vinegar extract with coagulation factors, we evaluated the effects of the extract on coagulation time by APTT, PT, and TT assays using PPP. The vinegar extract significantly prolonged APTT, TT, and PT, suggesting that the vinegar extract played a role in anticoagulation by inhibiting intrinsic and extrinsic coagulation factors. The prolongation of TT also indicated that the vinegar extract could inhibit the activity of thrombin.

We also investigated the fibinolytic activity of the vinegar extract. The fibrinolytic activity of the LMW fraction (MW < 5000 Da) from the vinegar extract was obvious (**Figure 2**). The result suggested that the vinegar extract, besides inhibiting platelet aggregation, also exerted its antithrombotic activity through fibrinolytic activiy. Antithrombotic agents acting in only one pathway of thrombosis formation have limited efficacy in treating arterial thrombotic diseases. Hence, the vinegar extract with a combination of antiplatelet aggregation, antithrombotic, and fibrinolytic activities may be effective in preventing thrombus formation through several pathways.

Several papers (*15*, *16*) revealed that rice vinegars contain phenolic acids, such as dihydroferulic acid, dihydrosinapic acid, ferulic acid, sinapic acid, and vanillic acid. These phenolic compounds were the major contributors to the antioxidant activity of rice vinegar. The major raw materials of sorghum vinegar in the present study are black sorghum (45%, w/w), wheat bran (14%, w/w), rice bran (14%, w/w), oat and pea (27%, w/w), which are all rich in phenolic compounds. Our research has revealed that the vinegar extract was rich in both phenolic acids (gallic acid, 3,4-dihydroxybenzoic acid, vanillic acid, and ferulic acid) and flavonoids (catechin, epigallocatechin, and epicatechin). A number of antioxidative phenolic compounds including quercetin and catechins are effective in inhibiting platelet aggregation in vitro and ex vivo. Human studies have also shown that diets with high contents of phenolic compounds may inhibit platelet aggregation in vivo (*26*−*30*). Therefore, although their antithrombotic potency is lower than that of aspirin, phenolics and flavonoids in the vinegar extract may be a potent source of antithrombotic agents.

Our experimental results suggest that aged vinegar could be a potent source of antithrombotic agents to help fight some cardiovascular diseases. In the present study, 1 mL of vinegar could produce 18.4 mg of vinegar extract. Considering the ratio of experimental rat weight to human weight, it would be necessary for a human to eat about 42−84 mL of initial vinegar/day/60 kg of body weight for the preventive purpose as a dietary supplement even though comparative investigation is lacking and the experiment was limited in this research. Because of the sourness of the initial vinegar, the vinegar extract might be made into troche for eating at 0.8−1.5 g/day/60 kg of body weight. In addition, the vinegar extract might also be made into powder injections considering its availability for injection.

In conclusion, the vinegar extract demonstrated potent antithrombotic activity in vivo and in vitro. The antithrombotic activity of the vinegar extract derived from its antiplatelet−aggregation and anticoagulation activities. The vinegar extract was important as an anticoagulant by significantly prolonging APTT, PT, and TT. In addition, the vinegar extract also has a potent fibrinolytic activity.

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