Inhibitory Effects of Two Ferulates from *Angelica Sinensis* on Platelet Aggregation and Oxytocin-induced Uterine Contraction

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Abstract: Ferulic acid (FA) is widely considered as a biologically active component in *Angelica sinensis*, and used as one of the marker compounds for the quality control of *Angelica sinensis*. However, in *A. sinensis*, FA mainly exists as its ester, coniferyl ferulate (CF). CF is unstable and readily hydrolyzed into FA during conventional extraction. Herein, their antiplatelet aggregation activities and relaxant effects on oxytocin-induced mouse uterine muscle contraction were investigated and compared. The results showed that FA inhibited arachidonic acid (AA), adenosine diphosphate (ADP) and thrombin (THR)-induced platelet aggregation with IC_{50} values of 974.8 ± 97.5, 737.9 ± 40.2 and 244.6 ± 25.6 μg/ml, respectively. The potency of CF is much higher than that of FA, and the IC_{50} values for AA, ADP and THR were 7.1 ± 0.3, 276.4 ± 53.4 and 77.5 ± 23.1 μg/ml, respectively. IC_{50} of FA was 23.8 ± 6.2 μg/ml for oxytocin-induced uterine contraction *in vitro*. CF could only be tested at low concentration and its IC_{50} could not be calculated thereafter because of its strong hydrophobic property. So CF has more potent antiplatelet aggregation activity, while FA has stronger inhibitory effect on oxytocin-induced uterine contraction *in vitro*.

Keywords: *Angelica sinensis*, coniferyl ferulate, ferulic acid, antiplatelet aggregation, oxytocin-induced uterine contraction.

INTRODUCTION

Angelica sinensis, known as Danggui in China, has been used for thousand years as traditional Chinese, Korean, and Japanese medicine. Nearly 19% of the traditional Chinese medicinal preparations recorded in Chinese Pharmacopeia (2005 edition) contain *A. sinensis*. *A. sinensis* can enrich blood, activate blood circulation, regulate menstruation, relieve pain, and relax bowels, and is used for the treatment of anemia with dizziness and palpitation, amenorrhea, dysmenorrhea, constipation, rheumatic arthralgia, traumatic injuries, carbuncles, boils and sores [1]. Modern studies have shown that *A. sinensis* has multiple pharmacological activities [2], including anti-inflammatory [3], antioxidant [4], immunostimulative [5], antiplatelet aggregation [6], antitumor [7, 8] and estrogenic [9] activities. Ferulic acid (FA) is usually considered as a biologically active component, and has been used as one of the marker compounds for the quality control of *A. sinensis* [1]. Indeed, FA has antioxidant [10], anti-inflammatory [11], antitumor [12], anti-diabetes [13], antimicrobial [14], anti-hypertension and anti-hyperlipidemia [15] activities, and protective effect on iron- and nicotine-induced toxicity [16, 17]. However, in *A. sinensis*, FA mainly exists as its ester, coniferyl ferulate (CF). CF is unstable and readily hydrolyzed into FA during conventional extraction [18, 19] although CF also has antineoplastic [20], antibacterial [21] and antioxidant [22] effects. So it is interesting to investigate and compare their potency of pharmacological activities, which will help to improve clinical uses of *A. sinensis*.

In the present study, antiplatelet aggregation and uterus-regulating activities of FA and CF, two ferulates related to *A. sinensis*, were investigated and compared.

MATERIALS AND METHODS

Materials

Arachidonic acid (AA), adenosine diphosphate (ADP) and FA (Fig. **1A**) were purchased from Sigma (St. Louis, MO, USA). Thrombin (THR) was the product of Dade Behring (Eschborn, Germany). Disposable cuvettes for antiplatelet aggregation assay were from SUCCESS Technology Development Co. Ltd (Beijing, China). Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Billerica, MA, USA). All other chemicals not mentioned here were analytical grade from standard sources. CF (purity > 95.0% determined by HPLC, Fig. **1B**) was separated and purified from essential oil of *A. sinensis* in our lab [23]. The structure of CF was confirmed by its UV, MS, ^{1}H-NMR and ^{13}C-NMR data [19, 24].

Animals

The experiments were performed in accordance with the Animal Ethics Committee of Nanjing University of Chinese Medicine. Male New Zealand white rabbits (1.8-2.5 kg) and non-pregnant sexually mature female Kunming mice (6-7 weeks, 18-22 g) were obtained from Experimental Animal Center, Nanjing University of Chinese Medicine. All animals were maintained on a pellet diet and water ad libitum

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1874-8473/09

and kept under the laboratory condition of light/dark cycle 12/12 hours.

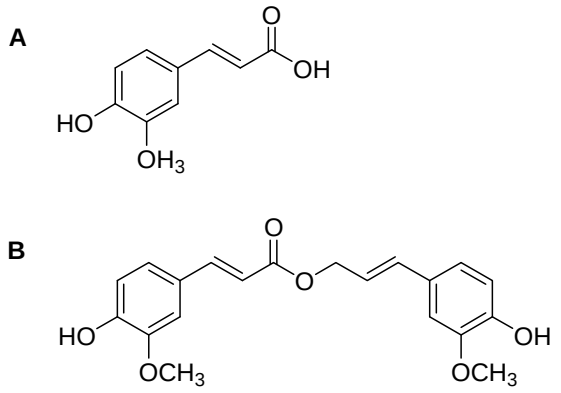

Fig. (1). Chemical structures of ferulic acid (FA) and coniferyl ferulate (CF).

Antiplatelet Aggregation Assay *in vitro*

Rabbit platelets were used for antiplatelet aggregation assays. Blood from the rabbit carotid artery was collected in plastic tubes and anticoagulated with 3.2% sodium citrate solution (9:1, v/v). The blood sample was centrifuged at 100 × g for 10 min, platelet rich plasma was obtained. Then the precipitate was centrifuged at 1,925 × g for another 10 min to obtain platelet poor plasma. The final count of platelets in platelet rich plasma was adjusted to 7×10^9 cells/ml with platelet poor plasma.

FA and CF were dissolved in dimethyl sulfoxide. Stock solutions of AA, ADP and THR were prepared with 1% sodium bicarbonate solution, phosphate buffer solution and deionized water, respectively.

Antiplatelet aggregation assays were performed by turbidimetric method [25] on a SC-2000 aggregometer (SUCCESS Technology Development Co. Ltd, Beijing). The test samples were pre-incubated with platelets for 5 min at 37 ℃. Then the platelet aggregation was induced by addition of AA, ADP or THR solution with final concentration of 330 μM, 15 μM and 0.3 IU, respectively. All tests were performed within 3 h after the collection of blood. Corresponding solvents were used as blank controls for the corresponding test. The antiplatelet aggregation potency is expressed as inhibition (%) which was calculated as follows:

$$\text{Inhibition\%} = (A - B) / A \times 100\%$$

where A and B were the absorbance values of corresponding blank controls and test samples, respectively.

Oxytocin-induced Uterine Contraction Assay *in vitro*

Oxytocin-induced female mice uterine contraction assay was tested *in vitro* [26]. In brief, synchronistically estrus mice were pretreated with estradiol benzoate (5 mg/kg, s.c.) 24 h prior to the study. On the third day, the mice were sacrificed by cervical dislocation and the uteri were isolated. The whole uterus was taken as one sample. The cervical end was tied to perspex holder, and the two ovarian ends were put together as another end tied to an isometric force transducer. A resting tension of 1 g was applied for superfusion with oxygenated Krebs (95% O_2, 5% CO_2, pH 7.3) at 37 ℃. Equilibration period was not less than 45 min. After establishing a steady basal tone, oxytocin (5 U/ml) was added to baths. Once the contraction reached a plateau, FA and CF of cumulative concentrations were added to organ bath with an interval of about 10 min. Dimethyl sulfoxide (3‰) was taken as vehicle control. Contractions were recorded by PowerLab/8s data recording system (AD Instruments, Australia) which was linked to a mackintosh computer (Powermac 7200/120, Apple, USA) where Chart software (v. 4.2.2 AD Instruments, Australia) was used to display and measure the tension changes of tissues. Antioxytocin effects were expressed as a percentage of relaxation based on 5 U/ml oxytocin-induced plateau contraction.

Statistical Analysis

Data were expressed as the mean ± SD and presented as an average of three replicates. The IC_{50} (the concentration that providing 50% inhibition) values were calculated based on linear regression of the inhibitory capacities to the concentrations or the logistics of concentration.

RUSULTS AND DISCUSSION

The Effects of FA and CF on Platelet Aggregation *in vitro*

Both FA and CF concentration-dependently inhibited AA, ADP and THR-induced platelet aggregations. As shown in Table **1**, FA inhibited platelet aggregation induced by AA, ADP and THR with IC_{50} values of 974.8 ± 97.5, 737.9 ± 40.2 and 244.6 ± 25.6 μg/ml, respectively. The potency of CF is much higher than that of FA, and the IC_{50} values for AA, ADP and THR were 7.1 ± 0.3, 276.4 ± 53.4 and 77.5 ± 23.1 μg/ml, respectively. The results of FA agreed with Ou's report which also showed that FA had antiplatelet aggregation effect [27]. The results accounted for the antiplatelet aggregation mechanism of *A. sinensis* injection [6] and activation blood circulation mechanism of *A. sinensis* [1]. CF had stronger effect than FA, but CF is unstable and readily hydrolyzed into FA during conventional extraction, which indicates that we should improve extraction method to reduce such hydrolysis when *A. sinensis* was mainly used to improve platelet aggregation.

The Effects of FA and CF on Oxytocin-induced Uterine Contraction *in vitro*

Oxytocin, a nonapeptide hormone, induces uterine and mammary gland contractions. Both tissues express oxytocin receptors. The number of these receptors is increased by estrogen and decreased by progesterone [28]. So the mice were pretreated with estradiol benzoate 24 h prior to the study to increase the oxytocin receptor numbers and thus increase the sensitivity of uterine to the stimulation of oxytocin *in vitro*.

The results showed that both FA and CF inhibited oxytocin-induced uterine contraction in a concentration-dependent manner, and FA had more potent inhibitory effect than CF (Fig. **2**). Actually, the concentration-dependent response of CF only could be found when the concentration beyond 25 μg/ml according to Fig. (**2**), and the concentrations of CF below 25 μg/ml had no obvious inhibition on oxytocin-induced mice uterine contraction. IC_{50} of FA was 23.8 ± 6.2

Table 1. Inhibitory Effects of FA and CF on Rabbit Platelet Aggregation Induced by AA (330 μM), ADP (15 μM) and THR (0.3 U/ml) *In Vitro*

	FA	CF
AA		
Test range (μg/ml)	101.0-1619.4[a]	2.3-37.6
Linear range (μg/ml)	101.0-1619.4	2.3-37.6
Regression equation	$Y^b = 53.14x - 1.86$	$Y^c = 58.63x + 0.08$
R^2	0.984	0.949
IC_{50} (μg/ml)	974.8 ± 97.5	7.1 ± 0.3
ADP		
Test range (μg/ml)	202.4-1215.0	19.0-303.2
Linear range (μg/ml)	202.4-1215.0	19.0-303.2
Regression equation	$Y^b = 92.63x - 8.52$	$Y^b = 147.51x + 9.67$
R^2	0.903	0.995
IC_{50} (μg/ml)	737.9 ± 40.2	276.4 ± 53.4
THR		
Test range (μg/ml)	25.2-403.9	19.0-303.2
Linear range (μg/ml)	25.2-403.9	19.0-303.2
Regression equation	$Y^b = 136.39x + 16.66$	$Y^c = 62.03x - 66.61$
R^2	0.950	0.939
IC_{50} (μg/ml)	244.6 ± 25.6	77.5 ± 23.1

IC_{50} values were expressed as the mean ± SD and presented as an average of three replicates.
[a] The actual tested samples were obtained with serial double dilution through the highest to lowest concentration of samples except FA (202.4, 404.8, 485.8, 809.7 and 1215.0 μg/ml) for ADP-induced platelet aggregation assay.
[b] Regression equations were expressed as inhibition% *vs.* concentration (mg/ml).
[c] Regression equation were as inhibition% *vs.* the logistic of concentration (μg/ml).

μg/ml. Potent prostaglandins and potent leukotrienes play an important role in generating primary dysmenorrhea symptoms [29], so the anti-inflammatory effect of *A. sinensis* may help to treat dysmenorrhea [3]. Moreover, in non-pregnant women, the oxytocin receptor density varies over the menstrual cycle and increases markedly at the onset of menstruation. So blocking the uterine oxytocin receptor can inhibit primary dysmenorrhoea [30]. Therefore, the potent antispasm activity of FA on oxytocin-induced uterine contraction could also partly elucidate the anti-dysmenorrhea mechanism of *A. sinensis*. Since CF has strong hydrophobic property, it could only be tested at low concentration and its IC_{50} could not be calculated thereafter. The results suggest that FA is a good form when *A. sinensis* is used to inhibit the oxytocin-induced uterine contraction.

In conclusion, the pharmacological activities of two ferulates, FA and CF, had their own characters. CF has more potent antiplatelet aggregation activity, while FA has stronger inhibitory effect on oxytocin-induced uterine contraction. The results suggest that different extraction methods of *A. sinensis* are necessary according to its different treatment purposes.

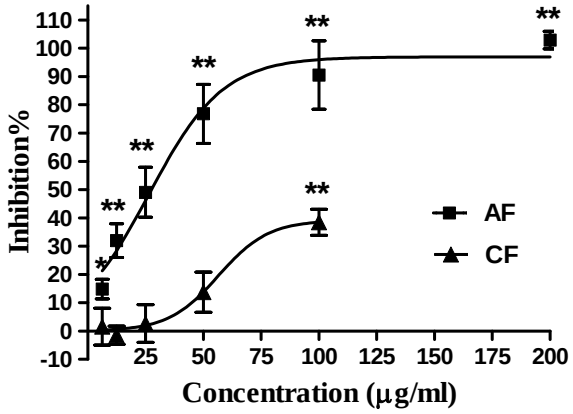

Fig. (2). The inhibitory effects of FA and CF on oxytocin-induced mice uterine muscle contraction *in vitro*. The data were expressed as mean ± SD of three replicates. **P*<0.05 and ***P*<0.01 *vs*. vehicle control.

ACKNOWLEDGEMENTS

The research was supported by grants from Jiangsu Key Laboratory for TCM Formulae Research, Nanjing University

of Chinese Medicine (No.022021014003) and University of Macau (RG083/07-08S) to S.P. Li.

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Received: February 14, 2009 Revised: July 30, 2009 Accepted: October 25, 2009