

# Therapeutic Effects of *Cyathula officinalis* Kuan and Its Active Fraction on Acute Blood Stasis Rat Model and Identification Constituents by HPLC-QTOF/MS/MS

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## ABSTRACT

**Background:** *Cyathula officinalis* Kuan is widely used in the clinics for the treatment of blood stasis in China. **Objective:** To evaluate the improving blood rheology and anti-inflammatory properties of *C. officinalis* Kuan extract (CO) and its active fraction (ACO) on acute blood stasis model Wistar rats and characterize the correlative constituents.

**Materials and Methods:** CO at 0.26, 0.53, and 1.04 g/kg and ACO at 0.38, 0.75, and 1.5 g/kg were administered to acute blood stasis model Wistar rats for 3 days. Whole blood viscosity, plasma viscosity, and the levels of interleukin-6 (IL-6), nitric oxide (NO), tumor necrosis factor alpha (TNF- $\alpha$ ), and cyclooxygenase-2 (COX-2) in the plasma were measured. HPLC-QTOF/MS/MS method was used to identify the major constituents of ACO; the properties of two representative components (cyasterone and chikusetsusaponin IV) from ACO on thrombin-induced human umbilical vein endothelial cells damage model were also assessed by the levels of thromboxane A2 (TXA2), endothelin (ET), malondialdehyde (MDA), COX-2, endothelial nitric oxide synthase (eNOS), and superoxide dismutase (SOD).

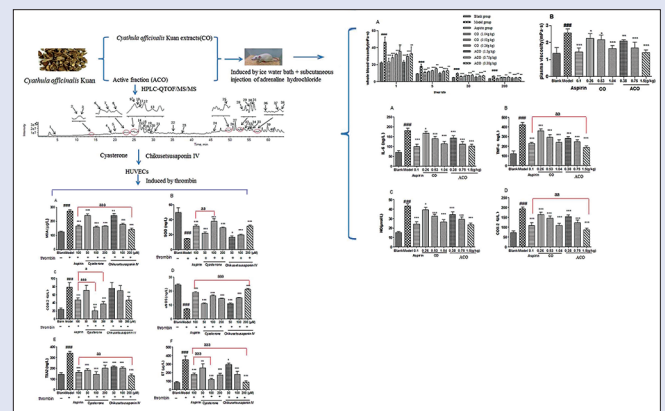
**Results:** CO and ACO significantly reduced whole blood viscosity, plasma viscosity, and levels of IL-6, NO, TNF- $\alpha$ , and COX-2 *in vivo*. Forty compounds were identified from ACO, mainly as phytoecdysteroids and saponins. Cyasterone and chikusetsusaponin IV could significantly inhibit levels of TXA2, ET, MDA, and COX-2 and promote the activities of eNOS and SOD *in vitro*. **Conclusion:** CO and ACO possessed significant improving blood rheology and anti-inflammatory effects on acute blood stasis model rats and the representative components Cyasterone and chikusetsusaponin IV showed significant anti-inflammatory, antioxidant, and anticoagulant effects *in vitro*.

**Key words:** Acute blood stasis model rats, anti-inflammatory, *Cyathula officinalis* Kuan, HPLC-QTOF/MS/MS, improving blood rheology

## SUMMARY

- Cyathula officinalis* Kuan is widely used in the clinic for the treatment of blood stasis in China
- The *C. officinalis* Kuan extract and the active fraction of *C. officinalis* Kuan (ACO) possessed significant improving blood rheology and anti-inflammatory effects on acute blood stasis model rats
- Forty compounds were identified from ACO, mainly as phytoecdysteroids and saponins

- The representative components cyasterone and chikusetsusaponin IV showed significant anti-inflammatory, antioxidant, and anticoagulant effects on thrombin-induced human umbilical vein endothelial cells damage model.



**Abbreviations used:** TCM: Traditional Chinese Medicine, CO: *Cyathula officinalis* Kuan extract, ACO: Active fraction of *Cyathula officinalis* Kuan, ROS: Reactive oxygen species, IL-6: Interleukin-6, TNF- $\alpha$ : Tumor necrosis factor alpha, NO: Nitric oxide, COX-2: Cyclooxygenase-2, TXA2: Thromboxane A2, ET: Endothelin, MDA: Malondialdehyde, eNOS: Endothelial nitric oxide synthase, SOD: Superoxide dismutase, ESI: Electronic spray ionization, ELISA: Enzyme-linked immunosorbent assay, HUVECs: Human umbilical vein endothelial cells, DMEM: Dulbecco's modified Eagle medium, MMP: Matrix metalloproteinase.

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## INTRODUCTION

*Cyathula officinalis* Kuan ("Chuan Niu Xi" in Chinese) deriving from the root of *C. officinalis* (Amaranthaceae family), which is a commonly used medicinal plant, plays an important role in the clinical treatment of Traditional Chinese Medicine (TCM) with functions of strengthening bones and muscles and activating blood and removing blood stasis.<sup>[1]</sup> It is a safe and effective herb widely used for the treatment of lower extremity joint pain, joint flexion, and extension negative, inducing urination and relieving stranguria with a medicinal history of more than 1000 years. It can be mainly used to activate blood and remove blood stasis in clinical application, such as treatment of cardiovascular and cerebrovascular

diseases, arthritis, and dysmenorrhea. *C. officinalis* Kuan has been listed in Chinese Pharmacopoeia as a blood-activating herb and is a health

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The aqueous extract of *C. officinalis* Kuan (CO) has been reported to exhibit anti-inflammatory activity through inhibiting the mice auricle tumefaction degree induced by xylol and rats' feet swelling caused by egg white.<sup>[2]</sup> Various components, such as phytoecdysteroids, saponins, flavones, and polysaccharides, have been identified from *C. officinalis* Kuan. Modern pharmacological studies are mostly focused on the activities of polysaccharides from *C. officinalis* Kuan. For example, the polysaccharides of *C. officinalis* Kuan have been reported to possess immune enhancement activity by upregulating humoral and cellular immune responses.<sup>[3]</sup> Besides, a water-soluble polysaccharide has been reported to have significant antioxidant activity, and a fructan CoPS3 could effectively inhibit the growth of Lewis pulmonary carcinoma implanted in mice.<sup>[4,5]</sup>

At present, there is no systematic research conducted on *C. officinalis* Kuan, especially the therapeutic effects on blood stasis model and correlative components. The active fraction of *C. officinalis* Kuan (ACO) responsible for anti-inflammatory and protective effects on endothelial cells (human umbilical vein endothelial cells [HUVECs]) injury has been investigated previously.<sup>[6]</sup> In this study, the effects of CO and ACO on improving blood rheology and anti-inflammatory properties were examined in acute blood stasis model rats, and the major constituents of ACO were identified by HPLC-QTOF/MS/MS method. Besides, the effects of two representative components (cyasterone and chikusetsusaponin IV) from ACO on thrombin-induced HUVECs damage model were also assessed by levels of thromboxane A2 (TXA2), endothelin (ET), malondialdehyde (MDA), cyclooxygenase-2 (COX-2), endothelial nitric oxide synthase (eNOS), and superoxide dismutase (SOD).

## MATERIALS AND METHODS

### Chemicals and reagents

Chloral hydrate was purchased from Sinopharm Chemical Reagent Beijing Co., Ltd; adrenaline hydrochloride injection was purchased from Shanghai Harvest Pharmaceutical Co., Ltd.; cyasterone (C<sub>26</sub>H<sub>44</sub>O<sub>8</sub>, MW 520.65) and chikusetsusaponin IV (C<sub>47</sub>H<sub>74</sub>O<sub>18</sub>, MW 927.08) with a purity of 98% as determined by HPLC were purchased from SenBeijia Biological Technology Co., Ltd. (Nanjing, China).

Ethanol, petroleum ether, dichloromethane and *n*-butanol (chemical pure), methanol (HPLC grade) were supplied by Tedia Company Inc. (Fairfield, USA). HPLC-grade formic acid was purchased from Merck Company (Darmstadt, Germany). Enzyme-linked immunosorbent assay (ELISA) kits for interleukin-6 (IL-6), nitric oxide (NO), tumor necrosis factor alpha (TNF- $\alpha$ ), COX-2, TXA2, ET, MDA, eNOS, and SOD were obtained from Roche Diagnostics (Mannheim, Germany). HUVECs and thrombin were purchased from Yi Fei Xue Biotechnology (Nanjing, China). Dulbecco's modified Eagle medium (DMEM) and fetal bovine serum were obtained from Invitrogen (Life Technologies Corporation, Carlsbad, CA, USA). All other reagents were of analytical grade, and distilled water was used to extract and prepare the samples.

### Plant material

*C. officinalis* Kuan was obtained in Sichuan Province, China, in December 2015 (No. 151201). It was identified and authenticated by Prof. Wu-De Kang of the School of Pharmacy of the Nanjing University of Chinese Medicine. A voucher specimen was deposited at the Nanjing University of Chinese Medicine, Nanjing 210023, PR, China.

### Extraction procedures

The dried *C. officinalis* Kuan (1.6 kg) was turned into powder form and refluxed with the 85% ethanol for 10 times (3 times  $\times$  1.5 h).

The extracts were concentrated under vacuum and dried, obtaining CO (79.2 g, with respect to 1.6 kg of the plant material). Then, CO (47 g, with respect to 950 g of the plant material) was resuspended in distilled water and submitted to sequential extraction with petroleum ether, dichloromethane, and *n*-butanol (5 times), obtaining *n*-butanol fraction. Then, *n*-butanol extract was concentrated under vacuum and dried, obtaining the ACO (33.5 g, with respect to 950 g of the plant material). The samples were stored at  $-4^{\circ}\text{C}$  until used.

### Animals

Male Wistar rats (200  $\pm$  30 g) were provided by the Shanghai Jiesijie Experimental Animal Center with animal license: Certificate No. SCXK (Hu) 2013-0006. Animals were housed in a standard breeding room (25°C, relative humidity, 12 h dark-light cycle). All animals were acclimated in the laboratory for at least 7 days before the experiment. The animals were given chow and water *ad libitum* but fasted overnight before the operation. The experiment was approved by the Animal Ethics Committee of Nanjing University of Chinese Medicine and was conducted in accordance with the international guidelines for laboratory animal use and care published by the US National Institutes of Health (NIH Publication No. 85-23, revised 1996).

### Experimental model and drug administration

Dosage of *C. officinalis* Kuan was determined based on the conversions from clinical adult dosage. Thus, the oral dosage in a rat of *C. officinalis* Kuan may be designed to be 5.3 g/kg (crude herbal dose). In the present study, the yields of CO and ACO were 4.95% and 3.53%, respectively. Meanwhile, the oral dosages of CO in the previous studies were taken into consideration in our present study. Therefore, we used three doses of CO at 0.26, 0.53, 1.04 g/kg (equivalent to crude herbal doses 5.3, 10.8, 21.3 g/kg, respectively) and ACO at 0.38, 0.75, 1.5 g/kg (equivalent to crude herbal doses 10.8, 21.3, 42.5 g/kg, respectively) in this study.

Seventy-two male Wistar rats approximately 7–8 weeks of age were randomly divided into nine groups ( $n = 8$ ), including blank group, model group, aspirin group (0.1 g/kg), CO (0.26 g/kg), CO (0.53 g/kg), CO (1.04 g/kg), and ACO (0.38 g/kg), ACO (0.75 g/kg), ACO (1.5 g/kg) groups. All drug treatments were administered, respectively, by gavage for 3 days. Rats of blank group and model group were only orally administered normal saline. The day before taking blood, rats except the blank group were hypodermically injected with adrenaline hydrochloride (0.8 mL/kg) twice at an interval of 4 h. Two hours after the first injection of adrenaline hydrochloride, the rats were kept in ice-cold water (0–2°C) to swim for 5 min.<sup>[7]</sup>

### Measurement of whole blood viscosity and plasma viscosity

All animals were anesthetized with 10% chloral hydrate before surgery. Blood (3 mL) was immediately collected in heparinized tubes from the abdominal aorta. Blood samples (1 mL) were used for measuring the whole blood viscosity. Plasma samples were prepared by centrifuging at 3000 rpm for 15 min. Whole blood viscosity and plasma viscosity were measured as soon as possible by a LG-R-80 B automated viscometer.

### Measurement of plasma interleukin-6, nitric oxide, tumor necrosis factor- $\alpha$ , and cyclooxygenase-2 values

Plasma samples were prepared by centrifuging at 3000 rpm for 15 min and the levels of IL-6, NO, TNF- $\alpha$ , and COX-2 were measured by ELISA kits (R and D Systems, Minneapolis, MN, USA) according to the manufacturer's instructions. Briefly, ELISA plates were coated with specific IL-6, NO, TNF- $\alpha$ , or COX-2 antibody. Diluted plasma samples were

added into the wells in duplicate, followed by adding IL-6, NO, TNF- $\alpha$ , or COX-2 to wells, and incubated for 1 h at 37°C. After washing, horseradish peroxidase-labeled IL-6, NO, TNF- $\alpha$ , or COX-2 monoclonal antibody was pipetted into the wells, and the plates were incubated for 1 h at 37°C. After washing completely, 3,3',5,5'-tetramethylbenzidine substrate solution was added to the wells. Color development was stopped with sulfuric acid solution, and the intensity of the color was measured at 450 nm by an ELISA plate reader. Concentrations of the samples were determined by comparison with standard curves generated with purified isotypes.

### The analysis of components of active fraction of *Cyathula officinalis* Kuan with HPLC-QTOF/MS/MS

Ten milligrams of ACO was dissolved in 1 mL methanol (HPLC grade) and filtered through a 0.45  $\mu$ m filter to the analysis. HPLC-QTOF/MS/MS analysis was performed on an LC-20a Shimadzu HPLC system coupled to the orthogonal AB SCIEX Triple TOFTM 5600 mass spectrometry equipped with an electrospray ionization source (ESI). The chromatographic separation was achieved with a Thermo C<sub>18</sub> ODS HYPERSIL column (150 mm  $\times$  4.6 mm, 5  $\mu$ m) at 30°C. A mixture of solvent A (0.5% formic acid; v/v) and solvent B (methanol) was used as the mobile phase at a flow rate of 1 mL/min. The gradient elution program was as follows: 0–5 min, 5%–5% B; 5–20 min, 5%–40% B; 20–40 min, 40%–50% B; 40–50 min, 50%–75% B; 50–65 min, 75%–90% B; 65–75 min, 90%–100% B. The injection volume was 2  $\mu$ L. ESI-MS spectra were operated in positive and negative ion modes, collecting full-scan MS data from 50 to 1500 *m/z*. The conditions of MS analysis were used as follows: capillary voltage, 2800 V; source temperature, 100°C; cone voltage, 20 V; collision energy, 35 V; spray voltage, 20 V; nebulizer, 55 psi; aux gas pressure, 60 psi; curtain gas, 40 psi; IS20V; gas flow 450 (L/HR); desolvation temperature 250°C; detector, time of flight mass spectrometer.

### Cell culture

HUVECs were cultured in DMEM containing 10% fetal bovine serum and 1% penicillin/streptomycin solution at 37°C in a humidified incubator with 5% CO<sub>2</sub>. The medium was changed every 48 h until cells reached 80%–90% confluency. After confluence, HUVECs were digested with 0.25% trypsin. Cells at passage 3–6 were used for the experiment.

### Enzyme-linked immunosorbent assay

Cells ( $2 \times 10^5$  cells/well) were seeded in wells of 24-well plates, pretreated with aspirin (100  $\mu$ M), cyasterone, or chikusetsusaponin IV (50, 100, and 200  $\mu$ M) for 24 h, and then stimulated with thrombin (15 U/mL)

for another 24 h. The levels of TXA<sub>2</sub>, ET, COX-2, MDA, eNOS, and SOD were measured in the supernatant using commercial ELISA kits.

### Statistical analysis

All data were represented as mean  $\pm$  standard deviation. Statistical analysis of data was performed with GraphPad Prism 5.0 software (GraphPad software, Inc., CA, USA) and was calculated using one-way analysis of variance (ANOVA). Values of  $P < 0.05$  were considered statistical significance.

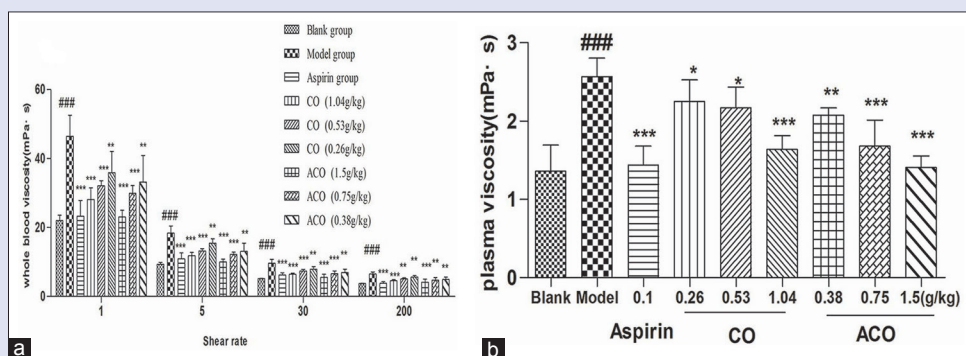
## RESULTS

### Improving blood rheology effects of *Cyathula officinalis* Kuan extract and active fraction of *Cyathula officinalis* Kuan on acute blood stasis model rats

As shown in Figure 1a and b, the values of whole blood viscosity under different shear rates and plasma viscosity of model group were obviously increased ( $P < 0.001$ ) compared with the blank group, indicating that the model was constructed successfully. Compared with the model group, whole blood viscosity and plasma viscosity were significantly decreased to various degrees ( $P < 0.05$  or  $P < 0.01$  or  $P < 0.001$ ) after the treatment with aspirin or CO (0.26, 0.53, 1.04 g/kg) or ACO (0.38, 0.75, 1.5 g/kg). Especially, at the same crude herbal dose, the improving blood rheology effects of ACO (0.75 g/kg, 0.38 g/kg) were same to CO (1.04 g/kg, 0.53 g/kg), respectively. Moreover, the effects of CO (1.04 g/kg) and ACO (0.75 g/kg, 1.5 g/kg) on improving blood rheology were same to aspirin group. The reduction of whole blood viscosity and plasma viscosity suggested that CO and ACO possessed a significant therapeutic effect on acute blood stasis model rats.

### Effects of *Cyathula officinalis* Kuan extract and active fraction of *Cyathula officinalis* Kuan on the plasma levels of interleukin-6, tumor necrosis factor- $\alpha$ , nitric oxide, and cyclooxygenase-2 of acute blood stasis model rats

As shown in Figure 2a-d, the levels of IL-6, TNF- $\alpha$ , NO, and COX-2 in the model group were significantly higher than those in the blank group ( $P < 0.001$ ). Compared with the model group, the levels of IL-6, TNF- $\alpha$ , NO, and COX-2 were significantly reduced ( $P < 0.001$ ) in the aspirin group. As shown in Figure 2a and c, the levels of IL-6 and NO were significantly decreased in CO ( $P_{0.26 \text{ g/kg}} < 0.05$ ,  $P_{0.53, 1.04 \text{ g/kg}} < 0.001$ ) and



**Figure 1:** Whole blood viscosity at different shear rates (a) and plasma viscosity (b) on acute blood stasis model rats of aspirin group, the CO (0.26, 0.53, 1.04 g/kg) and ACO (0.38, 0.75, 1.5 g/kg) dose groups assay at 30 min after the last oral administration. Data are expressed as mean  $\pm$  standard deviation ( $n = 8$ ).  $###P < 0.001$  as compared with the blank group.  $*P < 0.05$ ,  $**P < 0.01$ ,  $***P < 0.001$  as compared with the model group. CO (1.04 g/kg) and ACO (0.75 g/kg) have a same crude herbal dose (21.3 g/kg); CO (0.53 g/kg) and ACO (0.38 g/kg) have a same crude herbal dose (10.8 g/kg). CO: *Cyathula officinalis* Kuan extract, ACO: Active fraction of *Cyathula officinalis* Kuan



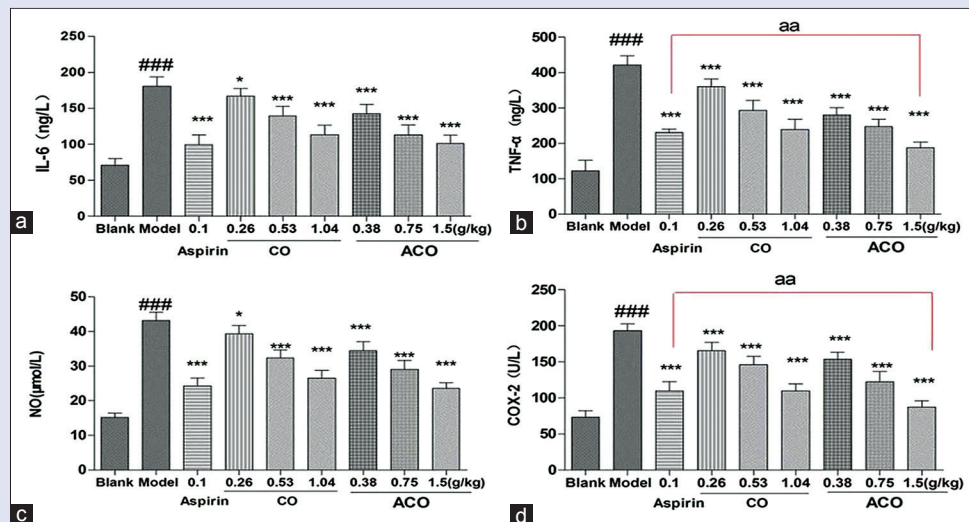
ACO ( $P_{0.38, 0.75, 1.5 \text{ g/kg}} < 0.001$ ) groups compared with the model group. As shown in Figure 2b and d, the levels of TNF- $\alpha$  and COX-2 were significantly lower ( $P < 0.001$ ) than those in the model group after treatment with CO (0.26, 0.53, 1.04 g/kg) and ACO (0.38, 0.75, 1.5 g/kg). Especially, at the same crude herbal dose, the inhibition of IL-6, TNF- $\alpha$ , NO, and COX-2 of ACO (0.75 g/kg, 0.38 g/kg) was same to CO (1.04 g/kg, 0.53 g/kg) groups, respectively. Moreover, the anti-inflammatory effects on the inhibition of IL-6, TNF- $\alpha$ , NO, and COX-2 in CO (1.04 g/kg) and ACO (0.75 g/kg) groups were same to aspirin group. What is noteworthy is that the levels of TNF- $\alpha$  and COX-2 in ACO (1.5 g/kg) group have significant differences ( $P < 0.01$ ) compared with aspirin group.

### HPLC-QTOF/MS/MS analysis

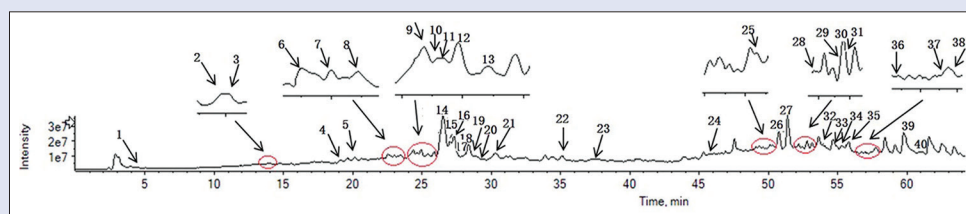
To accurately identify the components of ACO, HPLC-QTOF/MS/MS spectra in positive and negative ion modes were acquired. The total ion chromatogram in negative ion mode, which provided more compounds information, was shown in Figure 3. According to the extracted ion chromatogram and the ion fragments information as well as the corresponding standards or LC/MS data in the literature,<sup>[8-24]</sup> forty compounds were primarily identified [Table 1] and their structures [Figure 4] were speculated as follows, where the compounds are numbered according to their retention times in the total ion chromatogram. HPLC-QTOF/MS/MS analysis revealed that the major constituents of ACO were phytoecdysteroids and saponins.

A comparison of the retention time with the reference standards and MS data from the literature, compounds 14 and 30 were unambiguously identified as cyasterone and chikusetsusaponin IV, respectively. Compounds 2, 4, 6, 9-12, 15-18, 20, 22-25, 27-29, 32-34, 36, 38-40 were tentatively identified based on a comparison of their MS and MS/MS fragmentation data with relevant references.

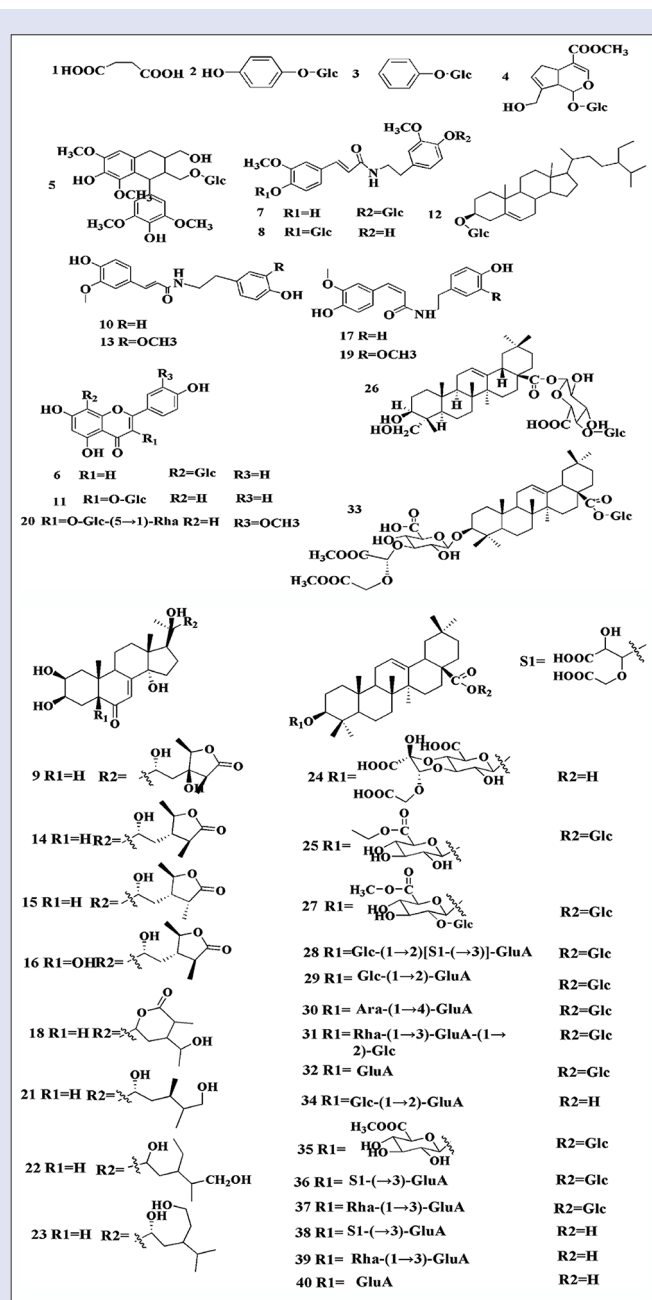
Compound 1 was inferred as succinic acid based on its ions at  $m/z$  117[M-H]<sup>-</sup> in the MS spectra<sup>[8]</sup> and  $m/z$  99[M-H-H<sub>2</sub>O]<sup>-</sup>, 73[M-H-CO<sub>2</sub>]<sup>-</sup> in the MS<sup>2</sup> spectra. Compound 3 was inferred as phenyl- $\beta$ -D-glucopyranoside by its ions at  $m/z$  301[M+HCOOH-H]<sup>-</sup> in the MS spectra<sup>[8]</sup> and  $m/z$  255[M-H]<sup>-</sup>, 161[M-H-C<sub>6</sub>H<sub>5</sub>O]<sup>-</sup>, and 93[M-H-Glc]<sup>-</sup> in the MS<sup>2</sup> spectra. Compound 5 was inferred as lyoniresinol-3 $\alpha$ -O- $\beta$ -D-glucopyranoside based on its ions at  $m/z$  581[M-H]<sup>-</sup> in the MS spectra<sup>[11]</sup> and  $m/z$  419[M-H-Glc]<sup>-</sup>, 371[M-H-Glc-C<sub>2</sub>H<sub>5</sub>O]<sup>-</sup>, 233[M-H-Glc-C<sub>9</sub>H<sub>14</sub>O<sub>4</sub>]<sup>-</sup> in the MS<sup>2</sup> spectra. Compounds 7 and 8 exhibited the same [M-H]<sup>-</sup> ion at  $m/z$  504 in the MS spectra, and the MS<sup>2</sup> spectrums gave fragment ions at  $m/z$  342[M-H-Glc]<sup>-</sup>, 327[M-H-Glc-CH<sub>3</sub>]<sup>-</sup>, 178[M-H-Glc-C<sub>10</sub>H<sub>12</sub>O<sub>2</sub>]<sup>-</sup>. Based on this information and literature,<sup>[13]</sup> they were tentatively attributed to *N-trans*-feruloyl-3-methoxytyramine-4'-O- $\beta$ -D-glucopyranoside and *N-trans*-feruloyl-3-methoxytyramine-4-O- $\beta$ -D-glucopyranoside, respectively. Compounds 13 and 19 exhibited [M-H]<sup>-</sup> ion at  $m/z$  342, 30 Da more than that of compounds 10 and 17. The MS<sup>2</sup> spectrums gave fragment ions at  $m/z$  148, 178, and 190. Hence, they were tentatively attributed to *N-trans*-feruloyl-3-methoxytyramine and *N-cis*-feruloyl-3-methoxytyramine, respectively.<sup>[13,15]</sup> Compound 21 was inferred as makisterone B by its ions at  $m/z$  539[M+HCOOH-H]<sup>-</sup> in the MS



**Figure 2:** Effects of the CO (0.26, 0.53, 1.04 g/kg) and the ACO (0.38, 0.75, 1.5 g/kg) dose groups and aspirin group on values of interleukin-6 (a), tumor necrosis factor- $\alpha$  (b), nitric oxide (c), and cyclooxygenase-2 (d) on acute blood stasis model rats at 30 min after last oral administration. Data are expressed as mean  $\pm$  standard deviation ( $n = 8$ ). ### $P < 0.001$  as compared to the blank group. \* $P < 0.05$ , \*\*\* $P < 0.001$  as compared with the model group. aa $P < 0.01$  as compared with aspirin group. CO (1.04 g/kg) and ACO (0.75 g/kg) have a same crude herbal dose (21.3 g/kg); CO (0.53 g/kg) and ACO (0.38 g/kg) have a same crude herbal dose (10.8 g/kg). CO: *Cyathula officinalis* Kuan extract, ACO: Active fraction of *Cyathula officinalis* Kuan



**Figure 3:** Total ion current chromatogram of the active fraction of *Cyathula officinalis* Kuan



**Figure 4:** Structures of the components identified from the active fraction of *Cyathula officinalis* Kuan

spectra<sup>[8]</sup> and  $m/z$  301, 319, and 475 in the MS<sup>2</sup> spectra. Compound 26 was inferred as 28-O- $\beta$ -D-glucuronopyranosyl-(1  $\rightarrow$  4)- $\beta$ -D-glucopyranosyl hederagenin by its ions at  $m/z$  809[M-H]<sup>-</sup> in the MS spectra<sup>[22]</sup> and  $m/z$  647[M-H-Glc]<sup>-</sup>, 471[M-H-Glc-GluA]<sup>-</sup> in the MS<sup>2</sup> spectra. Compound 31 was inferred as 3-O-[ $\beta$ -D-glucopyranosyl-(1  $\rightarrow$  2)- $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  3)- $\beta$ -D-glucuronopyranosyl]-28-O- $\beta$ -D-glucopyranosyl oleanolic acid by its ions at  $m/z$  1101[M-H]<sup>-</sup> in the MS spectra<sup>[22]</sup> and  $m/z$  955[M-H-Rha]<sup>-</sup>, 939[M-H-Glc]<sup>-</sup>, 777[M-H-2Glc]<sup>-</sup>, 715[M-H-2Glc-H<sub>2</sub>O-CO<sub>2</sub>]<sup>-</sup>, 455[M-H-2Glc-Rha-GluA]<sup>-</sup> in the MS<sup>2</sup> spectra. Compound 35 was inferred as chikusetsusaponin IVa methyl ester by its ions at  $m/z$  807[M-H]<sup>-</sup> in the MS spectra<sup>[22]</sup> and  $m/z$  645[M-H-Glc]<sup>-</sup>, 455 in the MS<sup>2</sup> spectra. Compound 37 was inferred as 3-O-[ $\alpha$ -L-rhamnopyranosyl-(1  $\rightarrow$  3)- $\beta$ -D-glucuronopyranosyl]-28-O- $\beta$ -D-glucopyranosyl oleanolic acid by its ions at  $m/z$  939[M-H]<sup>-</sup>

in the MS spectra<sup>[22]</sup> and  $m/z$  793[M-H-Rha]<sup>-</sup>, 757[M-H-Rha-2H<sub>2</sub>O]<sup>-</sup>, 595[M-H-Rha-2H<sub>2</sub>O-Glc]<sup>-</sup>, 455 in the MS<sup>2</sup> spectra.

## Effects of cyasterone and chikusetsusaponin IV on thrombin-induced human umbilical vein endothelial cells injury model

To determine the effects of cyasterone and chikusetsusaponin IV on thrombin-induced HUVECs injury model, the levels of TXA<sub>2</sub>, ET, MDA, COX-2, eNOS, and SOD in the supernatant were measured. Compared with blank group, when HUVECs were stimulated with thrombin (15 U/mL), the productions of MDA, COX-2, TXA<sub>2</sub>, ET were greatly increased ( $P < 0.001$ ) and of SOD, eNOS were markedly decreased ( $P < 0.001$ ). Cyasterone and chikusetsusaponin IV could effectively inhibit thrombin-stimulated TXA<sub>2</sub>, ET, COX-2, and MDA production and raise SOD and eNOS levels compared with the model group [Figure 5a-f]. Especially, the effect of chikusetsusaponin IV (200  $\mu$ M) on inhibiting the levels of MDA, TXA<sub>2</sub>, ET and promoting the activity of eNOS as well as cyasterone (100  $\mu$ M) on inhibiting the levels of COX-2, ET and promoting the activity of SOD was better than aspirin group (100  $\mu$ M). Taken together, the results profoundly suggested that cyasterone and chikusetsusaponin IV had significant protective effects against thrombin-induced HUVECs injury model.

## DISCUSSION

Inflammation can damage the lining of the arteries and affect the viscosity of the blood, which is the major culprit to blood stasis.<sup>[25]</sup> Moreover, normal blood flow is essential for conveying oxygen and nutrients to cells and is also critical for removing metabolic wastes and reactive oxygen species (ROS). Consequently, blood stasis promotes an oxidative state where ROS accumulate. Oxidative stress and inflammation also promote blood flow abnormalities, causing a vicious pathophysiologic cycle.<sup>[26]</sup> As a result, regulating inflammation and oxidative stress is the important therapeutic approach for blood stasis.<sup>[27]</sup>

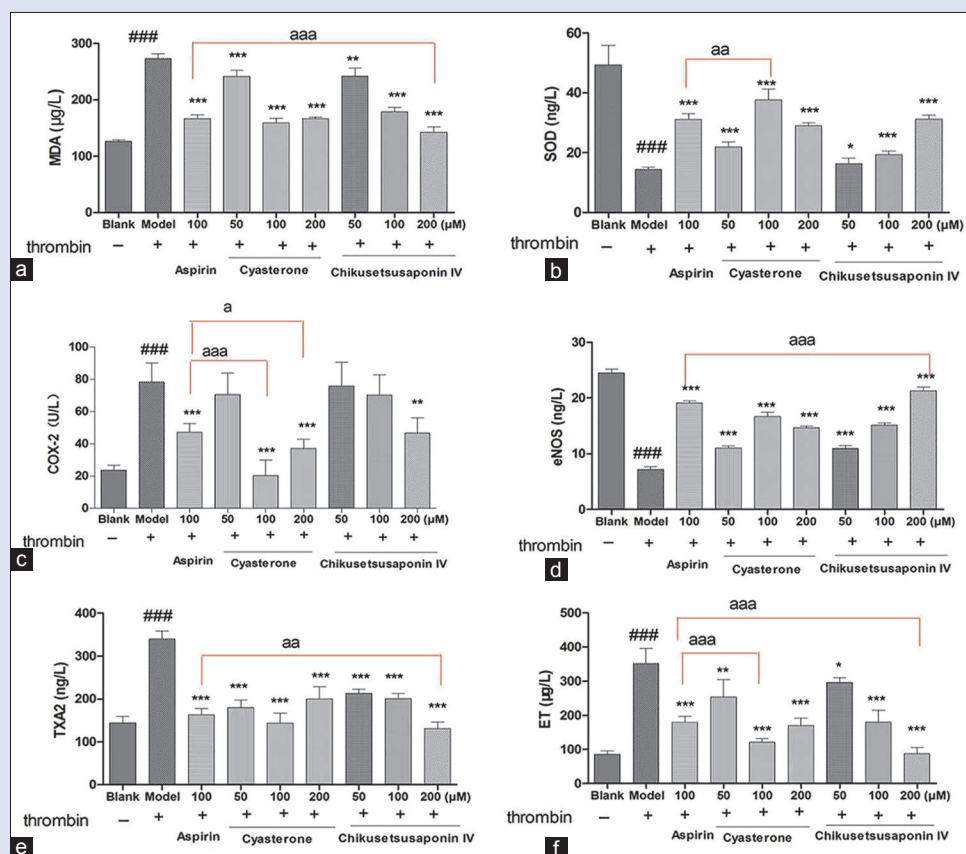
As we all know, *C. officinalis* Kuan is an effective blood-activating herb in clinical application in China with more than 1000 years of medicinal history. However, there is little information available on blood-activating effect and correlative ingredients of *C. officinalis* Kuan. Acute blood stasis rat model induced by ice water bath and subcutaneous injection of adrenaline hydrochloride is a common animal model *in vivo* studies.<sup>[28]</sup> Thus, this study systemically evaluated the improving blood rheology and anti-inflammatory properties of CO and ACO on acute blood stasis model rats by the assessment of whole blood viscosity, plasma viscosity, and levels of IL-6, TNF- $\alpha$ , NO, and COX-2. Endothelial dysfunction plays a pivotal role in the development of blood stasis,<sup>[29]</sup> and thrombin-induced HUVECs damage model is the most frequently used model to evaluate the protective effects of drugs *in vitro*. Hence, the effects of cyasterone and chikusetsusaponin IV on thrombin-induced HUVECs damage model were assessed by levels of TXA<sub>2</sub>, ET, MDA, COX-2, eNOS, and SOD.

IL-6 is an important marker for blood inflammation and the elevated level of IL-6 is associated with increased risk of blood stasis.<sup>[30]</sup> Besides, TNF- $\alpha$ , as a critical initiation factor and inflammatory peptide neurotransmitter released by macrophage, can cause fever, inflammation, and the production of a series of cytokines. Endothelial cell is an important target cell of TNF- $\alpha$ , and endothelial cell injury has important significance in the pathogenesis of blood stasis.<sup>[29]</sup> NO, as an important endothelium-derived substance, can relax blood vessel walls and improve blood circulation throughout the body. However, NO as a free radical is a vital mediator of both physiological and pathological reactions.<sup>[31]</sup> In addition, COX-2 is seldom expressed in most cells under

Table 1: Identified results of the constituents of the active fraction from *Cyathula officinalis* Kuan

n	Retention time (min)	ESI <sup>-</sup> (m/z)		MW	MF	Name	Reference
		MS	MS/MS				
1	4.65	117[M-H] <sup>-</sup>	7399	118	C <sub>4</sub> H <sub>6</sub> O <sub>4</sub>	Succinic acid	[8]
2	13.9	271[M-H] <sup>-</sup>	91,109	272	C <sub>12</sub> H <sub>16</sub> O <sub>7</sub>	Arbutin	[9]
3	14.05	301[M+HCOO] <sup>-</sup>	93,161,255	256	C <sub>7</sub> H <sub>16</sub> O <sub>6</sub>	Phenyl-β-D-glucopyranoside	[8]
4	18.99	387[M-H] <sup>-</sup>	163,207,225	388	C <sub>17</sub> H <sub>24</sub> O <sub>10</sub>	Geniposide	[10]
5	20.63	581[M-H] <sup>-</sup>	233,371,419	582	C <sub>20</sub> H <sub>30</sub> O <sub>13</sub>	Lyoniresinol-3α-O-β-D-glucopyranoside	[11]
6	22.5	431[M-H] <sup>-</sup>	283,311,341	432	C <sub>20</sub> H <sub>30</sub> O <sub>10</sub>	Vitexin	[12]
7	23	504[M-H] <sup>-</sup>	178,327,342	505	C <sub>25</sub> H <sub>31</sub> NO <sub>10</sub>	N-trans-feruloyl-3-methoxytyramine-4'-O-β-D-glucopyranoside	[13]
8	23.51	504[M-H] <sup>-</sup>	178,327,342	505	C <sub>25</sub> H <sub>31</sub> NO <sub>10</sub>	N-trans-feruloyl-3-methoxytyramine-4-O-β-D-glucopyranoside	[13]
9	24.31	581[M-H] <sup>-</sup>	373,391,499,535	536	C <sub>29</sub> H <sub>44</sub> O <sub>9</sub>	24-Hydroxycysterone	[8,14]
10	24.54	312[M-H] <sup>-</sup>	148,178,190,297	313	C <sub>20</sub> H <sub>30</sub> O <sub>4</sub>	N-trans-feruloyltyramine	[13,15]
11	24.7	447[M-H] <sup>-</sup>	153,267,285	448	C <sub>25</sub> H <sub>38</sub> O <sub>11</sub>	Kaempferol-3-O-glucoside	[16]
12	24.96	575[M-H] <sup>-</sup>	283,311,395,413	576	C <sub>35</sub> H <sub>60</sub> O <sub>6</sub>	β-daucosterol	[17]
13	25.12	342[M-H] <sup>-</sup>	148,178,190,327	343	C <sub>20</sub> H <sub>30</sub> O <sub>5</sub>	N-trans-feruloyl-3-methoxytyramine	[13,15]
14	26.5	565[M+HCOO] <sup>-</sup>	199,301,319,501	520	C <sub>29</sub> H <sub>44</sub> O <sub>8</sub>	Cysterone	[8,14]
15	27.15	565[M+HCOO] <sup>-</sup>	199,301,319,501	520	C <sub>29</sub> H <sub>44</sub> O <sub>8</sub>	Isocysterone	[8,14]
16	27.43	535[M-H] <sup>-</sup>	373,391,499	536	C <sub>29</sub> H <sub>44</sub> O <sub>9</sub>	Sengosterone	[8,14]
17	27.58	312[M-H] <sup>-</sup>	148,178,297	313	C <sub>16</sub> H <sub>19</sub> NO <sub>4</sub>	N-cis-feruloyltyramine	[13,15]
18	28.38	565[M+HCOO] <sup>-</sup>	199,301,319,501	520	C <sub>29</sub> H <sub>44</sub> O <sub>8</sub>	Precysterone	[8,14]
19	28.4	342[M-H] <sup>-</sup>	148,178,190,327	343	C <sub>20</sub> H <sub>30</sub> NO <sub>5</sub>	N-cis-Feruloyl-3-methoxytyramine	[13,15]
20	28.62	342[M-H] <sup>-</sup>	153,271,299,315	624	C <sub>28</sub> H <sub>32</sub> O <sub>16</sub>	Isorhamnetin-3-O-rutinoside	[12]
21	30.22	539[M+HCOO] <sup>-</sup>	173,301,319,475	494	C <sub>28</sub> H <sub>46</sub> O <sub>7</sub>	Makisterone B	[8]
22	35.02	553[M+HCOO] <sup>-</sup>	301,319,489,507	508	C <sub>29</sub> H <sub>48</sub> O <sub>7</sub>	Amarasterone A	[8,18,19]
23	37.21	553[M+HCOO] <sup>-</sup>	301,319,471,507	508	C <sub>29</sub> H <sub>48</sub> O <sub>7</sub>	Amarasterone B	[18]
24	45.83	837[M+HCOO] <sup>-</sup>	455,499,661,791	792	C <sub>41</sub> H <sub>60</sub> O <sub>15</sub>	Betavulgaroside II	[20]
25	50.33	821[M-H] <sup>-</sup>	455,645,659,821	822	C <sub>44</sub> H <sub>70</sub> O <sub>14</sub>	Chikusetsusaponin IVa ethyl ester	[21]
26	50.74	809[M-H] <sup>-</sup>	471,647,809	810	C <sub>42</sub> H <sub>66</sub> O <sub>15</sub>	28-O-β-D-glucuronopyranosyl-(1→4)-β-D-glucopyranosyl hederagenin	[22]
27	51.49	969[M-H] <sup>-</sup>	455,645,807	970	C <sub>40</sub> H <sub>78</sub> O <sub>19</sub>	Chikusetsusaponin V methyl ester	[21,23]
28	51.89	1117[M-H] <sup>-</sup>	455,613,793,955	1118	C <sub>53</sub> H <sub>82</sub> O <sub>25</sub>	Achyranthoside D	[24]
29	52.74	955[M-H] <sup>-</sup>	455,631,793	956	C <sub>48</sub> H <sub>76</sub> O <sub>19</sub>	Ginsenoside Ro	[24]
30	52.75	925[M-H] <sup>-</sup>	455,631,793	926	C <sub>47</sub> H <sub>74</sub> O <sub>18</sub>	Chikusetsusaponin IV	[24]
31	52.81	1101[M-H] <sup>-</sup>	455,793,955	1102	C <sub>54</sub> H <sub>86</sub> O <sub>23</sub>	3-O-β-D-glucopyranosyl-(1→2)-α-L-rhamnopyranosyl-(1→3)-β-D-glucuronopyranosyl]-28-O-β-D-glucopyranosyl]oleanolic acid	[22]
32	53.95	793[M-H] <sup>-</sup>	455,569,631	794	C <sub>41</sub> H <sub>66</sub> O <sub>14</sub>	Chikusetsusaponins IVa	[21,24]
33	54.98	953[M-H] <sup>-</sup>	455,791,807,909	954	C <sub>48</sub> H <sub>74</sub> O <sub>19</sub>	Achyranthoside E dimethyl ester	[23,24]
34	55.1	793[M-H] <sup>-</sup>	455,631,731	794	C <sub>42</sub> H <sub>66</sub> O <sub>14</sub>	Zingibroside R1	[21,24]
35	55.74	807[M-H] <sup>-</sup>	455,485,645,745	808	C <sub>43</sub> H <sub>68</sub> O <sub>14</sub>	Chikusetsusaponin IVa methyl ester	[22]
36	56.49	955[M-H] <sup>-</sup>	455,631,793,835	956	C <sub>47</sub> H <sub>72</sub> O <sub>20</sub>	Achyranthoside C	[24]
37	57.75	939[M-H] <sup>-</sup>	455,613,731,793	940	C <sub>48</sub> H <sub>76</sub> O <sub>18</sub>	3-O-β-D-glucopyranosyl-(1→3)-β-D-glucuronopyranosyl]-28-O-β-D-glucopyranosyl]oleanolic acid	[22]
38	57.84	793[M-H] <sup>-</sup>	455,631,673,793	794	C <sub>41</sub> H <sub>62</sub> O <sub>15</sub>	Betavulgaroside IV	[24]
39	60.17	777[M-H] <sup>-</sup>	455,631,701	778	C <sub>42</sub> H <sub>66</sub> O <sub>13</sub>	3-O-β-D-glucuronopyranosyl-(1→3)-β-D-glucuronopyranosyl]oleanolic acid	[22]
40	60.39	631[M-H] <sup>-</sup>	455,613,631	632	C <sub>36</sub> H <sub>56</sub> O <sub>9</sub>	Oleanolic acid 3-O-β-D-glucuronopyranoside	[24]

ESI: Electronic spray ionization



**Figure 5:** Effects of cyasterone and chikusetsusaponin IV on the levels of malondialdehyde (a), superoxide dismutase (b), cyclooxygenase-2 (c), endothelial nitric oxide synthase (d), thromboxane 2 (e), and endothelin (f) with cyasterone, chikusetsusaponin IV (50, 100, and 200  $\mu\text{M}$ ) for 24 h, and then stimulated without or with thrombin (15 U/mL) for 24 h. Aspirin (100  $\mu\text{M}$ ) was taken as a positive control. Results are expressed as mean  $\pm$  standard deviation of six independent experiments. ### $P < 0.001$  versus blank group; \* $P < 0.05$ , \*\* $P < 0.01$ , \*\*\* $P < 0.001$  versus Model group.  $^{\#}P < 0.05$ ,  $^{aa}P < 0.01$ ,  $^{aaa}P < 0.001$  as compared with aspirin group

normal conditions, while the elevated level of COX-2 could be caused by inflammation. TXA2 as a strong inducer of platelet aggregation and vasoconstriction obviously increases in blood stasis.<sup>[32]</sup> The release of COX-2 will increase the level of TXA2, which would ultimately promote platelet adhesion and aggregation and increase the risk of blood stasis.<sup>[33]</sup> The present study revealed that CO and ACO effectively reduced the levels of IL-6, NO, TNF- $\alpha$ , COX-2 and improved whole blood viscosity and plasma viscosity on acute blood stasis model rats. Therefore, our study demonstrated that CO and ACO have therapeutic effect on acute blood stasis model rats through improving blood rheology and reducing the levels of inflammatory cytokines.

In the current study, forty components were identified from ACO by HPLC-QTOF/MS/MS and most of them are phytoecdysteroids and saponins. As the main ingredient of ACO, Ginsenoside Ro was reported to have antioxidant activities through inhibiting the levels of MDA, ROS and promoting SOD activity.<sup>[34]</sup> What is more, Ginsenoside Ro and  $\beta$ -ecdysterone have been reported to possess anti-inflammatory activities via downregulating the expression of matrix metalloproteinase (MMP 3, MMP 9), COX-2 and inhibiting nuclear factor- $\kappa$ B p65 and I $\kappa$ B- $\alpha$  phosphorylation in IL-1 $\beta$ -induced rat chondrocytes.<sup>[35,36]</sup> Chikusetsusaponin IVa presented significant antithrombotic effects in a stasis model of venous thrombosis and anticoagulation effects *in vitro*.<sup>[37]</sup> Chikusetsusaponin IVa and chikusetsusaponin IVa methyl ester displayed suppressive effects on lipopolysaccharide-induced NO, prostaglandin E2, TNF- $\alpha$ , IL-6, and IL-1 $\beta$  production in RAW 264.7 cells and exhibited

potential anti-inflammatory effect.<sup>[38]</sup> Chinese Pharmacopoeia takes the content of cyasterone as the quality standard of *C. officinalis* Kuan, and chikusetsusaponin IV is one of the main saponins in ACO. However, there is seldom report on their pharmacological activities.

SOD is one of the major defense mechanisms to protect cells against ROS damage.<sup>[39]</sup> MDA is commonly used as a biomarker of oxidative stress.<sup>[40]</sup> ET and NO play key roles in regulating cardiovascular system function and are involved in a variety of pathological procedures of cardiovascular disease.<sup>[41]</sup> The damage of HUVECs enhances the release of ET and causes vasoconstriction. eNOS is primarily responsible for the endothelium-dependent vasodilatation, and the activation of eNOS promotes the production of NO.<sup>[42]</sup> Thrombin-induced HUVECs damage experiment demonstrated that cyasterone and chikusetsusaponin IV have significant protective effects on HUVECs through inhibiting levels of TXA2, ET, MDA, COX-2 and promoting the activities of eNOS and SOD.

Aspirin, a positive drug, is widely used as an anti-inflammatory, antithrombotic, and even an anti-malignant agent. Even though it possesses great efficacy, its adverse effects should not be ignored, such as allergic reaction and adverse reactions of the blood system, digestive system, and nervous system. Compared to aspirin, *C. officinalis* Kuan has great advantages, such as reliable efficacy, lower side effects, and toxicity. Especially, the effects of cyasterone (100  $\mu\text{M}$ ) on inhibiting levels of COX-2, ET and promoting the activity of SOD were better than aspirin (100  $\mu\text{M}$ ). Hence, *C. officinalis* Kuan as a clinically safe



and effective TCM may be considered as one of the useful drugs for the development of blood-activating agents.

## CONCLUSION

The present study has demonstrated that CO and ACO showed significant improving blood rheology and anti-inflammatory effects on acute blood stasis model rats by improving whole blood viscosity, plasma viscosity and reducing the levels of IL-6, NO, TNF- $\alpha$ , and COX-2. HPLC-QTOF/MS/MS was used to identify components of ACO, mainly as phytoecdysteroids (e.g., 24-hydroxycyasterone and cyasterone) and saponins compounds (e.g., Ginsenoside Ro and chikusetsusaponin IV). Cyasterone and chikusetsusaponin IV possessed significant protective effects on thrombin-induced HUVECs damage model by inhibiting levels of TXA<sub>2</sub>, ET, MDA, COX-2 and promoting the activities of eNOS and SOD. What is more, the inhibition of COX-2, ET and promotion of SOD in cyasterone (100  $\mu$ M) were better than aspirin group (100  $\mu$ M). The data and the result of our investigation showed that CO and ACO possessed a significant therapeutic effect on acute blood stasis model rats through improving blood rheology and reducing inflammation and the representative components cyasterone and chikusetsusaponin IV showed significant anti-inflammatory, antioxidant, and anticoagulant effects on thrombin-induced HUVECs damage model.

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## Conflicts of interest

There are no conflicts of interest.

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