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Interactions between thrombin and natural products of *Millettia nitita* var. *hirsutissima* using capillary zone electrophoresis

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ABSTRACT

A sensitive and selective high-performance analytical method based on capillary zone electrophoresis (CZE) was developed for investigating interactions between natural products isolated from *Millettia nitita* var. *hirsutissima* and thrombin qualitatively and quantitatively for the first time. The results showed that, compared with positive and negative control, the compounds ZYY-5 (genistein-8-C-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranoside), ZYY-6 (calycosin), ZYY-8 (isoliquiritigenin), ZYY-9 (formononetin), ZYY-12 (gliricidin), ZYY-13 (8-O-methylretusin), FJ-2 (dihydrokaempferol), FJ-3 (biochanin), FJ-5 (afromosin) and XC-2 (hirsutissimiside F) interacted with thrombin, while ZYY-1 (sphaerobioside), ZYY-2 (formononetin-7-O-β-D-apiofuranosyl-(1→6)-O-β-D-glucopyranoside), ZYY-3 (genistein-5-methylether-7-O-α-L-rhamnopyranosyl-(1→6)-O-β-D-glucopyranoside), ZYY-4 (retusin-7,8-O-β-D-diglucopyranoside), ZYY-7 (symplocoside), ZYY-10 (ononin), ZYY-11 (genistin), ZYY-14 (afromosin-7-O-β-D-glucopyranoside), ZYY-15 (lanceolarin), FJ-1 (liquiritigenin), FJ-4 (7,2-dihydroxy,4-methoxyisoflavan) and XC-1 (sphaerobioside) had no binding to thrombin. This indicated that the reported CZE method for the determination of compound–thrombin interactions is powerful, sensitive and fast, and requires less amounts of reagents, and further, it can be employed as a reliable alternative to other methods.

1. Introduction

Millettia nitita var. *hirsutissima* is a fabaceous plant in the Jiangxi Province originally. In folk, the vine stems of *M. nitita* var. *hirsutissima* is used as a substitute for Ji Xue Teng (Caulis Spatholobi, the stems of *Spatholobus suberectus* [1]) which is a traditional Chinese medicine for promoting blood circulation or relieving stasis [2,3]. This study is to appraise whether *M. nitita* var. *hirsutissima* is the eligible substitute for Ji Xue Teng by CZE method for the first time. Twenty-two ingredients were isolated from *M. nitita* var. *hirsutissima*, named ZYY-1, ZYY-2, ZYY-3, ZYY-4, ZYY-5, ZYY-6, ZYY-7, ZYY-8, ZYY-9, ZYY-10, ZYY-11, ZYY-12, ZYY-13, ZYY-14, ZYY-15, FJ-1, FJ-2, FJ-3, FJ-4, FJ-5, XC-1 and XC-2 and their structures as shown in Fig. 1. The structures of ZYY-1, ZYY-2, ZYY-3, ZYY-4, ZYY-5 and XC-2 were identified for the first time.

Thrombin is the final serine protease in the inactivation of the blood coagulation network. It initiates blood clotting by cleaving soluble fibrinogen and also activates other procoagulant enzymes including factors V, VIII, XI and XIII, and the anticoagulant enzyme protein C. Thrombin also has marked effects on a variety of cells including platelets, endothelial and smooth muscle cells, which seem to be transmitted via specific thrombin receptors called protease-activated receptors [4,5]. Consequently, most current antithrombotic treatment strategies are aimed at blocking the activity of thrombin, or preventing its generation [6]. It is an important target for therapeutic intervention in thrombotic disease, and a number of strategies have been successfully applied to the development of potent thrombin inhibitors. Screening of natural and synthetic compounds based on the concept of ligand/receptor interaction is an indispensable strategy to search for thrombin inhibitors.

Many methods have been performed to study the interactions between proteins and different compounds, such as nuclear magnetic resonance (NMR), Fourier transform infrared spectrometry (FTIR), affinity chromatography (AC), mass spectrometry (MS), X-ray, fluorescence, surface plasmon resonance spectrometry (SPR), polyacrylamide gel electrophoresis (PAGE), equilibrium dialysis, and capillary electrophoresis (CE) [7]. NMR, X-ray, MS, fluorescence, and FTIR require an experiment and elaborated procedures for sample purification before analysis. SPR, AC, PAGE, and equilibrium dialysis are time-consuming procedures and demand a large

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sample size for analysis. In this study, CE was chosen to investigate the interactions between the natural products and target proteins due to its high speed, excellent resolution, low sample size, high reproducibility and flexibility. CE is able to study the interaction of individual components in a mixture as well as to determine binding parameters in a single step, a unique feature as compared with other techniques for the study of noncovalent interactions [8].

CE has been used in a wide range of binding studies between biologically important molecules, such as protein–protein,

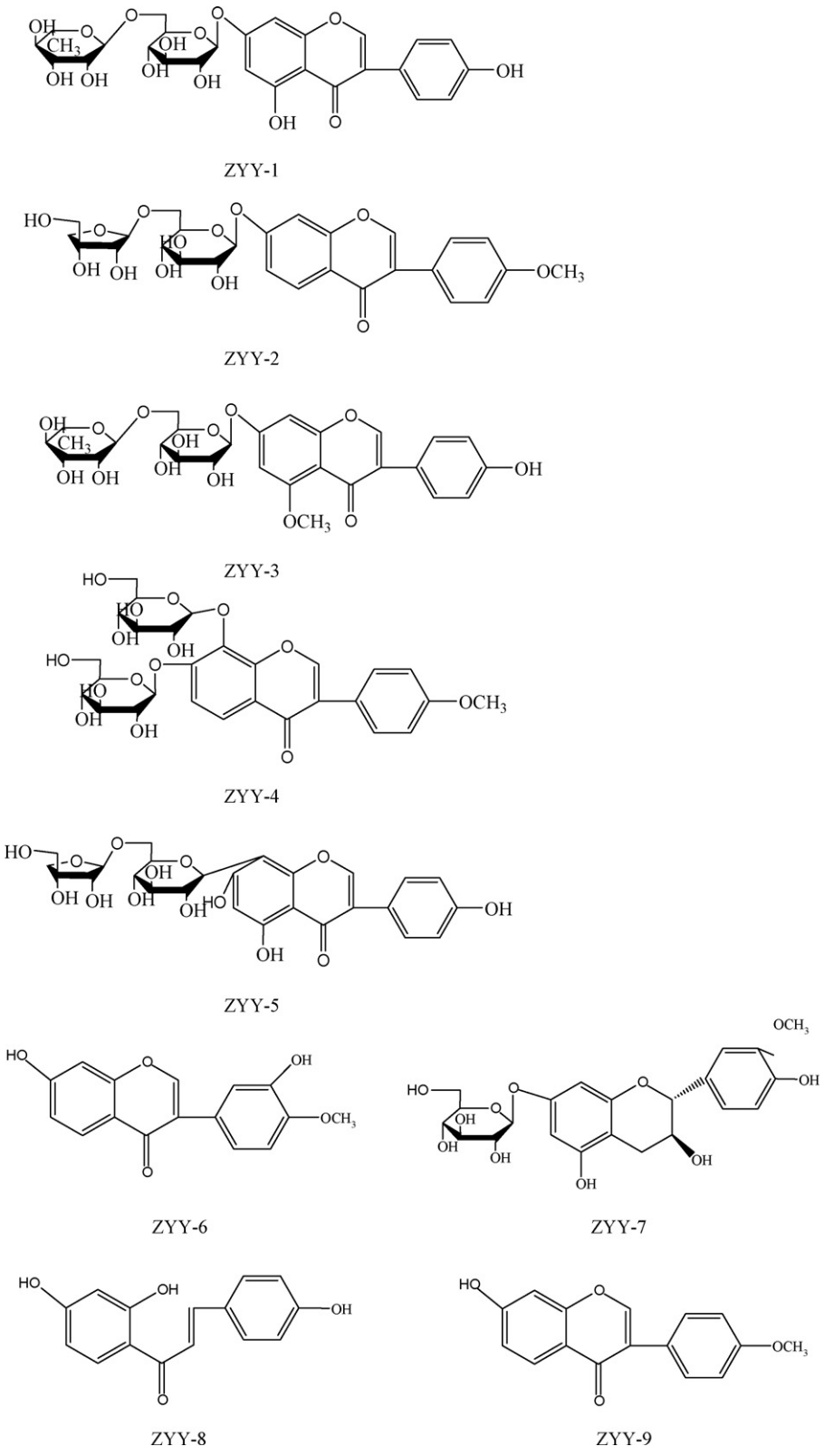


Fig. 1. Structures of the compounds.

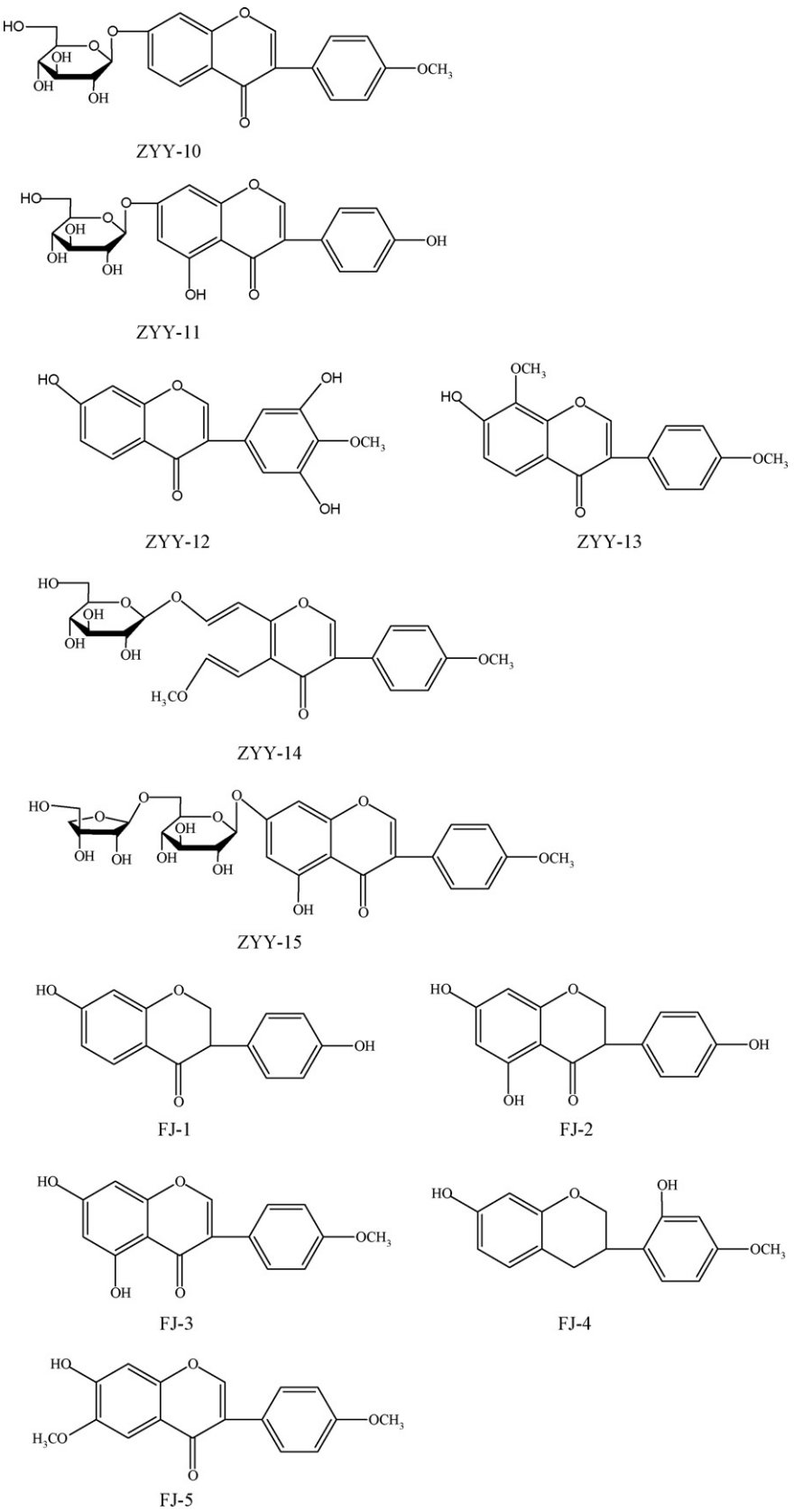
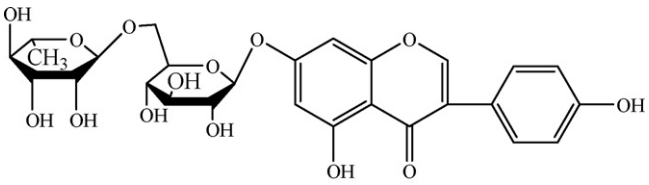
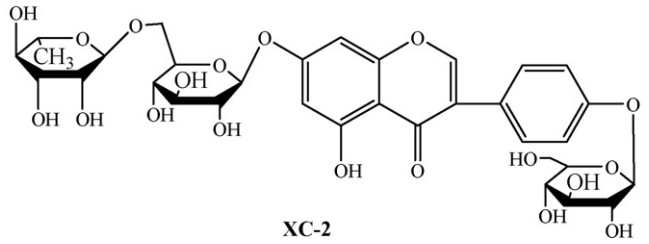


Fig. 1. (*Continued*).



XC-1



XC-2

Fig. 1. (*Continued*).

protein–DNA, protein–drug, protein–sugar, DNA–peptide, peptide–drug, peptide–peptide, antibody–antigen, and peptide–carbohydrate [9–15]. Among the various CE modes [16,17], CZE is the most appropriate one for the study of molecular interactions if only small amounts of samples are available.

2. Experimental

2.1. Chemicals and reagents

All chemicals were analytical grade unless otherwise indicated. Human Thrombin (3190 NIH units mg^{-1} protein) was purchased from Sigma (St. Louis, MO, USA). Trisbase (ultrapure) and acetic acid were from Beijing Chemical Reagent Factory (Beijing, China). Thrombin was dissolved in Tris–acetate buffer (30 mmol L^{-1} Tris, the pH was adjusted to 7.2 by acetic acid) at 1.595 mg mL^{-1} (2 NIH units μL^{-1}) as stocking solution. Deionized water was prepared using a Millipore Milli Q-Plus system (Millipore, Bedford, MA). All buffers and solutions used in the study were filtered through 0.45 μm membranes (Agilent, Germany) before using. Compounds ZYY-1, ZYY-2, ZYY-3, ZYY-4, ZYY-5, ZYY-6, ZYY-7, ZYY-8, ZYY-9, ZYY-10, ZYY-11, ZYY-12, ZYY-13, ZYY-14, ZYY-15, FJ-1, FJ-2, FJ-3, FJ-4, FJ-5, XC-1 and XC-2 were isolated from the natural plants and were dissolved in methanol at 1 mg mL^{-1} as stocking sample solution and diluted to different concentrations by adding appropriate amounts of Tris-HAc running buffer (30 mmol L^{-1} Tris, the pH was adjusted to 7.2 by acetic acid).

2.2. Instrumentation

All experiments were performed on a Beckman P/ACE™ MDQ system (Beckman Coulter, Inc., Fullerton, CA, USA) equipped with a photodiode array detector as well as the 32 Karat™ software version 5.0 (Beckman). A fused silica capillary tube (Yongnian Optical Fibre Corp., Hebei, China) with an internal diameter of 75 μm was used. The total and effective lengths of the capillary were 60.2 cm and 50 cm, respectively. Before using, the new capillaries were rinsed with 0.1 mol L^{-1} NaOH solution for 20 min, and subsequently with deionized water for 5 min.

2.3. Sample preparation

To investigate compound–thrombin interaction, different concentrations of thrombin were tested for the formation of complex, which was formed by mixing compounds with thrombin with the running buffer. It was incubated for 20 min at 25 °C before CE analysis [18]. All solutions were prepared with deionized water.

2.4. CZE conditions

To study the interaction of natural product and thrombin, the temperatures of the cartridge and sample room were kept at 25 °C and 4 °C, respectively. Before each measurement, the capillary was rinsed with running buffer (30 mmol L^{-1} Tris-HAc, pH 7.2). Samples containing the mixtures of compounds and thrombin were injected using the pressure injection mode at 0.5 p.s.i. for 5 s (1 p.s.i. = 6894.76 Pa). The applied voltage was 15 kV. The capillary was washed between runs with the running buffer for 5 min at 20 p.s.i. Each concentration was run in duplicate.

2.5. Quantitative model for the binding study

In the binding studies, the binding constant and the stoichiometry are important parameters to be determined. Scatchard analysis is a common way to linearize the binding data, as expressed in the following equation:

$$\frac{r}{C_f} = -Kr + nK \quad (1)$$

Assuming that there are n binding sites for the binding and each binding site does not influence the bindings on the other sites (non-cooperative binding), K is defined as the intrinsic association constant of certain type of binding, C_f as the concentration of unbound ligand, and r as the average number of bound ligand per receptor molecule. The plot of r/C_f versus r gives a linear curve [19]. In this study, r is the concentration ratio of the bound natural product compounds to the total natural product compounds and C_f is the unbound thrombin concentration.

In this method, the peak height linearly depends on the concentration of the analyte. Therefore, a standard curve for natural product compound concentration is created in order to measuring the concentration of unbound natural product compound by the peak height exhibited in the electropherograms. Usually, most of publications in binding studies with CE assume that the stoichiometry of the binding between receptor and ligand is 1:1 to establish

Table 1
Compounds and the binding constants.

Compounds	Regression equation	r	Binding constants ($\times 10^4$ M^{-1})	RSD
ZYY-5	$y = -1.8120x + 1.544$	0.8405	1.820	0.064
ZYY-6	$y = -0.6350x + 0.4309$	0.9784	0.6350	0.005
ZYY-8	$y = -2.356x + 2.156$	0.9023	2.356	0.148
ZYY-9	$y = -1.137x + 1.083$	0.9153	1.137	0.039
ZYY-12	$y = -1.896x + 1.391$	0.9327	1.896	0.006
ZYY-13	$y = -2.242x + 1.930$	0.8580	2.242	0.185
FJ-2	$y = -8.723x + 7.493$	0.8891	8.723	0.154
FJ-3	$y = -2.092x + 1.222$	0.9591	2.092	0.070
FJ-5	$y = -0.8918x + 0.3977$	0.8419	0.8918	0.041

a simple model ($n = 1$). In the following session, therefore, it is also assume the equations for the simple model to estimate binding constants so that the concentration of bound natural product compound can be gotten which equal the concentration of bound Thrombin according to the assumption ($n = 1$). Consequently, C_f as the concentration of unbound thrombin can be calculated, r is the concentration ratio of the bound natural product compound to the total natural product compound and the binding constant is calculated from the slope ($-K$) of the linear regression curve fitting from acquired data with Eq. (1).

3. Results and discussion

3.1. Optimization of separation conditions

We have tested the complex five times by the time point 20 min, 30 min, 40 min, 50 min and 60 min. There are no significant change of the complex which indicate this complex is steady at least in 1 h. In consideration that each run was completed within 5 min in our method, the stability of the complex during each running is believed to be unquestionable. All samples were,

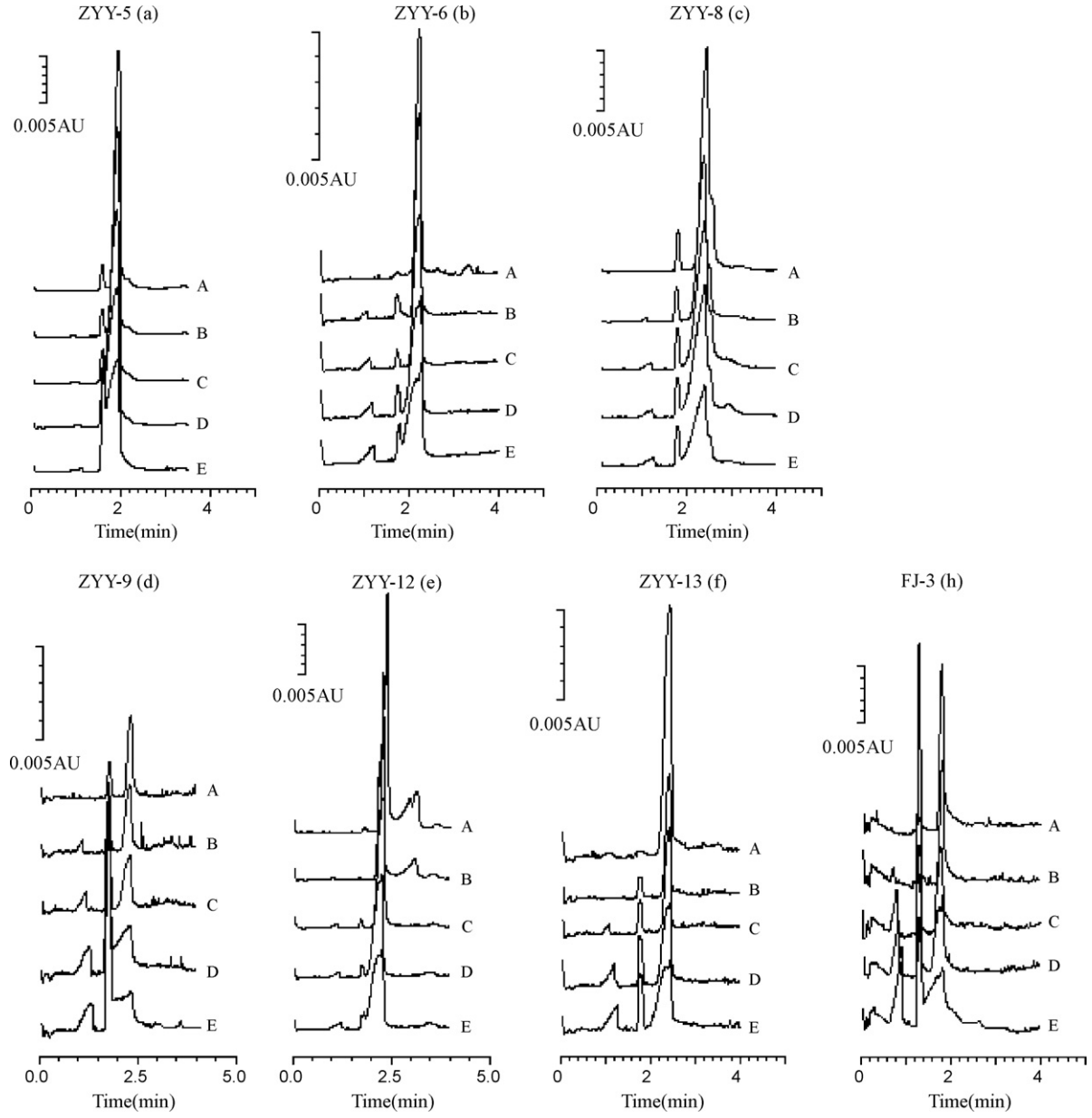


Fig. 2. Electropherograms of:
(a) ZYY-5–thrombin interaction, natural products: thrombin (mol/mol). (A) 1:0; (B) 1: 1.3; (C) 1:3.8; (D) 1:5.6; (E) 1:9.4.
(b) ZYY-6–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.9; (C) 1:2.3; (D) 1:6.8; (E) 1:9.1.
(c) ZYY-8–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.9; (C) 1:1.4; (D) 1:2.3; (E) 1:9.1.
(d) ZYY-9–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.4; (C) 1:1.1; (D) 1:2.1; (E) 1:4.3.
(e) ZYY-12–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.7; (C) 1:1.8; (D) 1:3.6; (E) 1:7.3.
(f) ZYY-13–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:2.0; (C) 1:4.0; (D) 1:7.9; (E) 1:11.9.
(g) FJ-2–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.6; (C) 1:1.3; (D) 1:4.3; (E) 1:13.
(h) FJ-3-20–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.6; (C) 1:1.6; (D) 1:2.4; (E) 1:6.1.
(i) FJ-5–thrombin interaction, natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.4; (C) 1:0.7; (D) 1:1.8; (E) 1:5.4.
The conditions used were as follows: Beckman P/ACE MDQ capillary electrophoresis system. Injection: 0.5 p.s.i. for 5 s. Applied voltage: 15 kV. Capillary: capillary of 60.2 cm (effective length 50 cm) × 75 μm i.d. detection at 214 nm.

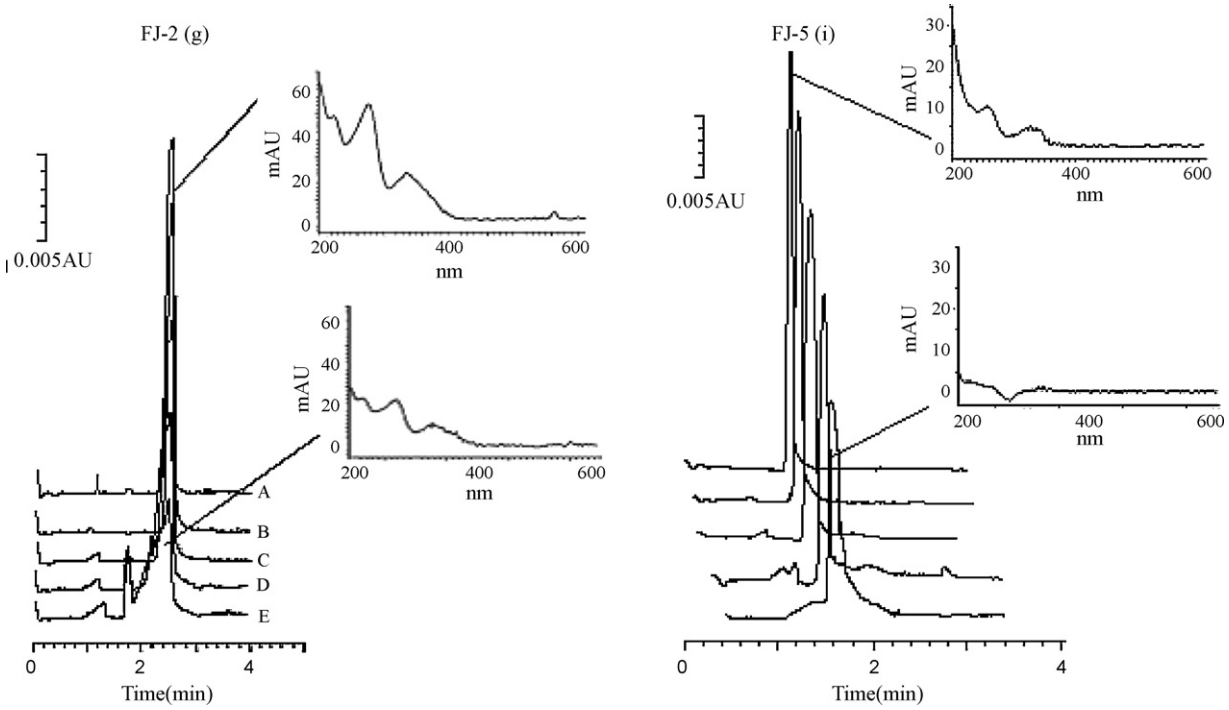


Fig. 2. (*Continued*).

therefore, incubated for at least 20 min before CE analysis. pH value was an another important factor for evaluating the interactions in capillary zone electrophoresis. Tris-HAc provided the better separation efficiency and resolution. Thus, 30 mmol L^{-1} pH 7.2 Tris-HAc solution was selected as the running buffer. Under the CE conditions employed in this paper, thrombin was not visible.

3.2. Interactions between compounds and thrombin by CZE

The interactions between different compounds and thrombin at pH 7.2 were studied. Fig. 2 shows the electropherograms of ZYY-5, ZYY-6, ZYY-8, ZYY-9, ZYY-12, ZYY-13, FJ-2, FJ-3, FJ-5 and their mixtures. Curve A is for each compound alone and curves B–D for the mixtures containing a fixed concentration of corresponding natural product with an increasing concentrations of thrombin. The first peaks of curves are from natural product-thrombin complexes, while the second peaks from the corresponding compounds in Fig. 2(a–i). The peak height of the natural product decreases with the increase in the concentration of thrombin and in front of the compound peaks the complex peaks can be observed. According to the absorption spectra of the ZYY-5, ZYY-6, ZYY-8, ZYY-9, ZYY-12, ZYY-13, FJ-2, FJ-3, FJ-5 and their complexes obtained from the PDA detector, the first peaks were also proved to be natural product–thrombin complexes and the second peak to be corresponding compounds.

The binding constant is an important quantitative parameter for characterizing the interaction between the compounds and thrombin. According to the aforementioned Scatchard analysis formula, the binding constants were calculated (Table 1).

Fig. 3 shows the electropherograms of XC-2 and its mixture. The main peak of curve A is for XC-2. The curves B–D are for the mixtures containing a fixed concentration of XC-2 with an increasing concentration of thrombin. In the electropherograms of XC-2 and its mixture, with increasing in thrombin concentration the height of main peaks increases simultaneously. The absorption spectra of the main peaks changed significantly, which shows there was the complex obtained. We suppose that the increase of the pick height caused because the natural product–thrombin complex peak has the same appearance time with the solo natural product which cannot be separated in the electrophoresis condition employed in this study. So the concentration of unbound natural product compound which calculated form the decrease of the natural product cannot be gotten. Therefore the binding constants cannot be calculated by Scatchard analysis.

Fig. 4 is the electropherograms of FJ-1 and its mixture. The main peak of curve A is compound FJ-1. While the curves B–D shows the mixture containing a fixed concentration of FJ-1 with an increasing concentration of thrombin. Fig. 4 shows that the peak height of the corresponding compound does not change obviously with increasing the thrombin concentration. According to the absorption spectra from the PDA detector, UV of FJ-1 and its mixtures had no obvious difference. No interactions were obtained. Natural products ZYY-1, ZYY-2, ZYY-3, ZYY-4, ZYY-7, ZYY-10, ZYY-11, ZYY-14, ZYY-15, FJ-4 and XC-1 shared the same results.

Screening of natural and synthetic compounds based on the concept of ligand/receptor interaction is an indispensable strategy to search for receptor inhibitors, and a number of strategies have been successfully applied. Thrombin is an important target for therapeutic intervention in thrombotic disease. The compounds, which have good thrombin-binding capacity, could be potential thrombin inhibitors. Compared with positive and negative control [20], the compounds ZYY-5, ZYY-6, ZYY-8, ZYY-9, ZYY-12, ZYY-13, FJ-2, FJ-3, FJ-5 and XC-2 interacted with thrombin, while ZYY-1, ZYY-2, ZYY-3, ZYY-4, ZYY-7, ZYY-10, ZYY-11, ZYY-14, ZYY-15, FJ-1, FJ-4 and XC-1 had no binding to thrombin.

The present study showed that it was possible to characterize interactions between compounds and thrombin by using CZE as a new technique. An attractive direction was to apply CE to high-throughput drug screening for products of combinatorial chemistry as well as components of traditional Chinese medicine whereas conventional drug screening techniques could only provide the IC_{50} values [21,22]. With the aid of CE, binding constants and stoichiometry data could be obtained. An important practical aspect is that

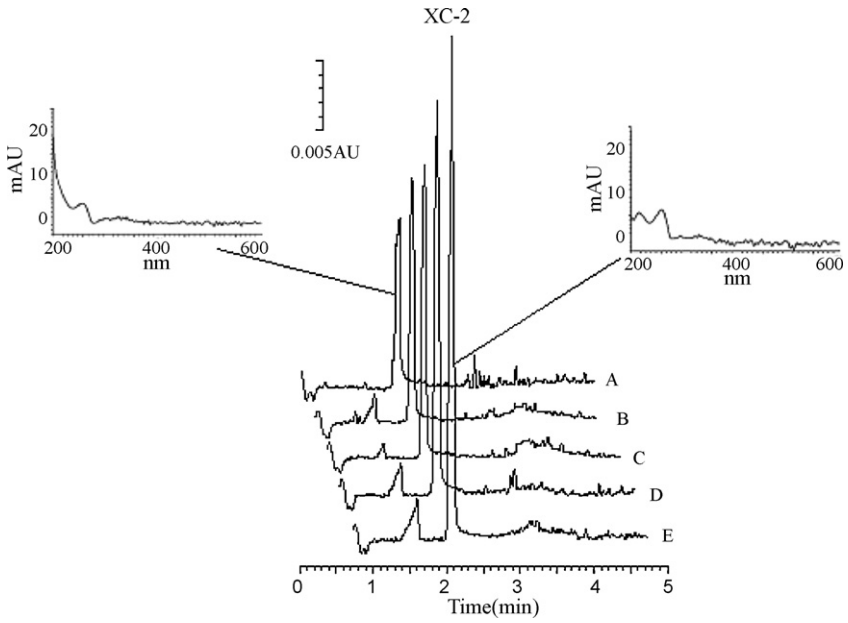


Fig. 3. Electropherograms of XC-2–thrombin interaction. The conditions used were as follows: Beckman P/ACE MDQ capillary electrophoresis system. Injection: 0.5 p.s.i. for 5 s. Applied voltage: 15 kV. Capillary: capillary of 60.2 cm (effective length 50 cm) 75 μm i.d. Natural products: thrombin (mol/mol) (A) 1:0; (B) 1:1.0; (C) 1:2.0; (D) 1:4.0; (E) 1:8.0. Detection at 214 nm.

the sample may not be purified prior to CE separation. Therefore, a large number of samples can be screened by a single CE run for biological activity.

Meanwhile the anti-blood coagulation property was determined by thrombin time (TT) tests *in vitro*. The results showed that ZYY-5, ZYY-6, ZYY-8, ZYY-9, ZYY-12, ZYY-13, FJ-2, FJ-3, FJ-5 and XC-2 possessed different levels of anticoagulant activity. Thereinto, FJ-2 had the largest binding constant and obvious anti-blood coagulation property. Compared with negative control, ZYY-1, ZYY-2, ZYY-3, ZYY-4, ZYY-7, ZYY-10, ZYY-11, ZYY-14, ZYY-15, FJ-1, FJ-4 and XC-1 had no anticoagulant activity by showing no significant difference in TT test.

Isoflavones, as natural dietary phytochemicals, are the focus of much current nutritional and therapeutic interest [23]. Research shows that *M. nitita* var. *hirsutissima* is of flavonoids-rich, especially of isoflavone-aglycones [24,25]. Many studies are beginning to suggest that isoflavones may be involved in many bioactivities. And the strength of biological activity is affected by the type and concentration of the isoflavones. In this study, some compounds with the structure of isoflavones can be observed to have the interaction with thrombin which may demonstrate some possible relations between the binding activity and structure. Most of the isoflavone-aglycones were observed in varying degrees of thrombin-binding activities, while the isoflavone-glycosides rarely have the binding activities. And substituting with hydroxyl at C-7 position in the structure of isoflavone is necessary for the interaction activity, when being substituted with glycosylation, it may lead to the loss of activity. The phenolic hydroxyl group may be indispensable [26]. In

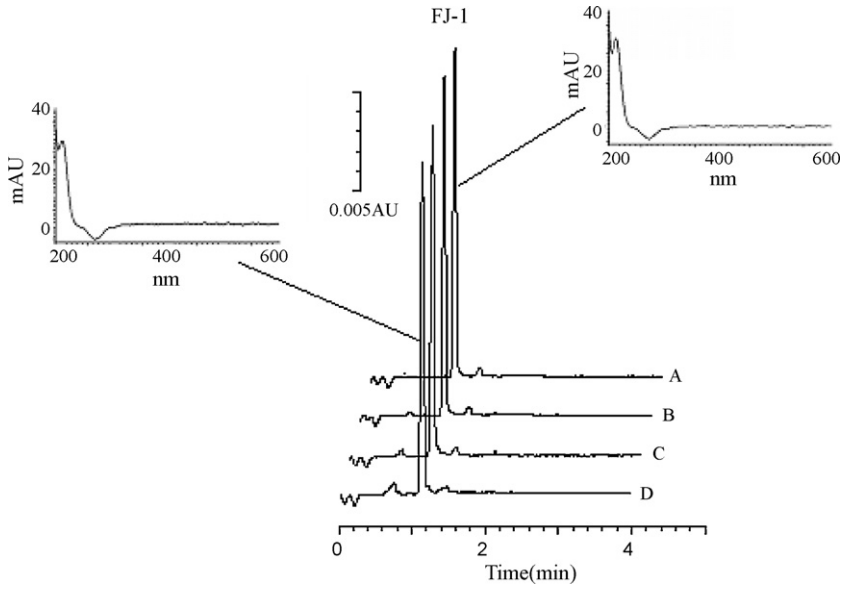


Fig. 4. Electropherograms of FJ-1–thrombin interaction. The conditions used were as follows: Beckman P/ACE MDQ capillary electrophoresis system. Injection: 0.5 p.s.i. for 5 s. Applied voltage: 15 kV. Capillary: capillary of 60.2 cm (effective length 50 cm) 75 μm i.d., natural products: thrombin (mol/mol) (A) 1:0; (B) 1:0.5; (C) 1:2.0; (D) 1:4.0. Detection at 214 nm.

addition, in study of another substitute of Xi Jue Teng, which is *Millettia speciosa* Champ., it is also indicated the important of phenolic hydroxyl at C-7 position [27].

4. Conclusion

The interactions between natural product of *M. nitita* var. *hirsutissima* and thrombin were determined for the first time. Twenty-two compounds were isolated from the natural plant and ten of them had the interactions with thrombin. Nine binding constants of the compounds to thrombin were calculated and the binding constant of FJ-2 was the largest among them [$(8.723 \pm 0.1540) \times 10^4\ M^{-1}$]. The anti-blood coagulation property was determined by TT tests *in vitro* at the same time. FJ-2 had the largest binding constant, which indicated it had obvious anti-blood coagulation property. It was concluded that there was a corresponding relation between interaction activity and anticoagulant activity. The larger the binding constant was, the stronger the anticoagulant would be. Based on above analysis, since *M. nitita* var. *hirsutissima* contains these compounds which have a different levels of anticoagulant activity, it might be the eligible substitute for Suberect Spatholobus Stem. Our results show that CZE provides a highly efficient, fast, quantitative, and sensitive method for studying the interactions between natural product compounds and thrombin. Furthermore, this method can be employed to screen a series of compounds in the drug discovery.

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