ORIGINAL RESEARCH
PAPER

CF-PK-PD analysis of natural hemostatic compounds from *Toddalia asiatica* (Linn) Lam root bark in rats

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ABSTRACT

Natural hemostatic compounds from *Toddalia asiatica* (Linn) Lam (*T. asiatica*) root bark had been investigated by a novel strategy, chemical fingerprint–pharmacokinetic–pharmacodynamic (CF-PK-PD) for the first time in this study. The extract sample of *T. asiatica* root bark was subdivided into petroleum ether (PE), ethyl acetate (EA) and n-butanol (n-B) sample by reagent extraction, EA sample showed significant hemostatic activity using prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (FIB) as evaluation indexes from rat plasma of PK experiment in hemorrhagic rat model. CF analysis was adopted to assist us to discover six natural compounds from *T. asiatica* root bark in actual rat plasma after sample treatment by Ultra Performance Liquid Chromatography-Electrospray Ionization (UPLC-ESI) MS, there were only lomatol and 5-methoxy-8-hydroxy psoralen showing significant hemostatic effect ($P < 0.05$) mainly through endogenous coagulation pathway and fibrinolytic system. In PK-PD study, six compounds in EA sample exhibited relatively rapid absorption and slow elimination characteristics. The mean T_{max} and $t_{1/2\beta}$ of isopimpinellin and pimpinellin were 1.74 and 0.59 h, 5.31 and 6.89 h in rats. On the basis of Sigmoid- E_{max} model, PK-PD related curves of FIB in hemorrhagic rat model after treatment of *T. asiatica* root bark were obtained. Predicted E_{max} , EC_{50} and k_{e0} of FIB under isopimpinellin were 4.87 mg/mL, 1.39 μ g/mL and 0.81 1/h; predicted E_{max} , EC_{50} and k_{e0} of FIB under pimpinellin were 4.29 mg/mL, 2.47 μ g/mL and 0.77 1/h. In conclusion, hemostatic compounds from *T. asiatica* root bark had been materialized, there were lomatol, isopimpinellin, pimpinellin and 5-methoxy-8-hydroxy psoralen at least as its main active substances through coagulation pathways and fibrinolytic system. CF-PK-PD method as a promising method was worthy of follow-up opening, application in pharmaceutical research.

KEYWORDS

Toddalia asiatica (Linn) Lam, CF-PK-PD, LC-MS, pharmacokinetics, pharmacodynamics, hemostatic activity

INTRODUCTION

Toddalia asiatica (Linn) Lam (*T. asiatica*) was a widely-applied natural medicine of Miao minority in Guizhou province, the southwest of China. In addition to having anti-bacterial, anti-oxidant activities and other conventional pharmacological actions [1, 2], hemostasis was its most significant pharmacological effect, had been applied and validated in thousands of years of Chinese folk history, which also led to derive its Chinese name Feilong Zhangxue or Jianxuefei [3–5]. Our research group earlier found the anti-inflammatory, analgesic, hemostatic and anti-tumor effects of *T. asiatica* which were experimentally verified [6–8], a large number of characteristic compounds including coumarins, alkaloids and flavonoids were

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Fig. 1. The original plant (A) and external form (B) of *T. asiatica* root bark

isolated from ethyl acetate (EA) part, n-butanol (n-B) part in *T. asiatica* root bark which were also presumably deduced by high resolution mass spectrometry and chemical databases [9–12], most of these compounds were coumarins, more specifically furan coumarin. *T. asiatica* as a kind of natural medicines, it itself couldn't escape the congenital deficiency of these drugs which were rich in a variety of natural chemical compounds, diverse and complex pharmacological mechanisms to ultimately achieve the same therapeutic effect. Chemical fingerprint–pharmacokinetics–pharmacodynamics (CF–PK–PD) analytical method was first proposed by our research group in 2013 [13], this research strategy was applied to closely link CF, PK and PD, combine time–concentration and concentration–effect, facilitate the description and prediction of the time course and drug effect resulting from a certain dosing regimen. It was particularly suitable for studying material basis and pharmacological mechanisms of natural medicines or traditional Chinese medicines (TCMs) in China. Our group had carried out a series of research work on this traditional herbal medicine (Fig. 1), in this study, *T. asiatica* root bark was chosen as a case to be analyzed in this new research strategy–CF–PK–PD, we aimed to more scientifically explain its material basis and hemostatic mechanisms by CF–PK–PD, effectively promote the development and utilization of the medicinal resources, new drug research process.

EXPERIMENTAL

Materials and reagents

T. asiatica root bark was collected from mountain slopes in Huaxi district, Guiyang, China in January 2014, which was authenticated by Professor Deyuan Chen from Guiyang College of traditional Chinese medicine (TCM). This voucher specimen was stored at Standard Library of traditional Chinese medicine and ethnic medicine, Guizhou Medical University. Isopimpinellin, pimpinellin were isolated from *T. asiatica* root bark by the authors in our lab, their purities were above 98% according to HPLC/UV analyses. Yunnan baiyao powder (Yunnan Baiyao Group Co., Ltd, lot no. ZDA1507); Aspirin enteric-coated tablets (CSPC Ouyi Pharmaceutical Co., Ltd., lot no. 286160208). Acetonitrile, methanol, formic acid (HPLC-pure, Sinopharm Chemical, China); pure distilled water (Watsons, Guangzhou, China). All other chemicals were of analytical grade. One-time vacuum blood collection tube, sodium citrate (1:9) (2ml, Aosaite Medical, Shandong, China). STA-R evolution coagulation analyzer (STAGO, France), JN300-2 nitrogen concentration (Ji Mino, Suzhou, China), 80-2B low-speed centrifuge (Anting Scientific, Shanghai, China), TGL-16G high-speed centrifuge (Anting Scientific, Shanghai, China), RE-201D rotary evaporator (Yuhua, Zhengzhou, China), SK-1 vortex mixer (Jintan, Jiangsu, China).

Drug preparation

The dried *T. asiatica* root bark 10 kg was firstly extracted in the solid-to-liquid ratio of 1:5 by 95% ethanol maceration, about one week time was needed per maceration extraction. After repeated extraction, each batch of *T. asiatica* root bark had been finally extracted for nine times. All combined extraction solutions were then filtered by Buchner funnel and concentrated in vacuo to yield the total dry extracts, and then these extracts were suspended in pure water. Sequential liquid–liquid extraction was carried out for different polar sample extracts through small polar reagents to large polar reagents: petroleum ether (PE, bp 60–90 °C), EA and n-B. Each extract sample was dissolved in 0.5% carboxymethyl-cellulose sodium (CMC-Na) aqueous solution at the final concentration of 80 mg/mL.

Drug administration and sampling

For the PK study, all Sprague-Dawley (SD) rats were housed at the Experimental Animal Laboratory of Department of Pharmaceutical Analysis, School of Pharmacy, Guizhou Medical University. All experimental procedures and protocols were reviewed and approved by the Animal Care and Use Committee of Guizhou Medical University, and were in accordance with the Guide for the Care and Use of Laboratory Animals. A total of 36 SD rats with body weight 180–250 g were randomly separated into 6 groups, half male and half female in each group: blank control group, hemorrhagic model group, positive control group, PE group, EA group and n-B group. In the first week, hemorrhagic rat model should be established at the beginning before the formal drug administration in rats. All selected SD rats for this experiment were orally given aspirin solution at a dose of 3.0 mg/kg except blank control group, once a day, continuous administration for 7 days[14]. On the eighth day, the second week, positive control group began to be orally given Yunnan Baiyao solution in 0.35 g/kg (Table 1), PE group, EA group and n-B group were treated with intragastric administration (IG) corresponding sample solutions (0.08 g/mL) respectively, IG volume to rats was 12.5 mL/kg. At the same time, blank control group, model group were administered orally by 0.9% saline solution. Once a day, continuous administration for 7 days. On the 15th day, the third week, the target dose of 1.0 g/kg extract sample was

administered by oral gavage to the rats, the rats had free access to water after oral administration. Blood samples were obtained from the tail vein according to specific schedules (0.25, 0.5, 0.75, 1.0, 1.5, 2.5, 4.0, 6.0, 8.0, 10.0 h). Sodium citrate anticoagulated blood was immediately centrifuged at 3,500 rpm for 15 min. The supernatant layer of the blood was thus collected as plasma, 50 µL of each separated plasma sample was stored at –20 °C prior to liquid chromatography-mass spectrometry (LC-MS) assay, the remaining plasma samples prepared for the hemostatic activity test were all stored at 4 °C before this biochemical analysis. After the completion of this experiment, all remaining rats were euthanized with chloral hydrate followed by cervical dislocation.

Biosample pretreatment

A 50 µL plasma sample was added to 150 µL blank methanol, vortexed for 20 s, then the samples were centrifuged at 13,000 rpm for 5 min. All supernatants of each 1.5 mL Ep tube were transferred to a new 1.5 mL Ep tube, respectively. These preliminarily-purified samples were further dried in nitrogen concentration, the residue in each tube was redissolved in 100 µL methanol, finally centrifuged at 13,000 rpm for 5 min. The supernatant liquid was biologically tested sample we want for UPLC-MS analysis.

Hemostatic activity test

According to the previously-scheduled plasma collection point, SD rats from each group were given orally to corresponding drugs, and then anticoagulated blood was collected, centrifuged. After subtracting the samples for chromatographic analysis, the supernatant layers were stored at 4 °C for hemostatic activity analysis, all tests had been completed on the same day. prothrombin time (PT), activated partial thromboplastin time (APTT) and fibrinogen (FIB) were chosen as indexes of hemostatic activity of plasma samples of all sampling time points, which were tested by automated coagulation analyzer. Each data as average obtained from six rat experiments, the final values of these indexes in the form of mean ± standard deviation were derived from the average of all data at 1, 4 and 8 h sampling time points in each group. The significant differences were calculated by independent-samples *t*-test for comparing

Table 1. Effect of different polarity fractions of 95% ethanol extract on hemostatic activity of *T. asiatica* root bark (mean ± standard deviation, *n* = 6)

	Dose (g/kg)	PT (s)	APTT (s)	FIB (mg/mL)
Blank	–	20.80 ± 2.50	39.07 ± 5.19	2.71 ± 0.32
Model	–	34.50 ± 6.22 ^{△△}	47.43 ± 3.42 [△]	2.13 ± 0.38 [△]
Positive	0.35	20.90 ± 3.43 ^{**}	34.83 ± 2.57 ^{**}	2.46 ± 0.34
PE	1.0	28.29 ± 3.51	43.86 ± 2.37	2.04 ± 0.22
EA	1.0	22.05 ± 4.05 [*]	35.23 ± 6.69 [*]	2.51 ± 0.33 [*]
n-B	1.0	25.94 ± 3.07 [*]	32.00 ± 9.55 [*]	2.29 ± 0.39

Compared with model group ^{*}*P* < 0.05, ^{**}*P* < 0.01.

Compared with blank group [△]*P* < 0.05, ^{△△}*P* < 0.01.

between the two groups. $P < 0.05$ was considered to indicate a statistically significant difference.

Chromatographic separation

Chromatographic separation was performed on a Thermo Hypersil GOLD C18 column (50×2.1 mm, $1.9 \mu\text{m}$). Accela 1250 UPLC system coupled to a photodiode array detector (PDA), a binary pump, an autosampler and a column compartment. A two-component mobile phase consisting of 0.1% (v/v) formic acid in acetonitrile (A): 0.1% (v/v) formic acid in water (B) was used following the elution program: 0 min, A:B (5:95, v/v); 20 min, A:B (90:10, v/v); 25 min, A:B (90:10, v/v). The flow rate was 0.2 mL/min, column temperature was maintained at 25 °C, injection volume was 5 μL by automatic sampling system at 20 °C.

UPLC-ESI MS conditions

A Thermo TSQ Quantum Ultra Triple-Quadrupole Mass Spectrometer (Thermo Fisher, USA) instrument system hyphenated with Thermo Accela 1250 UPLC equipped with an ESI source was applied for qualitative and quantitative analysis of plasma samples from rats. Mass spectrometer and UPLC system were controlled by Thermo Xcalibur® V3.0 software (Thermo, USA). The UPLC effluent after chromatographic separation was introduced into the ESI source, each sample was determined in positive ion, Total Ion Chromatography (TIC) modes. The parent ion signals of m/z 100 to 1,000 were all collected. The sheath gas was 35 Arb, auxiliary gas was 15 Arb. Ion monitoring conditions were set

with 3000 V for spray voltage, 500 °C for vaporizer temperature, and 350 °C for capillary temperature.

Data analysis

CF analysis was based on bivariate correlation analysis in this study. Pharmacokinetic parameters of the determined compounds were processed by the non-compartmental method using Data Analysis System (DAS) 3.2 (Center for Drug Clinical Research, Shanghai University of Traditional Chinese Medicine, China). Linear trapezoidal integration was used to calculate areas under the concentration–time curves (AUCs). The maximum plasma concentration (C_{max}), the time to reach C_{max} (T_{max}) and other parameters following the administration of *T. asiatica* root bark were determined from the observed data. Based on the PK and PD data, a PK–PD fitted mathematic model was established to investigate the relationship between the active compounds in *T. asiatica* root bark and the pharmacodynamic indexes, Sigmoid- E_{max} model (Hill equation, $E = (E_{\text{max}} \times C^s) / (EC_{50}^s + C^s)$) as a classic PK–PD model was applied here. All the data were expressed as the mean $\pm$ standard deviation. A P -value less than 0.05 were considered statistically significant.

RESULTS AND DISCUSSION

Screening of hemostatic activity of different samples

Aspirin-induced hemorrhagic rats as a kind of pathological model to investigate the hemostatic activity of different

Fig. 2. TIC chromatograms of blank rat plasma sample (A), model rat plasma sample (B), positive control group plasma sample (C), PE group plasma sample (D), EA group plasma sample (E) and n-B group plasma sample (F)

Fig. 3. EIC chromatograms of six specific molecular ions in rat plasma sample after single oral administration of EA sample for 2.5 h. (A) m/z 260 (Rt 9.3 min), (B) m/z 230 (Rt 9.8 min), (C) m/z 246 (Rt 10.1 min), (D) m/z 246 (Rt 10.6 min), (E) m/z 246 (Rt 11.1 min), (F) m/z 233 (Rt 14.0 min). EIC chromatograms of rat blank plasma spiked with isopimpinellin (G) or pimpinellin (H) reference solution

polarity sample extracts from *T. asiatica* root bark were used in this study, these hemostatic rat model were divided into several groups, these rats in the same group received the same drug treatment, but different drugs between group and group. After one week of continuous drug treatment, conventional PK studies were conducted. All plasma samples at sampling time points within 10 h were collected since the last IG drug administration to rats, timely purified and tested. PT and APTT levels of the model group were significantly higher than that of the blank control group ($P < 0.05$), meanwhile, FIB was exactly lower with statistical difference ($P < 0.05$). These results indicated that the

aspirin-induced hemorrhagic rat model was successful (Table 1). The hemostatic indexes of the positive control group had been reversed and improved to a great extent under the treatment of classic drug, Yunnan Baiyao. The results from PE group, EA group and n-B group reflected the therapeutic effect of each test drug sample, hemostatic differences between the different groups were obvious. The hemostatic activity of EA sample was the strongest among these three tested extracts, followed by n-B sample, PE sample. Compared with PT, APTT and FIB of model group, PT value and APTT value of EA group were reduced by 12.45 s ($P < 0.01$), 12.20 s ($P < 0.05$) respectively, its FIB value was

Fig. 4. Plasma drug concentration-versus-time curve after single oral administration of EA sample 1.0 g/kg to rat model ($n = 6$). (A) Marker 1, (B) Marker 2, (C) Marker 3, (D) Marker 4, (E) Marker 5, (F) Marker 6

Table 2. Main pharmacokinetic parameters of isopimpinellin and pimpinellin after oral EA sample 1.0 g/kg of *T. asiatica* root bark in hemorrhagic rat model

Parameters	Isopimpinellin	Pimpinellin
$t_{1/2\beta}$ (h)	5.31 ± 1.87	6.89 ± 0.34
V_1/F (L/kg)	14.76 ± 4.84	15.89 ± 12.95
CL_1/F (L/h/kg)	3.86 ± 0.38	3.56 ± 0.89
AUC_{0-t} ($\mu\text{g h/mL}$)	26.81 ± 5.79	99.31 ± 25.55
K_{10} (1/h)	0.28 ± 0.09	1.12 ± 0.71
K_{12} (1/h)	0.82 ± 0.46	13.23 ± 9.16
K_{21} (1/h)	1.35 ± 1.07	5.58 ± 4.58
K_a (1/h)	1.62 ± 0.35	9.52 ± 6.47
T_{lag} (h)	0.09 ± 0.04	0.17 ± 0.07
T_{max} (h)	1.74 ± 0.30	0.59 ± 0.51
C_{max} ($\mu\text{g/mL}$)	4.28 ± 0.91	16.60 ± 2.31

increased by 0.38 mg/mL accordingly ($P < 0.05$). The overall pharmacological effect indicated the hemostatic activity of *T. asiatica* root bark could activate exogenous and endogenous coagulation pathways, also increased the hemostatic sensitivity of the fibrinolytic system. And this natural medicine ultimately reflected its own good hemostatic activity.

Chemical fingerprint analysis

In order to understand in depth hemostatic material basis of *T. asiatica* root bark, chemical fingerprint (CF) technique based on UPLC-ESI MS was applied in the analysis of rat plasma samples. The typical TIC chromatograms of blank rat plasma sample, model rat plasma sample, and different polarity plasma samples were listed in Fig. 2. As very high detection sensitivity of MS and unusually complex chemical composition of rat plasma and natural medicines, independent data acquisition (IDA) was adopted in chemical fingerprint analysis, which can be good at excluding most of the substances with low content, weak MS signal, MS fragments or biological endogenous components. By repeated comparison and analysis of blank control group, PE group, EA group

and n-B group, there were six characteristic ions, m/z 260 $[M+H]^+$ (Marker 1), 230 $[M+H]^+$ (Marker 2), 246 $[M]^+$ (Marker 3), 247 $[M+H]^+$ (Marker 4), 247 $[M+H]^+$ (Marker 5), 233 $[M+H]^+$ (Marker 6) in PE, EA and n-B samples completely from *T. asiatica* root bark, first found in rat plasma samples (Fig. 3). By searching reported known natural compounds from this herbal medicine in Shanghai Institute of Organic Chemistry (SIOC) chemical database and CNKI, combining with research literature and database data, the possible natural compounds were initially inferred, they were skimmianine (RN 83-95-4, Marker 1), gamma-fagarine (RN 524-15-2, Marker 2), lomatol (RN 19380-05-3, Marker 3), isopimpinellin (RN 482-27-9, Marker 4), pimpinellin (RN 131-12-4, Marker 5), 5-methoxy-8-hydroxy psoralen (RN 1603-47-0, Marker 6). Through further comparison by reference substance, Marker 4 and Marker 5 were finally identified as isopimpinellin and pimpinellin according to the same chromatographic retention time, molecular ion peaks. Isopimpinellin and pimpinellin were analyzed and found 0.17 and 0.56 g/g respectively in EA sample of *T. asiatica* root bark. Because of EA sample's significant hemostatic activity on hemorrhagic rat model rather than PE and n-B samples, we decided to do bivariate correlation analysis of PT, APTT, FIB and Markers 1–6 in LC-MS chromatograms. The statistical analysis results suggested that Markers 2, 3, 5, 6 had their own degree of negative correlation with PT, but without statistical differences ($P > 0.05$). Markers 1, 3, 4 also existed obviously negative correlation with APTT, especially Marker 3 with correlation coefficient 0.864 ($P < 0.05$). While FIB as an index for evaluation of test sample activity, Markers 3, 4, 5, 6 showed positively correlated with this pharmacological index, the correlation coefficient of Markers 3 and 6 were 0.750 ($P < 0.05$) and 0.681 ($P < 0.05$) respectively. Based on the above findings and analytical data, we can confirm that these were close relationship between Markers 3, 6 and hemostatic activity of *T. asiatica* root bark, lomatol and 5-methoxy-8-hydroxy psoralen as natural compounds in *T. asiatica* root bark

Fig. 5. Schematic diagram of PK-PD model in this study (A). Efficacy-versus-time curve after single oral administration of EA sample 1.0 g/kg to rat model ($n = 6$), (B) PT, (C) APTT, (D) FIB

Fig. 6. PK-PD related mean curves of FIB after single oral administration of EA sample to rat model ($n = 6$). (A) isopimpinellin, (B) pimpinellin

Table 3. Pharmacodynamic parameter of FIB with isopimpinellin or pimpinellin after oral EA sample 1.0 g/kg of *T. asiatica* root bark in hemorrhagic rat model

Parameters	Isopimpinellin	Pimpinellin
$E_{\max}$ (mg/mL)	4.87 ± 0.19	4.29 ± 0.75
EC_{50} ($\mu\text{g/mL}$)	1.39 ± 0.85	2.47 ± 1.27
s	0.24 ± 0.08	0.38 ± 0.14
k_{e0} (1/h)	0.81 ± 0.45	0.77 ± 0.31

ensured this pharmacological effect through activating endogenous coagulation pathway and fibrinolytic system.

PK-PD analysis

The actual plasma pimpinellin concentration and plasma isopimpinellin concentration can be directly calculated through their reference substance solutions and verified linear equations. Systematic LC-MS method validation had been done before, intra- and inter-day precision of the method was assessed by testing the quality control stock solution in the same day for five times and on three consecutive days. The Relative Standard Deviation (RSD) values for intra- and inter-day precisions ranged within 15.0%, indicating the acceptable precision of the method. Stability of the analytes was evaluated by analyzing the same sample solution stored at 4 °C within 24 h. RSD of the analytes ranged from 0.62 to 21.62%, indicating the analytes were stable. The recovery was validated by spiking the reference solutions to a blank rat plasma at three concentration levels. Recovery of the 2 analytes varied from 86.05 to 113.82% with RSD between 0.57 and 18.24%, indicating the method was accurate.

All markers' peak area in rat plasma-versus-time curves after single oral administration of EA sample to rat model had been fitted out in Fig. 4, the curves of Marker 4 and Marker 5 were their own actual plasma concentration-versus-time curves ($n = 6$). It demonstrated that those six compounds in EA sample exhibited relatively rapid absorption and slow elimination characteristics. The PK model and the parameters were calculated using the PK software. The mean $T_{\max}$ and $t_{1/2\beta}$ of isopimpinellin and pimpinellin were 1.74 and 0.59 h, 5.31 and 6.89 h in rats. The main PK parameters of isopimpinellin and pimpinellin in hemorrhagic rat model after oral administration were summarized in Table 2.

The hemostatic effect of tested sample orally administered to rat model was recorded synchronously, strictly abiding by time schedule of PK experiment. Efficacy-versus-time curve after single oral administration, Fig. 5 showed the therapeutic effect of EA sample with the time after oral administration. As PT and APTT values were both negatively correlated with the concentrations of tested compounds, these two types of efficacy data needed to be converted into positive correlation data before their PK-PD analysis. Different conversion methods would result in

different analytical data, and then getting diverse research results. Therefore, here FIB as a suitable index of hemostatic activity of *T. asiatica* root bark was further investigated in the light of Sigmoid- $E_{\max}$ model. PK-PD related curves of FIB in hemorrhagic rat model after treatment of *T. asiatica* root bark were drawn in DAS system, including $C-T$, $\ln C-T$, $C_{\text{obs}}-E_{\text{obs}}$, C_e-E_{pre} curves in Fig. 6. Pharmacodynamic parameter of FIB were also calculated out, the establish PK-PD model had predicted $E_{\max}$ 4.87 mg/mL of FIB under isopimpinellin, EC_{50} 1.39 $\mu\text{g/mL}$ of isopimpinellin, k_{e0} 0.81 1/h; $E_{\max}$ 4.29 mg/mL of FIB under pimpinellin, EC_{50} 2.47 $\mu\text{g/mL}$ of pimpinellin, k_{e0} 0.77 1/h (Table 3). These data also indirectly certified that isopimpinellin and pimpinellin obtained a certain degree of in-vivo pharmacological activity in hemostasis.

DISCUSSION

CF-PK-PD as a novel method was the first attempt to be proposed and applied in fundamental research on pharmacodynamic substances of natural medicines or traditional Chinese medicines. CF-PK-PD is to add CF analysis to the recognized PK-PD technique in pharmacometrics. Compared to classical PK-PD method, CF-PK-PD method can fully embodies systematicness, characteristics and stability of complex drugs, help our researchers to efficiently find out effective chemical constituents of complex drugs, describe and predict time course, drug effect and pharmacological mechanism in response to administration of a drug dose. But there is a prerequisite for the smooth implementation of CF-PK-PD technology in my opinion which was timely feedback of efficacy of test drugs in relatively limited time of drug research.

By screening and discovering of hemostatic compounds from *T. asiatica* root bark in this study, EA sample of *T. asiatica* root bark was once again confirmed to obtain the most significant hemostatic activity relatively, this extract sample could activate exogenous, endogenous coagulation pathways and fibrinolytic system [15]. Through CF analysis, six main natural compounds from *T. asiatica* root bark were found in the plasma of hemorrhagic rat model receiving treatment of *T. asiatica* root bark, they were skimmianine, gamma-fagarine, lomatin, isopimpinellin, pimpinellin and 5-methoxy-8-hydroxy psoralen successively. Lomatin and 5-methoxy-8-hydroxy psoralen as natural compounds in *T. asiatica* root bark expressed their hemostatic effect mainly through endogenous coagulation pathway and fibrinolytic system. These results of CF analysis of *T. asiatica* (Linn) Lam root bark provided the necessary and important specific pharmacodynamics substances for subsequent PK-PD analysis.

Since only pimpinellin and isopimpinellin of six markers in rat plasma could be calculated for drug concentration in rat plasma, the subsequent PK-PD analysis was around the two markers, main PK and pharmacodynamic parameters,

C_e-E_{pre} curves had been provided. But due to limited collection volume of rat plasma samples in this CF-PK-PD study, thrombin time (TT) as the last one had been hard to detect in actual experiment after completing the first three indexes of the test program settings. Anyway, the increase of FIB content indicated fibrin system had been regulated, TT level should be lowered back in contrast to the level of the hemorrhagic rat model.

In conclusion, hemostatic compounds from *T. asiatica* root bark had been materialized. Lomatin, isopimpinellin, pimpinellin, and 5-methoxy-8-hydroxy psoralen played more important roles in hemostasis of *T. asiatica* root bark through activating coagulation pathways and fibrinolytic system.

Conflict of interests: The authors declare that they have no competing interests.

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ABBREVIATIONS

PE	petroleum ether
EA	ethyl acetate
n-B	n-butanol
CMC-Na	carboxymethylcellulose sodium
IG	intragastric administration
IDA	independent data acquisition
TCM	traditional Chinese medicine
PK	pharmacokinetic
PT	prothrombin time
APTT	activated partial thromboplastin time
FIB	fibrinogen
TT	thrombin time
LC-MS	liquid chromatography-mass spectrometry
TIC	total ion current
EIC	extracted ion chromatogram

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