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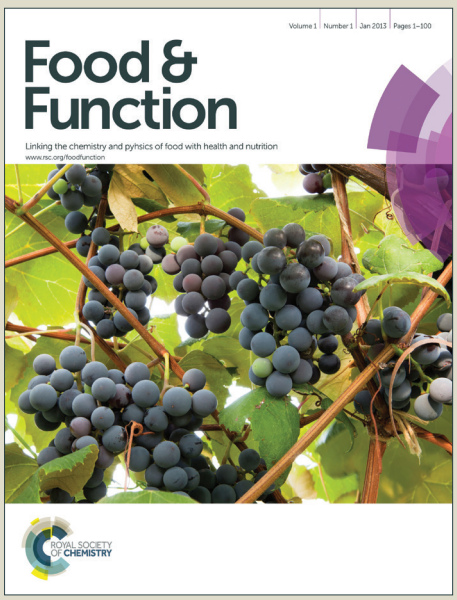


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# Acetic acid in aged vinegar affects molecular targets for thrombus disease management

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## Abbreviations

AA, arachidonic acid; ADP, adenosine diphosphate; BN-PAGE, bule native-polyacrylamide gel electrophoresis; BSA, bovine serum albumin; COX1, Cyclooxygenase-1; LDH, lactate dehydrogenase; NADH, nicotinamide adenine dinucleotide; NS, normal saline; PPP, platelet-poor plasma; PRP, platelet-rich plasma; $TXA_2$, thromboxane-$A_2$; $TXB_2$, thromboxane-$B_2$; WP, washed platelet.

**ABSTRACT**

To elucidate the mechanism underlying the action of dietary vinegar on antithrombotic activity, acetic acid, the main acidic component of dietary vinegar, was used to determine antiplatelet and fibrinolytic activity. The results revealed that acetic acid significantly inhibits adenosine diphosphate (ADP)-, collagen-, thrombin-, and arachidonic acid (AA)-induced platelet aggregation. Acetic acid (2.00 mM) reduced AA-induced platelet aggregation to approximately 36.82 ± 1.31%, and vinegar (0.12 mL/L) reduced the platelet aggregation induced by AA to 30.25 ± 1.34%. Further studies revealed that acetic acid exerts its effects by inhibiting cyclooxygenase-1 and the formation of thromboxane-$A_2$. Organic acids including acetic acid, formic acid, lactic acid, citric acid, and malic acid also showed fibrinolytic activity; specifically, the fibrinolytic activity of acetic acid amounted to 1.866 IU urokinase/mL. Acetic acid exerted its fibrinolytic activity by activating plasminogen during fibrin crossing, thus leading to crosslinked fibrin degradation by the activated plasmin. These results suggest that organic acids in dietary vinegar play important roles in the prevention and cure of cardiovascular diseases.

**Key words:**

Thrombus; cardiovascular disease; platelet; collagen; cyclooxygenase-1; plasminogen

**Chemical compounds studied in this article:**

Acetic acid (PubChem CID: 176); Adenosine diphosphate (PubChem CID: 6022); Arachidonic acid (PubChem CID: 444899); Citric acid (PubChem CID: 311); Collagen (PubChem CID: 6913668); Formic acid (PubChem CID: 284); Lactic acid (PubChem CID: 612); Malic acid (PubChem CID: 525); Tartaric acid (PubChem CID: 875); Thrombin (PubChem CID: 16133232); Thromboxane-$B_2$ (PubChem CID: 5283137); Thromboxane-$A_2$ (PubChem CID: 5280497).

## 1. Introduction

Arterial thrombosis induced by platelet aggregation may cause life-threatening disorders, such as unstable angina and reocclusion after angioplasty. During the initial stage of thrombosis, blood vessel damage leads to the production of adhesive proteins such as collagen and von Willebrand factor, and soluble agonists including adenosine diphosphate (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 (Jackson *et al.*, 2003). Meanwhile, a variety of coagulation factors release and activate endogenous and exogenous coagulation systems, leading to the generation of thrombin. Thrombin subsequently cleaves fibrinogen into fibrin and crosslinks platelets, resulting in thrombus formation (Fu, 2005; Tang, 2013). Therefore, platelet aggregation and fibrin crosslinking play key roles in thrombus formation. Most of the current antithrombotic researches focus on antiplatelet aggregation and fibrin degradation (Fan *et al.*, 2009; Ryu *et al.*, 2009). Although there are many specific pharmacological agents (Zhang, 2012), existing therapies have significant drawbacks, including issues related to limited efficacy and safety (Yu *et al.*, 2011). Plant-derived food has a nature-friendly image and various safety merits. Recently, efforts have been made to identify bioactive compounds from food for the prevention of thrombosis and atherosclerotic cardiovascular diseases (Ryu *et al.*, 2009; Endale *et al.*, 2012).

Vinegar derived from grain fermentation (sorghum or rice is generally employed as a raw material) is used as a common seasoning in China, and can be used for treating diseases in traditional Chinese medicine (TCM) since it is rich in physiological substances with multiple health benefits (Xu *et al.*, 2003). Shizhen Li (a Chinese medical scientist who lived from 1518 to 1593) stated in his book *Compendium of Materia Medica* that vinegar can disperse blood stasis, cure jaundice, attenuate yellow perspiration, and treat inflammatory diseases (Shen, 2005). The other traditionally important medicinal property of vinegar involved its use

in combination with herbs, a technique that was extensively applied by the ancient Chinese to treat numerous diseases, for detoxification and anti-inflammatory purposes, and to improve the efficacy of herbs (Liu, 2010). Recent studies have also shown that dietary vinegar contains many bioactive substances, including organic acids, saccharides, proteins, amino acids, minerals, phenolics, alkaloids, saponins, *etc*. (Fan *et al.*, 2011). The main bioactive compound in vinegar is acetic acid, the content of which ranges from 5.7% (w/v) to 9.8% (w/v) (Fan *et al.*, 2011). In addition, vinegar contains formic acid, lactic acid, citric acid, malic acid, tartaric acid, *etc*. (Yu *et al.*, 2010). It has been reported that vinegar possesses a wide spectrum of physiological effects, can affect serum triglyceride levels (Kondo *et al.*, 2009), act as an antiglycemic agent (Johnston & Gass, 2006), and exert antioxidant activity (Xiang *et al.*, 2013). Vinegar has also been reported to improve blood fluidity, leading to the prevention of hypertension (Yamagishi *et al.*, 1998). The methanolic extract of aged vinegar has potent antiplatelet and antithrombotic activity *in vitro*, which is highly correlated with polyphenols and low molecular weight substances (Fan *et al.*, 2009; He *et al.*, 2012). Recently, we determined that, in sorghum vinegar, alditols and monosaccharides demonstrate antiplatelet activity (Li *et al.*, 2014).

Organic acids endow vinegar with an acidic flavor. In vinegar, acetic acid has been shown to inhibit disaccharidases in Caco-2 cells, which contain key diabetes management markers (Ogawa *et al.*, 2000). Recently, studies have shown that acetic acids exhibit important physiological activities, including the promotion of metabolism, detoxification, improvement of liver function (Chen *et al.*, 2009), and especially antihypertensive and antihyperglycemic effects (Fan *et al.*, 2011). Thus, the role of vinegar in the prevention and treatment of cardiovascular disease has recently received considerable attention. However, little information is available regarding antiplatelet aggregation and the fibrinolytic activity of organic acids in vinegar.

In this study, we evaluated the antiplatelet and fibrinolytic activities of the organic acids in vinegar. To clarify the mechanism of the effects of acetic acid, we studied the effects of the inhibition of acetic acid on COX1 and $TXA_2$, the key enzyme and metabolite, respectively, in the AA metabolic pathway. In addition, we further studied the effect of acetic acid on plasminogen during fibrin crosslinking to evaluate its fibrinolytic activity.

## 2. Materials and methods

### 2.1. Animals and Materials

Male New Zealand white rabbits weighing 2.3–3.0 kg 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-bottomed cages in an air-conditioned room at controlled ambient temperature (22 ± 1°C) and humidity (50 ± 10%) and a 12-h light/dark cycle. The experiment was carried out according to the European Community guidelines for the use of experimental animals and was approved by the Beijing Forestry University Committee on Animal Care and Use.

Blood was collected from healthy volunteer donors aged 20 to 25 years upon receipt of informed consent, with approval from the Beijing Forestry University Ethical Review Committee (Permission number: 2013XL001-3). Volunteer donors fasted overnight before collecting blood from the vein in the morning. Fresh blood was collected into plastic tubes containing 3.8% sodium citrate and used within 3 h.

Aged vinegar was purchased from Ziyuan Microorganism R&D Co., Ltd. (Shanxi, China). Acetic acid, formic acid, lactic acid, citric acid, malic acid, and tartaric acid were all purchased from Beijing Chemical Works (Beijing, China). Heparin was purchased from Beijing Ke-bai-ao Biotechnology Co. (Beijing, China). Heparin, arachidonic acid (AA),

collagen, adenosine diphosphate (ADP), thrombin, bovine serum albumin (BSA), β-nicotinamide adenine dinucleotide (reduced form; β-NADH), and pyruvic acid were purchased from Sigma Chemical Co. (St. Louis, MO, USA). Human cyclooxygenase-1 (COX1) EIA and thromboxane-$B_2$ ($TXB_2$) EIA kits were purchased from Feng-xiang Biological Co. (Shanghai, China). Bovine fibrinogen, urokinase standard, and thrombin were purchased from National Institutes for Food and Drug Control (Beijing, China). A protein marker (4.6–300 kDa) was purchased from ProSieve QuadColor$^{TM}$ Lonza (China). All other chemicals and reagents were of analytical grade.

### 2.2. Preparation of platelet-rich plasma

Platelet-rich plasma (PRP) was prepared as described previously, with slight modifications (Kim *et al.*, 2011; Yu *et al.*, 2011). Fresh blood was collected in plastic tubes containing 3.8% sodium citrate. The ratio of blood to sodium citrate was 1:9 (v:v). PRP was obtained by centrifugation of blood at 150 × *g* for 10 min at room temperature (25 °C). PRP supernatants were used for the aggregation study. To prepare washed platelets (WP), blood was directly collected into plastic tubes containing anticoagulant citrate dextrose (ACD: 2.5% trisodium citrate, 2% dextrose, and 1.5% citric acid), and adjusted to a ratio of 1:9 (v:v). After centrifugation at 150 × *g* for 10 min at room temperature, platelets were isolated and centrifuged again at 150 × *g* for 10 min in washing buffer (Tyrode's solution containing 10% ACD buffer and 0.3% BSA). Platelets were diluted with Tyrode's solution (11.9 mM NaCl, 2.7 mM KCl, 2.1 mM $MgCl_2$, 0.4 mM $NaH_2PO_4$, 11.9 mM $NaHCO_3$, 11.1 mM glucose, and 3.5 mg/mL BSA, pH 7.2) containing 0.3% BSA. To make platelet-poor plasma (PPP), PRP was centrifuged at 1,200 × *g* for 10 min at room temperature and supernatants were obtained as PPP.

### 2.3. Preparation of vinegar samples, organic acid samples, and heparin

Acetic, formic, lactic, citric, malic, and tartaric acid were dissolved in 0.9% NS to yield concentrations of 0.50, 1.00, and 2.00 mM. Vinegar and heparin were also dissolved in 0.9% NS to obtain concentrations of 0.12 mL/L. Another 10 mL of acetic, formic, lactic, citric, malic, or tartaric acid was neutralized by sodium hydroxide (1.5 mM) to obtain neutralized organic acid.

### 2.4. Antiplatelet activity of acetic acid

The antiplatelet activity of acetic acid was determined as described previously, with slight modifications (Seo *et al.*, 2012). PRP was adjusted with PPP to obtain a platelet count of $2 \times 10^8$ platelets/mL. PRP was pre-incubated with vinegar (0.12 mL/L), acetic acid (0.50, 1.00, and 2.00 mM), heparin (2.00 mM), or 0.9% NS (vehicle) at 37°C in a LBY-NJ4 aggregometer (Precil, Beijing, China) for 5 min prior to induction by various aggregating agents at 37°C with stirring at 1,000 rpm. Aggregating agents were present at the following final concentrations: AA, 100 μM; collagen, 0.1 mg/mL; ADP, 2.5 μM; and thrombin, 0.5 U/mL. Platelet aggregation was recorded for 5 min after stimulation, and maximum aggregations were obtained. The extent of platelet aggregation was expressed as percent change in light transmission with respect to PPP.

### 2.5. Determination of cytotoxicity

To determine the cytotoxicity of acetic acid, lactate dehydrogenase (LDH) release from platelets was measured as described previously (Seo *et al.*, 2011). After incubating platelet rich plasma (PRP; $2 \times 10^8$ platelets/ml) with vinegar (0.12 mL/L), acetic acid (0.50, 1.00, and 2.00 mM), heparin (2.00 mM), or 0.9% NS for 5 min at 37 °C, PRP was centrifuged at room temperature at 10,000× *g* for 1 min. A 25-μL aliquot of the supernatant was mixed with 100 μL of NADH solution (0.03% β-NAD, a reduced form of the disodium salt found in

phosphate buffer) and 25 μL of pyruvate solution (22.7 mM pyruvic acid in phosphate buffer) at room temperature. The reduction in absorbance at 340 nm due to the conversion of NADH to $NAD^+$ was measured to determine LDH activity in supernatants. LDH release is expressed as the percentage of the total enzyme activity in platelets completely lysed with 0.2% triton X-100.

### 2.6. COX1 activity and $TXA_2$ production

COX1 and $TXB_2$ EIA kits were used to determine the inhibitory effect of acetic acid on COX1 activity and $TXA_2$ production. PRP was incubated with acetic acid (0.50, 1.00, and 2.00 mM), heparin (2.00 mM), or 0.9% NS (as vehicle) for 5 min prior to induction by AA (100 μM) at 37°C with stirring at 1,000 rpm. Sample plasmas were then transferred to the kits according to the manufacturer's instructions. The activity of acetic acid on COX1 was expressed as the percent of inhibition (% inhibition). The content of $TXB_2$ was calculated according to the manufacturer's instructions and expressed as ng/L.

### 2.7. In vivo bleeding time assay

Vinegar (0.12 mL/kg), acetic acid (0.50, 1.00, and 2.00 mL/kg), heparin (2.00 mg/kg), or 0.9% NS (vehicle) was administered, 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 it was no longer stained with blood. When necessary, bleeding was manually stopped after 20 min to prevent the rat's death.

### 2.8. Fibrinolytic activity of organic acids

Fibrin plates were prepared according to the method reported previously (Liu *et al.*, 2008). Briefly, 0.5 g agarose was added into 45 mL phosphate-buffered saline (PBS), pH 7.4, and incubated in a boiling water bath for 30 min to completely dissolve the agarose. After cooling

to 50-60°C, the agarose solution was mixed with fibrinogen solution (prepared by dissolving 25 mg fibrinogen in 5 mL PBS buffer, pH 7.4). One milliliter of thrombin (20 IU/mL) was then added to the mixture to induce fibrin crosslinking. The solution was then rapidly poured into a 9 cm petri dish and shaken for 3-5 s. Fibrin plates were obtained 20 min later.

Fibrin plates were drilled to create six apertures with 2-mm diameters. Organic acid, neutralized-organic acid, or urokinase was added into the aperture. Plates were then incubated at 37°C for 18 h. Fibrinolytic activity per milliliter of organic acid was measured by the transparent circle diameter and expressed as units (U) of urokinase.

To determine whether acetic acid can activate plasminogen, plates were heated to 80°C for 30 min before addition of acetic acid (we refer to this as 'heated plate').

### 2.9. Bule native polyacrylamide gel electrophoresis

Bule native polyacrylamide gel electrophoresis (BN-PAGE) was performed as described previously with a slight modification (Hu *et al.*, 2011). In this study, 7.5% acrylamide separating gel, 5% acrylamide stacking gel, 1.5 M Tris-HCl, pH 8.8, 0.05 M Tris-HCl, pH 6.8, and 10% AP were used. Samples were prepared according to the conditions used for fibrin plate preparation without the addition of agarose. After the addition of acetic acid or 0.9% NS to the holes and incubation at 37 °C for 18 h, a fibrin sample with either acetic acid or NS (10 μL) was loaded onto each lane (Ying, 2008). Electrophoresis was performed at 20 mA. Gel slabs were fixed and stained with 0.25% Coomassie Brilliant Blue (R-250) in 10% acetic acid (methanol: acetic: water, 50:10:40 (v/v/v)) and destained in 7% acetic acid (methanol: acetic: water, 125:35:340 (v/v/v)). The electrophoresis pattern of the experimental lane and control lane were compared.

### 2.10. Statistical analysis

Data are expressed as the means of independent experiments with at least three replications.

Statistical analysis was conducted using the proc GLM procedures of SAS version 9.3 (SAS Institute, Inc., Cary, NC). Group mean comparisons were conducted using LSD means and were considered significant at $P < 0.05$, based on minimum significant differences from one-way analysis of variance (ANOVA) with $\alpha = 0.05$. Correlations were performed using Pearson's correlation values, with $P < 0.05$.

## 3. Results

### 3.1. Acetic acid markedly inhibits platelet aggregation induced by multiple agonists

The inhibitory activity of acetic acid on platelet aggregation is shown in Fig.1. Vinegar (0.12 mL/L) and acetic acid (0.50, 1.00, and 2.00 mM) significantly inhibited collagen-, AA-, ADP-, and thrombin-induced platelet aggregation ($p < 0.05$). Agonist-induced platelet aggregation was reduced by vinegar (0.12 mL/L) to 65.35 ± 0.92%, 30.25 ± 1.34%, 22.36 ± 1.18%, and 2.26 ± 1.01%, respectively. At a concentration of 2.00 mM, acetic acid reduced the collagen-, AA-, ADP-, and thrombin-induced aggregation rates to 72.14 ± 0.62%, 36.82 ± 1.31%, 29.67 ± 1.19%, and 3.20 ± 0.82%, respectively. The same concentration of heparin, a clinical medical standard, decreased agonist-induced platelet aggregation by collagen, AA, ADP and thrombin to 26.95 ± 0.11%, 15.01 ± 1.13%, 71.57 ± 1.21%, and 4.07 ± 0.66%, respectively. We also determined that the antiplatelet activity of acetic acid occurred in a concentration-dependent manner, which suggested that acetic acid strongly inhibits platelet aggregation induced by multiple agonists, and the inhibitory activity was considerable with respect to heparin, especially thrombin- and AA-induced platelet aggregation.

### 3.2. An appropriate concentration of acetic acid has no cytotoxic effect on platelets.

LDH release is used as an index of cellular injury, including injury to platelets (Moon *et al.*, 2000). To examine the cytotoxicity of vinegar and acetic acid, LDH release in platelets was measured. As shown in Fig. 2, there was no significant LDH release in platelets treated with

vehicle (0.9% NS), vinegar (0.12 mL/L), acetic acid (0.50, 1.00 and 2.00 mM), or heparin (2.00 mM). These findings suggest that the antiplatelet effect of acetic acid was not the result of cytotoxic effects, such as platelet membrane disruption.

### 3.3. Acetic acid mediates the AA metabolic pathway by inhibiting COX1 and decreasing $TXA_2$ production

As shown in Table 1, acetic acid (0.50, 1.00, and 2.00 mM) significantly inhibited COX1 activity ($p < 0.05$). At 2.00 mM, the COX1 inhibition rate of acetic acid reached 53.32 ± 5.17%, which was slightly lower than that of 2.00 mM heparin (80.44 ± 7.18%). $TXA_2$ is unstable and can rapidly convert into its stable form $TXB_2$. For this reason, $TXA_2$ production was conducted using a $TXB_2$ EIA kit. AA-induced $TXB_2$ production (100 μM) was significantly inhibited by acetic acid ($p < 0.05$). $TXB_2$ production reached 87.39 ± 1.76 ng/L after incubation of PRP with 0.09% NS, while acetic acid (2.00 mM) reduced $TXB_2$ production to 47.78 ± 1.14 ng/L. These results suggest that acetic acid exerts a strong antiplatelet effect through COX1 inhibition, causing a subsequent reduction in $TXA_2$ production.

### 3.4. Effect of acetic acid on tail bleeding time in rats

To investigate whether vinegar and acetic acid affect normal platelet function in an *in vivo* model, a mouse tail bleeding time experiment was conducted. The bleeding time assay is widely used to evaluate platelet function during normal hemostasis (Cho et al., 2008). The effects of the vinegar and acetic acid extract on tail bleeding time are shown in Table 2. Vinegar (0.12 mL/kg) and acetic acid (0.50, 1.00, and 2.00 mL/kg) extracts significantly prolonged ($p < 0.05$) the bleeding time, by 105.55 s, 100.79 s, 107.01 s, and 123.78 s, respectively, compared to 0.9% NS injection (73.60 ± 2.04 s). The tail bleeding time of rats administered heparin (2.00 mg/kg) was similar to that of rats given vinegar and acetic acid.

These results suggest that acetic acid may be suitable for use as an anti-platelet agent.

### 3.5. Organic acids in vinegar exhibit strong fibrinolytic activity

In this study, the transparent circle diameter on fibrin plates represented the fibrinolytic activity of urokinase; a larger diameter indicated higher activity. The correlation between transparent circle diameter and urokinase concentration is shown in Fig.3. In the range of 0.1–0.8 IU, urokinase concentration and transparent circle diameter exhibit a linear relationship. The fibrinolytic activity of organic acid was calculated according to the curve regression equation, where $y = 2.281x + 0.322$ ($R^2 = 0.9769$), and is expressed as IU urokinase equivalent/mL.

As shown in Fig.4, acetic acid, formic acid, lactic acid, citric acid, malic acid, and tartaric acid exhibited significant, and concentration-dependent, fibrinolytic activity ($p < 0.05$). Formic acid demonstrated the highest fibrinolytic activity, and tartaric acid the lowest (68.54 IU/mL). The fibrinolytic activity of acetic acid (186.64 IU/mL) was the second highest among all agents. At an acetic acid concentration <0.30 mM, no transparent circle was generated on the fiber plate.

### 3.6. $H^+$ plays an important role in the fibrinolytic activity of acetic acid

To determine the action of $H^+$ in the fibrinolytic activity of acids, we compared the fibrinolytic activity of neutralized and non-neutralized acetic acid (Fig.5). Acetic acid (0.3, 0.5, and 1.0 mM) demonstrated strong fibrinolytic activity, while the neutralized acetic acid (0.3, 0.5, and 1.0 mM) did not generate a transparent circle. Therefore, the $H^+$ in acetic acid plays an important role in its fibrinolytic activity.

### 3.7. Organic acids degrade crosslinked fibrin by activating plasminogen

To understand the mechanism underlying the organic acid-induced depolymerization of blood clots, we examined the effects of acetic acid, formic acid, lactic acid, citric acid, malic

acid, and tartaric acid on plasminogen activation through comparison of transparent circle sizes on non-heated and heated fibrin plates. As shown in Fig.6, all organic acids (1.0 mM) produced transparent circles on the non-heated fibrin plate, but not on the heated fibrin plate, suggesting that organic acids degrade crosslinked fibrin by directly activating plasminogen.

The electrophoretic patterns of crosslinked fibrin and fibrin polymers degraded by acetic acid (1.0 mM) are shown in Fig.7. Crosslinked fibrin generated a band with a molecular weight of 284 kDa (lane 1), whereas crosslinked fibrin less than 200 kDa (176 kDa and 69 kDa, lane 2). This finding confirmed that acetic acid exerts fibrinolytic activity by activating plasminogen and ultimately depolymerizing crosslinked fibrin.

## 4. Discussion

Organic acids, such as acetic acid, are important components of dietary-aged vinegar. We first investigated the antiplatelet aggregation and fibrinolytic activity of organic acids in dietary vinegar. We found that acetic acid inhibited the platelet aggregation induced by AA, collagen, ADP, and thrombin. Acetic acid also strongly inhibited COX1 and reduced the formation of $TXA_2$, thereby inhibiting platelet aggregation. Additionally, acetic acid activated plasminogen in fibrin and then cleaved crosslinked fibrin, resulting in a dissolved thrombus.

After vascular damage, collagen binds to the glycoprotein VI (GPVI) receptor on the platelet membrane and induces platelet activation, resulting in increased $[Ca^{2+}]_i$. The activated platelet then changes shape and releases ADP and $TXA_2$ granules (Lee *et al.*, 2006). ADP and $TXA_2$ bind to the $P2Y_1$ and TP receptors, respectively, and can then activate additional platelets (Broos *et al.*, 2012). Arachidonic acid (AA) metabolism can begin once $Ca^{2+}$ increases and $TXA_2$ is produced (Jackson *et al.*, 2003). Thrombin is produced during platelet activation; it initiates platelet activation by cleaving the N-terminal exodomain of the

protease-activated receptor (De-Candia, 2012), inducing the membrane receptor GPIb/V/IX complex (Woulfe, 2005). Heparin is commonly used in clinics, since it exerts anticlotting activity by interacting with clotting factors, thus significantly inhibiting the occurrence of thrombosis (Wang & Zhao, 2007; Zhang, 2012). To examine the effect of aged vinegar and acetic acid on platelet aggregation, PRP was stimulated with AA (100 μM), collagen (0.1 mg/mL), ADP (2.5 μM), or thrombin (0.5 U/mL) to induce platelet aggregation. Interestingly, agonist-induced platelet aggregation was significantly inhibited by acetic acid in a concentration-dependent manner ($p < 0.05$). At a concentration of 0.12 mL/L, vinegar reduced platelet aggregation induced by collagen, AA, ADP, and thrombin to 65.35 ± 0.92%, 30.25 ± 1.34%, 22.36, ± 1.18%, and 2.26 ± 1.01%, respectively. Acetic acid (2.00 mM) reduced platelet aggregation induced by collagen-, AA-, ADP-, and thrombin, to 72.14 ± 0.62%, 36.82 ± 1.31%, 29.67 ± 1.19%, and 3.20 ± 0.82%, respectively. However, heparin (2.00 mM), a medical standard, decreased collagen-, AA-, ADP-, and thrombin-induced platelet aggregation to 26.95 ± 0.11%, 15.01 ± 1.13%, 71.57 ± 1.21%, and 4.07 ± 0.66%, respectively. Acetic acid in aged vinegar usually ranges between 5.7% and 9.8% (w/v), thereby representing its major bioactive compound (Fan et al., 2011). These results indicate that acetic acid is a multiple-site inhibitor of platelet aggregation. Acetic acid in aged vinegar may have a therapeutic advantage with respect to preventing thrombus resulting from platelet aggregation.

It is well known that the AA metabolic pathway plays a key metabolic role in platelet aggregation. In this pathway, COX1 is the most important enzyme in AA metabolism associated with the production of $TXA_2$ production (Wang *et al.*, 2011). Phospholipids on the platelet membrane are activated by phospholipase A2 and phospholipase C to release AA. Free AA is then converted to prostaglandin $G_2$ ($PGG_2$) and prostaglandin $H_2$ ($PGH_2$), which is mediated by COX1. Immediately, $PGG_2$ and $PGH_2$ are transformed by thromboxane-$A_2$

synthase (TXS) to $TXA_2$. Upon interaction with the Tp receptor on the platelet membrane, $TXA_2$ plays a key role in platelet shape change and fibrinogen receptor (GPIIb/IIIa) activation, subsequently leading to platelet aggregation (Lee *et al.*, 2013). In this study, COX1 was significantly inhibited by acetic acid at concentrations of 0.50, 1.00, and 2.00 mM ($p < 0.05$). As a result, $TXB_2$ production was reduced significantly. We found that acetic acid (2.00 mM) reduced $TXB_2$ to 47.78 ± 1.14 ng/L, and heparin (2.00 mM) reduced $TXB_2$ production to 33.38 ± 2.71 ng/L. These findings suggest that acetic acid plays an important role in the AA metabolic pathway and exhibits an inhibitory effect on platelet aggregation.

Thrombin cleaves peptides A and B of fibrinogen to produce fibrin; fibrin monomers are then polymerized through hydrogen bonds and hydrophobic bonds to the fibrin polymer. Crosslinked fibrin can then recruit platelets and other blood molecules, leading to the formation of thrombus (Liu *et al.*, 2008). Plasminogen is activated to generate plasmin, which can directly degrade crosslinked fibrin in plasma, and indirectly inhibit platelet aggregation (Yang, 2011). Therefore, plasminogen is the key to preventing thrombotic disorders. Generally, commercial fibrinogen contains little plasminogen. In the present study, plasminogen was inactivated when the plate was heated, and the fibrinolytic activity of organic acids disappeared. We also found that neutralized acetic acids did not demonstrate fibrinolytic activity. These results indicated that organic acids are plasminogen activators. Electrophoresis experiments showed that acetic acid degraded crosslinked fibrin, cleaving it into two small proteins. These results further suggest that the fibrinolytic activity of acetic acid is mediated mainly by its activation of plasminogen; the plasmin thus generated degrades crosslinked fibrin, thereby preventing thrombosis.

The organic acids in dietary vinegar include acetic acid, lactic acid, formic acid, citric acid, malic acid, and tartaric acid. Our data indicated that these organic acids possess considerable fibrinolytic activity. Acetic acid accounts for 90% of all organic acids in vinegar (Wang *et al.*,

2013). We determined that the fibrinolytic activity of acetic acid is equivalent to 186.64 U of urokinase per milliliter, a considerable amount. When ingested, food containing acetic acid within the range 0.61-9.08 mg/mL can lead to levels of 0.61-6.05 mg/mL in the small intestine (Ogawa *et al.*, 2000). The present study found that the lowest concentration of acetic acid that inhibited thrombosis was 0.3 mM, (pH 3.63); this concentration has no effect on the body. Currently, we have no data on the absorption rate of vinegar from the intestine to plasma. Here, we hypothesize that the absorption rate is 10%. According to this hypothesis, the acetic acid content in food must be at least 864 mg to prevent thrombosis (presuming that the body weight of a normal individual is 60 kg and blood volume is 4.8 L). In China, the total acid content must reach levels of 35 mg acetic acid equivalent/mL (Chinese National standard, GB18187-2000), indicating that humans must ingest at least 25 mL vinegar to yield an acetic acid level of 0.30 mM. This concentration represents the lowest plasma acetic acid level for degradation of crosslinked fibrin and prevention of thrombosis. Although direct oral intake of vinegar is not possible due to its high acidity, our results strongly suggest that daily ingestion of vinegar could prevent thrombotic disorders.

## 5. Conclusions

In conclusion, this study was the first to demonstrate that acetic acid has considerable antiplatelet activity and could be a multiple-step inhibitor of platelet aggregation. Vinegar and acetic acid inhibited the activity of COX1, reduced $TXA_2$ production, and attenuated platelet aggregation. Acetic acid was also found to be an activator of plasminogen; fibrin is then degraded by plasmin resulting in a fibrin monomer. These results indicate that organic acids from aged vinegar, especially acetic acid, have a therapeutic effect with respect to preventing thrombosis due to their potential to inhibit multiple steps in the platelet aggregation pathway and degrade crosslinked fibrin by activating plasminogen.

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**Captions**

**Fig.1** Effect of acetic acid on platelet aggregation induced by AA (A), collagen (B), ADP (C) and thrombin (D). Data are expressed as means ± standard deviation (SD) (n = 3). Different letters indicate significant differences among groups ($p < 0.05$).

**Fig.2** The effect of acetic acid on LDH release by platelets. LDH release was measure after platelet was treated with either vehicle (0.9% NS) or samples for 5 min. Date are expressed as means ±standard deviation (SD) (n = 3). Different letters indicate significant differences among groups ($p < 0.05$).

**Fig.3** Fibrinolytic activity of urokinase

**Fig.4** Fibrinolytic activity of organic acids. Data are expressed as means ± SD (n = 3). Different letters indicate significant differences among groups ($p < 0.05$).

**Fig.5** Fibrinolytic activity of neutralized and non-neutralized acetic acid. Holes 1, 2 and 3 represent 0.3, 0.5 and 1.0 mM non-neutralized acetic acid, respectively. Holes 4, 5 and 6 represent 0.3, 0.5 and 1.0 mM neutralized acetic acid, respectively.

**Fig.6** Heat treatment to test fibrinolytic activity of organic acids. A, plate heated to 80˚C for 30 min; B, plate not heated. Formic acid (1.0 mM), acetic acid (1.0 mM), lactic acid (1.0 mM), citric acid (1.0 mM), malic acid (1.0 mM), and tartaric acid (1.0 mM) were added to holes 1-6 on plates A and B, respectively.

**Fig.7** BN-PAGE electrophoretic patterns of crosslinked fibrin (lane 1) and fibrin polymers

depolymerized by acetic acid (lane 2).

**Table 1**-Effect of acetic acid on COX1 and $TXB_2$ production[a, b]

| | COX1 inhibition (%) | $TXB_2$ concentration (ng/L) |
|---|---|---|
| Heparin (2.00 mM) | 80.44 ± 7.18 A | 33.38 ± 2.71 A |
| Acetic acid (0.50 mM) | 20.16 ± 4.02 B | 68.69 ± 2.28 B |
| Acetic acid (1.00 mM) | 32.37 ± 6.32 C | 60.76 ± 1.60 C |
| Acetic acid (2.00 mM) | 53.32 ± 5.17 D | 47.78 ± 1.14 D |

[a] Data are expressed as means ± SD (n = 4).
[b] Data within the same column with different letters are significantly different ($p < 0.05$).

**Table 2**-Effect of vinegar, acetic acid on tail bleeding time of rats[a, b, c]

| Sample | Dose | Tail bleeding time (s) | n |
|---|---|---|---|
| 0.9% NS | 2.00 (mL/kg) | 73.60 ± 2.04 | 10 |
| Heparin | 2.00 (mg/kg) | 168.13 ± 1.25 A | 9 |
| Vinegar | 0.12 (mL/kg) | 179.15 ± 0.95 B | 9 |
| | 0.50 (mL/kg) | 174.39 ± 1.18 C | 9 |
| Acetic acid | 1.00 (mL/kg) | 180.61 ± 1.60 D | 9 |
| | 2.00 (mL/kg) | 197.38 ± 1.34 E | 9 |

[a] Samples were administered intraperitoneally once a day for 7 days. Heparin was injected intravenously 2 min before thrombin.
[b]Data are expressed as means ± SD.
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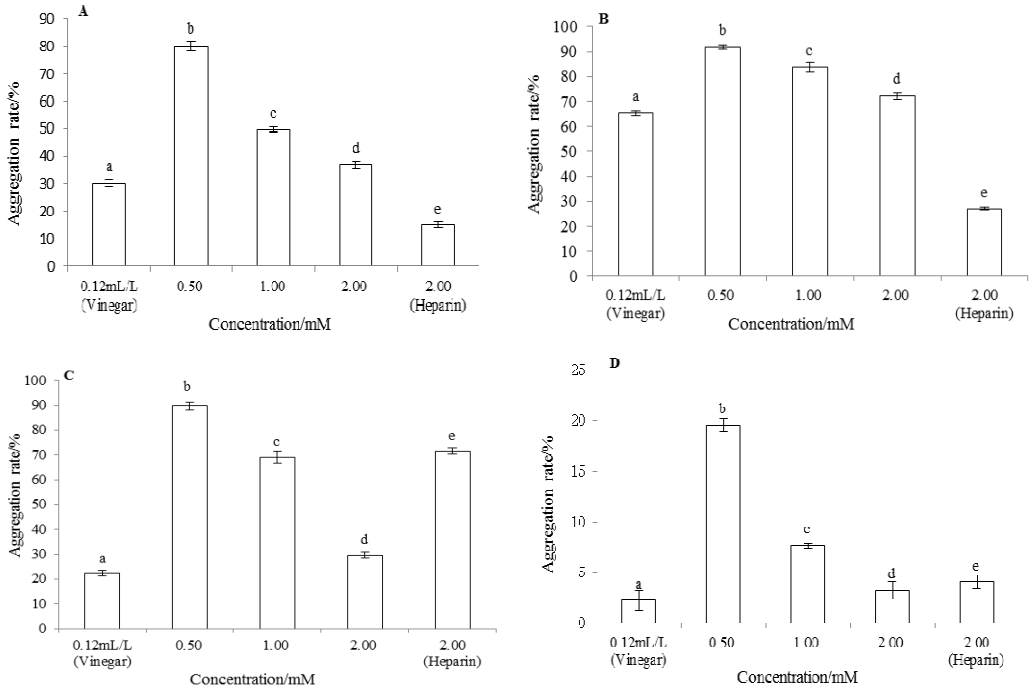


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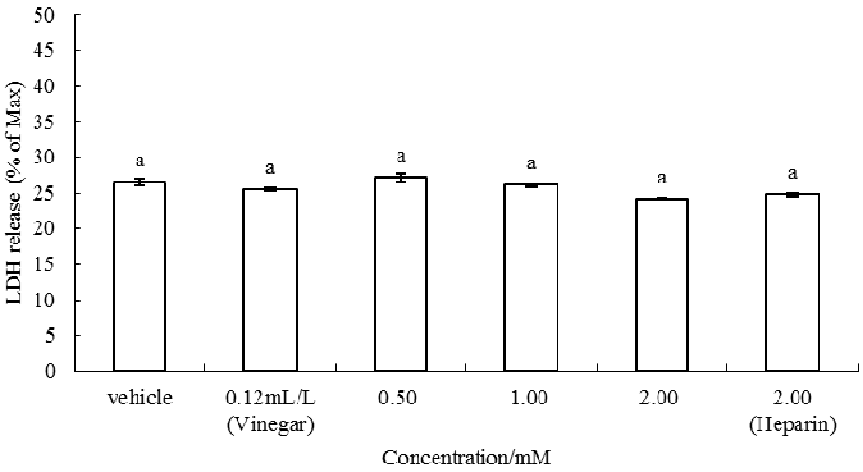


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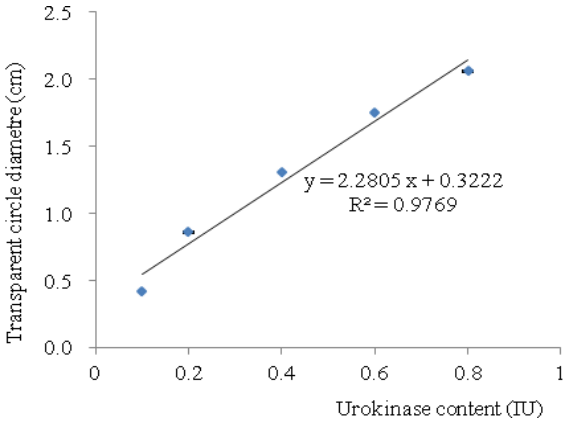


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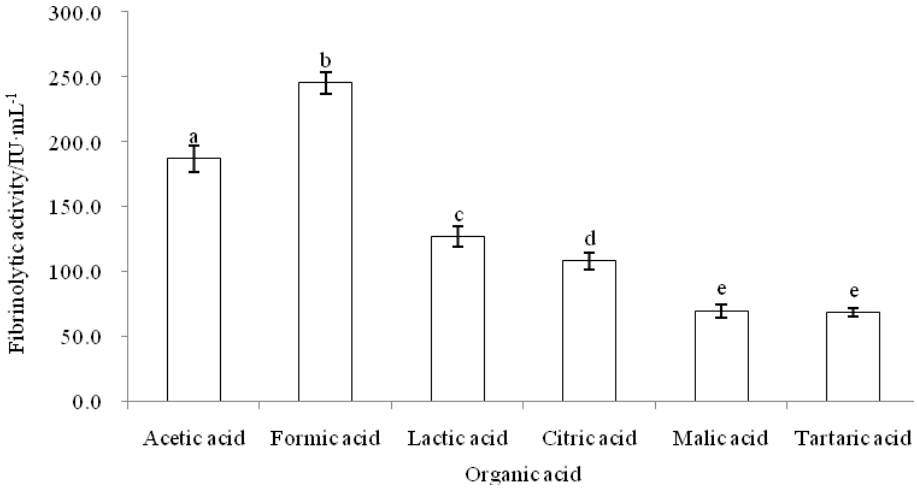


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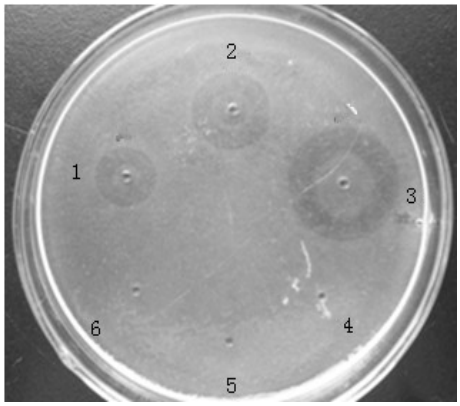


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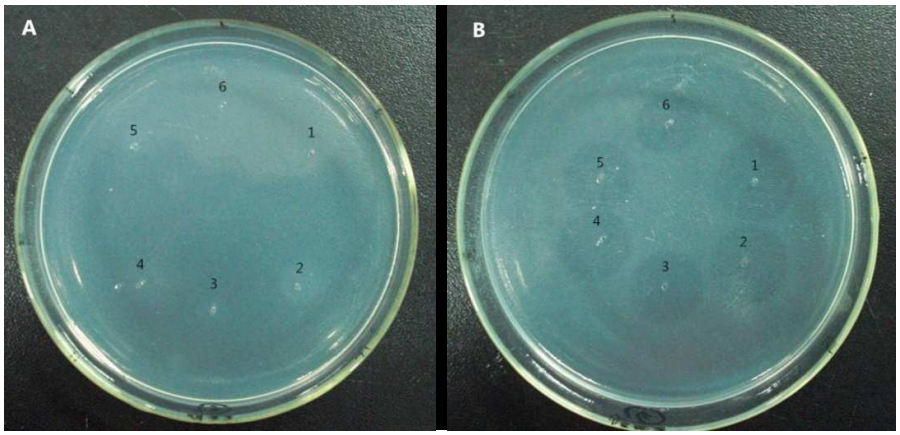


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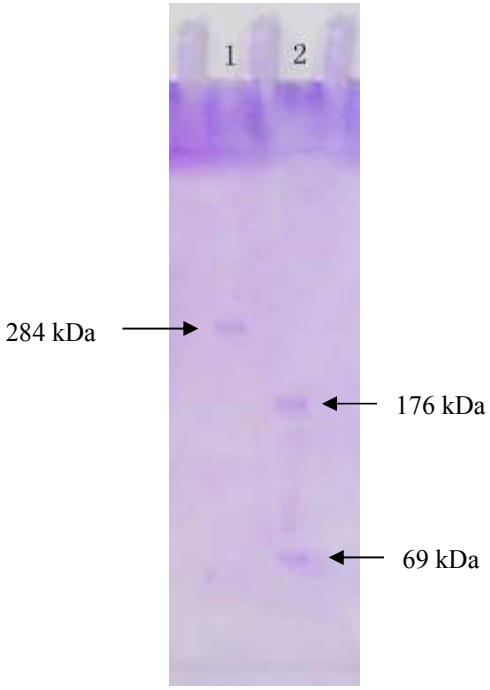


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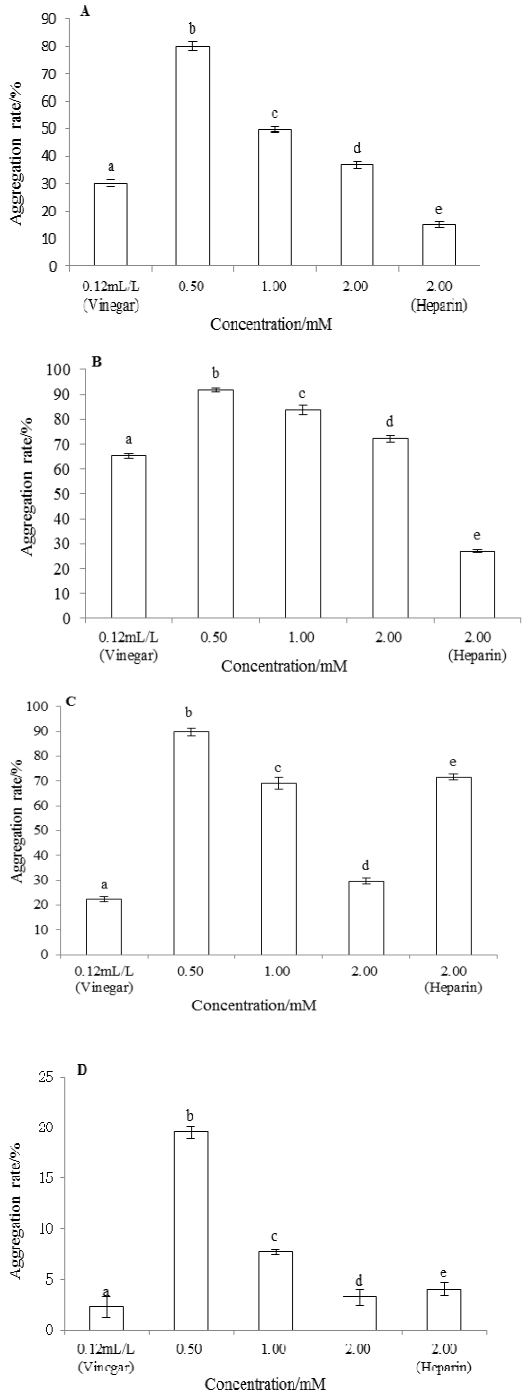


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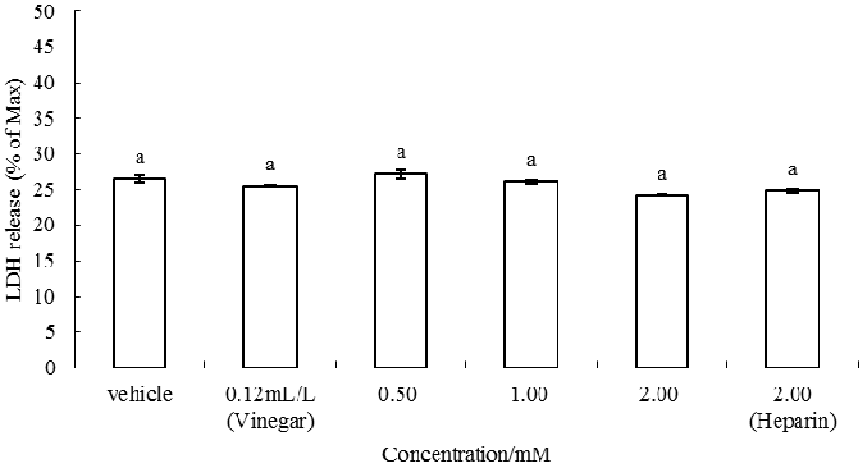


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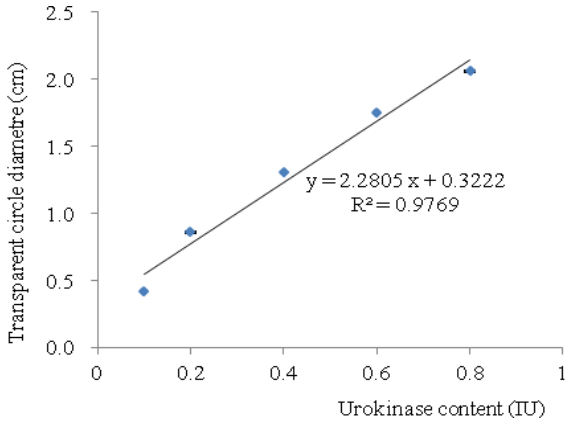


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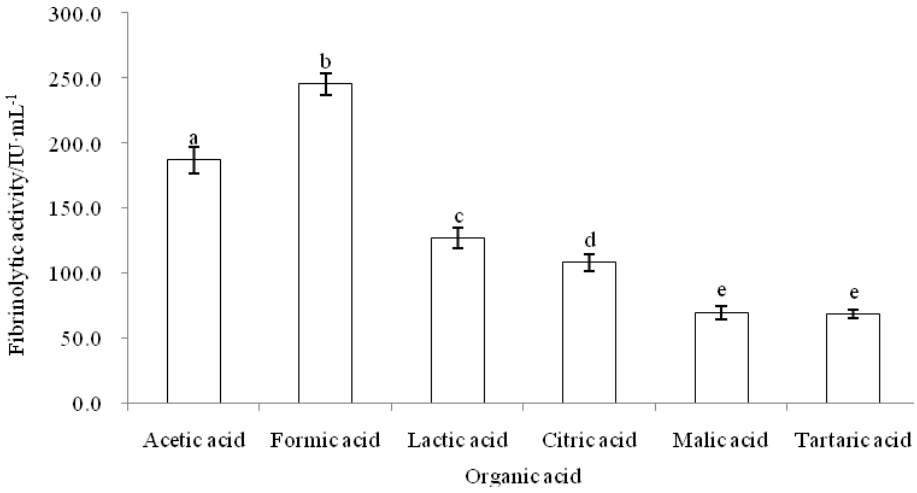


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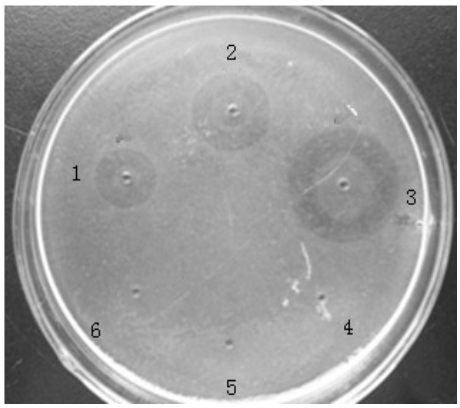


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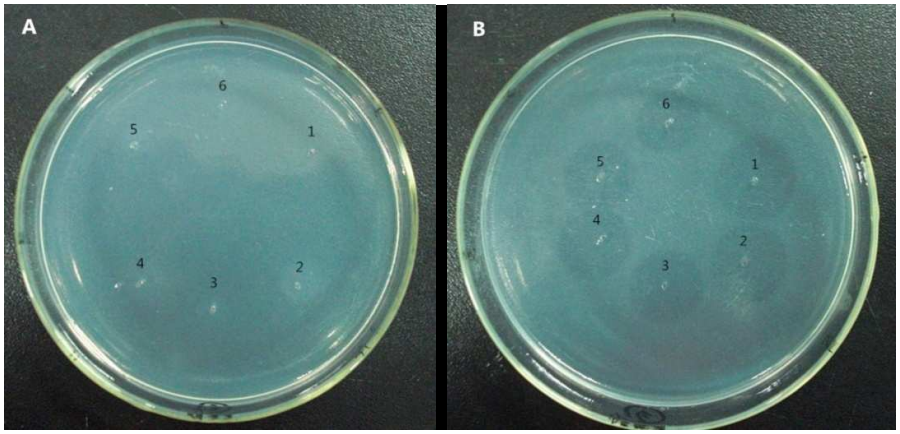


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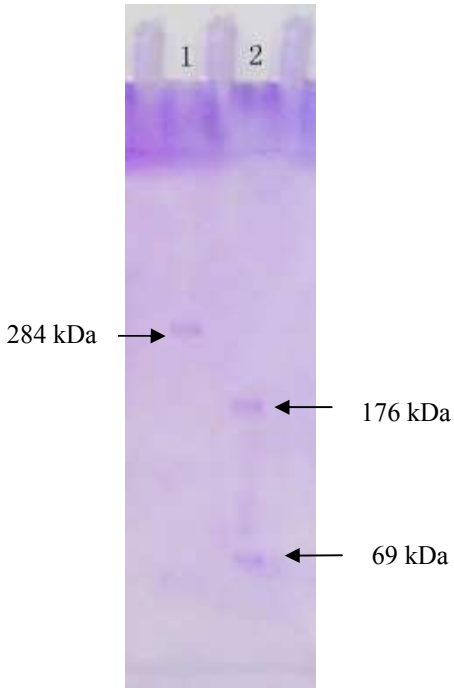


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