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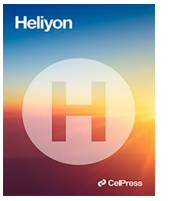

Research article

Comparative pharmacokinetic study on phenolic acids and flavonoids in normal and microcirculation dysfunction rats plasma by UPLC-TQ/MS/MS after oral administration of *Salvia miltiorrhiza* stem-leaf extracts

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ABSTRACT

According to the Standard of Chinese Medicinal Materials of Shaanxi Province (2015 edition), *Salvia miltiorrhiza caulis et folium* is the dried stems and leaves of *Salvia miltiorrhiza*, which could activate blood and dispell blood stasis, clear the mind and remove annoyance. In this study, the dynamic absorption changes of phenolic acids (FS) and phenolic acids-flavonoids (FT) in rats after oral administration were studied by UPLC-TQ/MS/MS, to elucidate the pharmacokinetics of seven major bioactive components of the stem-leaf of *Salvia miltiorrhiza in vivo*. The results showed that the pharmacokinetic parameters of FS and FT were significantly different in normal rats and model rats. Compared with the control group, after injecting 10 % polymer dextran 500 into the tail vein to establish a model of microcirculation disturbance, the C_{max} of caffeic acid decreased. The C_{max} of rosmarinic acid and lithospermic acid increased. Danshensu showed a decrease in CLz/F, accompanied by an increase in both $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$. The $AUC_{0\text{-}t}$ of lithospermic acid was also increased. These results indicated that microcirculation disturbance could decrease the absorption of caffeic acid while increasing the absorption of danshensu, rosmarinic acid and lithospermic acid. After oral administration of FT, the C_{max} of danshensu and the $AUC_{0\text{-}t}$ of caffeic acid were increased significantly, suggesting that the presence of flavonoids may promote the absorption and exposure of phenolic acids *in vivo*. This study provides a reference for the elucidation of the *in vivo* substances and the mechanisms of action of FS and FT from the stem-leaf of *Salvia miltiorrhiza*.

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1. Introduction

Salvia miltiorrhiza is the dried roots and rhizomes of *Salvia miltiorrhiza Bge.*, a species of plant in the *Lamiaceae* family, which has the efffect of activating blood circulation to dissipate blood stasis, regulating menstrual pain, clearing the mind to dispel irritation and cooling blood to eliminate carbuncles [1]. According to the Standard of Chinese Medicinal Materials of Shaanxi Province (2015 edition) [2], *Salvia miltiorrhiza caulis et folium* is the dried stems and leaves of *Salvia miltiorrhiza*, which has the effect of activating blood and dispelling blood stasis, clearing the mind and removing annoyance. Modern studies have shown that the stem-leaf part of *Salvia miltiorrhiza* account for more than 2/3 of the whole plant, and are rich in salvianolic acids and flavonoids [3]. Salvianolic acid is the most abundant component in *Salvia miltiorrhiza* and plays a major role in pharmacological activities, such as antioxidant, anti-inflammatory, anti-tumor and so on [4–9]. The preliminary study found that the composition of salvianolic acids in the stem-leaf of *Salvia miltiorrhiza* was similar to that in roots, whereas, the content and proportion of specific components were different [3,10,11].

Due to the complex process *in vivo* after administration, the exact therapeutic mechanism of the stem-leaf part of *Salvia miltiorrhiza* remains unclear [12]. At present, the metabolic kinetics and metabolites of total phenolic acids, monomeric phenolic acids, and monomeric flavonoids of *Salvia miltiorrhiza* in rats have been studied [13–18]. After oral administration of rosmarinic acid (RA) at doses ranging from 12.5 to 50 mg kg^{-1}, the results showed that the systemic exposure of it in rats was not dose-dependent. The pharmacokinetic characteristics of RA were fast absorption, moderate elimination and low absolute bioavailability [19]. After administration, four tanshinones, cryptotanshinone, tanshinone IIA, dihydrotanshinone I and tanshinone I can be widely distributed in lung, heart, liver, kidney and brain tissues, and the *AUC* and C_{max} of them in rats showed a significant increase in tanshinone extract group compared with tanshinone alone group [20]. Nevertheless, there are few studies on the absorption and metabolism of phenolic acids and flavonoids in the stem-leaf of *Salvia miltiorrhiza*.

Our previous study showed that salvianolic acids and flavonoids, the active components of the stem-leaf of *Salvia miltiorrhiza*, could

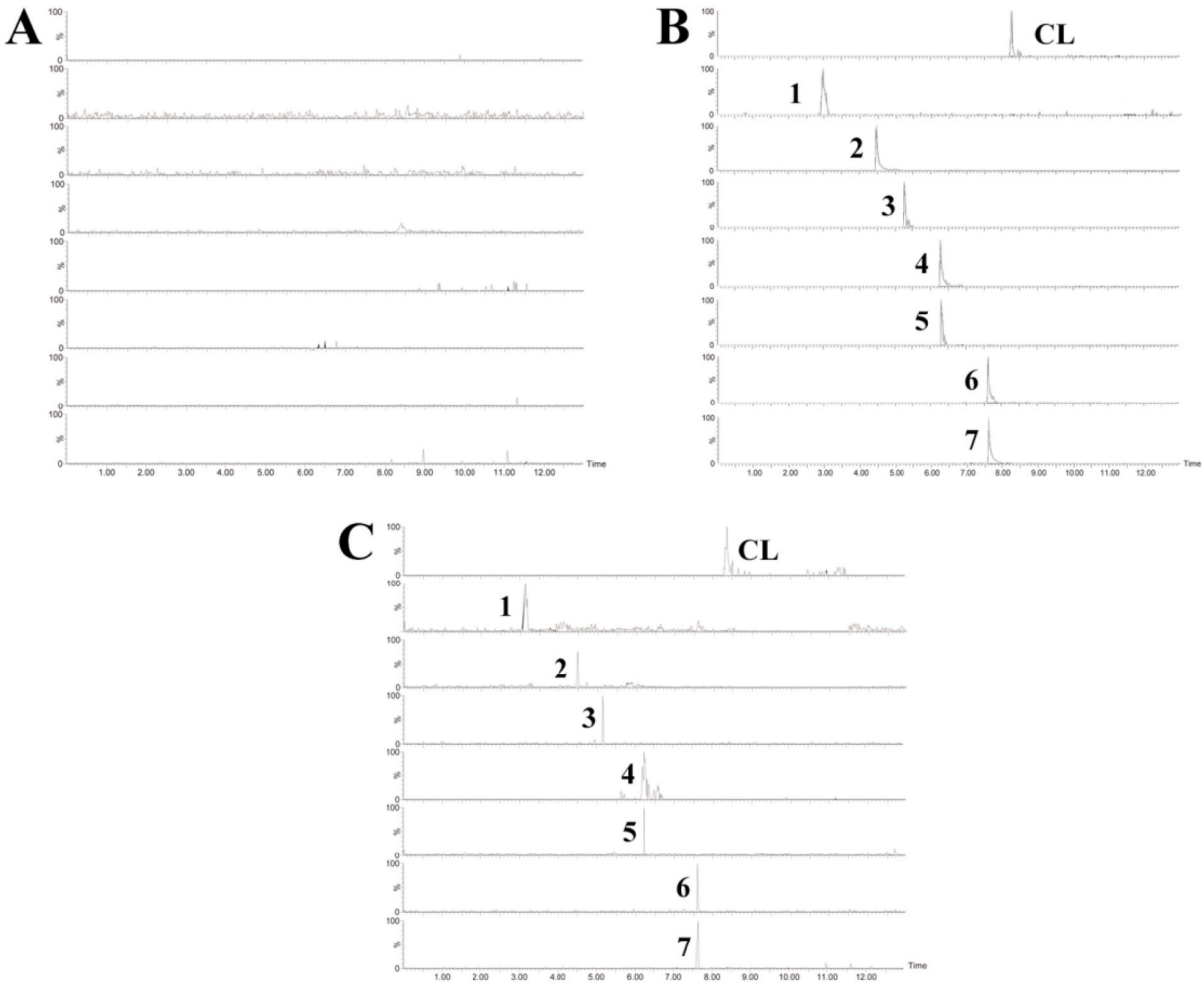

Fig. 1. Blank plasma (A); blank plasma added with internal standard and standards (B); rat plasma sample 20 min after FT administration (C). CL: clarithromycin; 1: Danshensu; 2: Caffeic acid; 3. Rutin; 4: Rosmarinic acid; 5: Lithospermic acid; 6: Salvianolic acid A; 7: Salvianolic acid C.

improve the hemorheological disorder and vascular endothelial function in microcirculatory dysfunction model rats [21]. In this study, two effective parts were prepared from the stem-leaf of *Salvia miltiorrhiza*. One was the active part containing low-purity phenolic acids and flavonoids (FT), with the contents of phenolic acids and flavonoids being 46.77 % and 4.72 %, respectively. The other was further purified on the basis of FT, and the effective part containing high-purity phenolic acids was obtained (FS), with the phenolic acid content being 67.48 %. On the basis of our previous study [21], seven bioactive components in FS and FT were selected for pharmacokinetic study. We analyzed and compared the pharmacokinetics of phenolic acids and flavonoids in the plasma of rats in normal group and microcirculatory dysfunction model group by triple-quadrupole tandem mass spectrometry (UPLC-TQ/MS/MS), after oral administration of two kinds of stem-leaf extracts of *Salvia miltiorrhiza*, attempting to explore the characteristics of pharmacokinetic parameters and lay a foundation for further elucidation of the pharmacological mechanism of *Salvia miltiorrhiza* stem-leaf.

2. Results

2.1. Methodology investigation

2.1.1. Specificity investigation

Blank plasma, blank plasma with seven standard products and rat plasma samples administered after 20 min were processed as described in "4.5". The MRM chromatograms of the three samples were analyzed by UPLC-TQ/MS/MS. The results showed that there were no significant endogenous or other interfering substances in the retention time of the internal standard and the seven standards in blank plasma, as each component tested had a good peak shape. The MRM diagrams of blank plasma, blank plasma added with internal standard and standards, and rat plasma sample 20 min after FT administration can be seen in Fig. 1 A-C.

2.1.2. Investigation of linearity and LOQ

The solutions of mixed standards at different concentrations were detected and analyzed. The linear regression was performed using the ratio of the peak area of the components to the peak area of the internal standard (y) and the corresponding mass concentration (x, ng·mL^{-1}). The calibration curve was drawn, and the limit of quantitation (LOQ) of the components to be tested was calculated when the S/N was 10. The regression equations and correlation coefficients of the seven phenolic acids and flavonoids in the stem-leaf of *Salvia miltiorrhiza* are shown in Table 1. The results showed that there was a good linear relationship in the concentration ranges of the seven active components ($R > 0.9811$), while LOQ value was between 0.8 and 3.6 ng mL^{-1}.

2.1.3. Investigation of precision and accuracy

Mixed standards solutions of high, medium and low concentrations were added to blank plasma of rats, and then six quality control (QC) samples with corresponding concentrations were processed and prepared according to the method of "4.6". Precision was evaluated by relative standard deviation (RSD, %), and accuracy was evaluated by relative error (RE, %). The intra-day and inter-day precision and accuracy results of QC samples with high, medium and low concentrations were shown in Table 2. The precisions of the seven components were between 1.99 % and 13.76 %, and the accuracies were between −13.64 % and 12.93 %.

2.1.4. Extraction recovery and matrix effect

QC samples of different concentrations were prepared by taking 90 μL of blank rat plasma and adding 10 μL of high, medium and low concentration standard solutions. Prepare six copies of the QC samples of each concentration in parallel and process them according to the method in "2.7". Then the extraction recovery rate was detected and calculated. Take 90 μL of blank plasma and ultrapure water and add 10 μL of high, medium and low concentration standard solutions to prepare QC samples with different concentrations. Then prepare six copies of each concentration in parallel, and the plasma samples were treated in accordance with the "4.6" Processing method: the samples were treated and tested, and the matrix effects of each compound were investigated by comparing the peak area ratios of the two detection methods. As shown in Table 3, the results showe that the extraction recovery rates of the seven components are between 86.01 % and 97.03 %, and the matrix effects are between 87.10 % and 98.75 %, indicating that the extraction recovery rates of these seven components are good and are not affected by the matrix effect.

2.1.5. Stability test

The stability of QC samples with high, medium and low concentrations was evaluated under the following four conditions: three

Table 1
Regression equation and limit of quantitation (LOQ) of seven phenolic acids and flavonoids in the stem-leaf of *Salvia miltiorrhiza* (FT).

Chemical compound	Regression equation	Linear range /ng•mL^{-1}	R value	LOQ/ng•mL^{-1}
Danshensu	$y = 3.5291x - 8.8408$	410.15–26250.00	0.9997	3.6
Caffeic acid	$y = 410.67x - 780.13$	94.73–6062.50	0.9966	0.8
Rutin	$y = 0.6825x - 2.7358$	125.00–8000.00	0.9938	1.0
Rosmarinic acid	$y = 5.135x - 24.28$	328.13–21000.00	0.9992	2.8
Lithospermic acid	$y = 0.35x - 2.5577$	218.75–14000.00	0.9926	2.0
Salvianolic acid A	$y = 0.3123x - 1.3441$	146.48–9375.00	0.9811	1.1
Salvianolic acid C	$y = 3.8385x - 6.2541$	148.44–9500.00	0.9998	1.0

Table 2
Precision and accuracy for the determination of the seven compounds.

Chemical compound	Concentration (ng·mL^{-1})	intra day		inter day	
		Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)
Danshensu	6562.50	9.90	4.52	5.45	−7.38
	1640.63	10.69	1.71	9.82	−9.84
	410.16	10.23	−12.12	4.32	−7.44
Caffeic acid	1515.63	7.46	−7.57	3.72	2.20
	378.91	7.63	12.68	3.88	10.70
	94.73	4.17	2.06	5.33	−7.01
Rutin	2000.00	13.76	−5.89	5.40	9.81
	500.00	5.54	−13.19	3.78	9.55
	125.00	11.14	−13.64	3.95	−9.49
Rosmarinic acid	5250.00	6.01	5.36	5.28	6.28
	1312.50	6.20	−3.11	10.57	12.03
	328.13	7.96	12.93	7.04	11.10
Lithospermic acid	3500.00	8.10	3.62	4.71	−4.96
	875.00	11.74	0.50	10.74	−10.40
	218.75	7.96	6.12	12.03	6.23
Salvianolic acid A	2343.75	10.66	12.61	7.38	2.00
	585.94	5.65	5.16	2.24	6.07
	146.48	7.53	−9.92	9.50	5.71
Salvianolic acid C	2375.00	5.31	−4.03	1.99	−3.35
	593.75	5.20	6.17	4.96	−4.93
	148.44	7.93	5.52	5.45	−13.61

Table 3
Recoveries and matrix effects of the seven compounds.

Chemical compound	Mass concentration (ng·mL^{-1})	Extraction recovery	Matrix effect
		(%, Mean ± S.D.)	(%, Mean ± S.D.)
Danshensu	22.82	87.93 ± 3.68	98.75 ± 4.35
	365.20	86.07 ± 4.43	88.23 ± 6.74
	2922.00	90.09 ± 5.34	92.76 ± 3.11
Caffeic acid	22.94	86.01 ± 6.28	91.65 ± 7.50
	367.00	92.08 ± 4.85	87.68 ± 5.43
	2936.00	87.96 ± 3.28	96.72 ± 6.84
Rutin	23.17	93.05 ± 3.25	87.10 ± 7.02
	370.60	93.28 ± 8.07	97.29 ± 5.06
	2965.00	87.56 ± 3.25	88.90 ± 2.15
Rosmarinic acid	22.71	87.08 ± 8.68	96.53 ± 0.89
	363.40	88.28 ± 2.96	93.13 ± 6.91
	2907.00	89.26 ± 6.38	97.63 ± 5.20
Lithospermic acid	23.73	91.22 ± 7.03	94.67 ± 3.39
	379.10	93.55 ± 5.63	89.67 ± 4.37
	3038.00	89.34 ± 6.73	88.06 ± 7.34
Salvianolic acid A	22.94	87.40 ± 1.92	93.46 ± 3.07
	367.00	92.53 ± 9.82	98.55 ± 8.41
	2936.00	96.56 ± 4.77	96.74 ± 1.52
Salvianolic acid C	23.39	86.63 ± 3.38	90.44 ± 7.12
	374.30	97.03 ± 7.35	91.57 ± 2.09
	2994.00	91.99 ± 4.23	88.33 ± 6.49

freeze-thaw cycles, 12 h at room temperature, 20 days at −20 °C, 24 h at 4 °C [22]. The results are shown in Table 4: the QC samples with high, medium and low concentrations of the seven components are relatively stable under the four conditions, with %RSD less than 15 % and %RE within ±15 %.

2.2. Pharmacokinetic results

2.2.1. Model validation results

The expression levels of inducible nitric oxide synthase (iNOS), P-selectin and vascular endothelial growth factor (VEGF) in plasma and microvessel density (MVD) in the lung, thymus and brain were measured to verify whether the model of microcirculation disturbance was successfully established. As showed in Fig. 2 A-B, the expression levels of iNOS, P-selectin and VEGF in the model group are significantly higher than those in the control group ($p < 0.05$), and the MVD of the three tissues in the model group is significantly lower than that in the control group ($p < 0.01$, 0.05), which prove that the model is successfully established.

Table 4
Stability of the seven compounds.

Chemical compound	Mass concentration ($ng \cdot mL^{-1}$)	Three freeze-thaw cycles		Store at room temperature for 12 h		Store at 4 °C for 24 h		Store at −20 °C for 20 days	
		Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)	Precision (RSD, %)	Accuracy (RE, %)
Danshensu	6562.50	3.91	−3.63	3.16	9.76	1.05	6.29	10.00	−8.83
	1640.63	4.86	−10.44	1.33	1.50	4.50	5.99	3.87	0.73
	410.16	8.21	2.54	1.44	−2.61	11.73	−5.50	5.46	6.50
Caffeic acid	1515.63	1.64	12.71	0.77	5.38	1.60	2.95	2.46	3.39
	378.91	4.18	11.31	0.38	2.52	0.53	−2.07	1.61	0.41
	94.73	2.05	3.68	3.41	−2.45	6.10	6.27	3.76	−12.26
Rutin	2000.00	2.30	4.10	3.53	11.51	1.33	0.42	2.76	−1.16
	500.00	9.66	−5.39	4.02	0.45	10.49	7.93	8.87	−5.30
	125.00	8.34	−8.01	5.58	−6.74	2.27	3.07	9.55	7.31
Rosmarinic acid	5250.00	0.49	−4.84	0.94	−10.92	0.63	5.57	1.41	0.99
	1312.50	3.81	−0.56	2.80	−0.80	1.53	0.63	4.01	6.02
	328.13	5.87	1.82	4.94	−3.95	1.81	−6.18	6.50	1.19
Lithospermic acid	3500.00	2.75	−10.67	2.61	11.92	3.65	7.65	0.80	−0.73
	875.00	2.37	9.01	0.38	0.69	14.98	6.58	4.21	0.15
	218.75	9.22	6.56	5.57	−5.80	3.81	6.82	13.88	−8.45
Salvianolic acid A	2343.75	6.74	−10.32	1.33	3.31	1.07	−0.93	2.69	2.45
	585.94	3.65	11.33	3.38	−3.89	1.39	11.49	4.07	−9.12
	146.48	8.30	1.08	7.94	−1.96	0.64	1.81	8.69	7.09
Salvianolic acid C	2375.00	4.72	−3.54	7.62	−13.32	0.19	7.30	6.47	−1.60
	593.75	3.59	7.92	0.78	1.94	1.18	−3.01	3.99	4.74
	148.44	1.83	−5.18	2.02	4.15	2.54	−9.02	3.66	−1.94

2.2.2. Pharmacokinetic study

After UPLC-TQ/MS analysis, the pharmacokinetic parameters of six active components in FS and seven active components in FT in nomal and model rats included C_{max}, CLz/F, T_{max}, $T_{1/2}$ and $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ (Table 5). The plasma concentration-time curve was shown in Fig. 3 A-C.

The results showed that in the nomal group, $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of rosmarinic acid, C_{max} of caffeic acid, rosmarinic acid and salvianolic acid C in FT were significantly lower than those in FS ($p < 0.01$, 0.05), and T_{max} of each component in FT was also lower than that in FS. While CLz/F of rosmarinic acid in FT was higher than that in FS ($p < 0.01$). After modeling, $AUC_{0\text{-}t}$, $AUC_{0\text{-}\infty}$ and T_{max} of all active ingredients increased in varying degrees. Among them, $AUC_{0\text{-}t}$ of danshensu and lithospermic acid and $AUC_{0\text{-}\infty}$ of danshensu in model group were significantly higher than those in control group ($p < 0.01$, 0.05). C_{max} of all components increased to varying degrees except caffeic acid in FS, lithospermic acid in FT and salvianolic acid A in FS. CLz/F of all components decreased except caffeic acid in FS and danshensu in FT.

3. Discussion

The stem-leaf of *Salvia miltiorrhiza* is rich in a variety of components, including phenolic acids, flavonoids, triterpenoids, polysaccharides, remainers, coumarin, amino acids, proteins and volatile oils [23], among which the most important active components are salvianolic acids and flavonoids. Li et al. [24] found that the contents of phenolic acids and flavonoids in the stem-leaf of *Salvia miltiorrhiza* were equal to or higher than those in the roots of *Salvia miltiorrhiza*, with the total phenolic acids content was about one times that of the roots and the flavonoids content was more than four times that of the roots. Danshansu can produce vasodilation by inhibiting calcium influx of vascular smooth muscle cells [25]. Hyperhomocysteinemia is one of the risk factors for cerebrovascular diseases, and systemic inflammation is its core feature. While caffeic acid can inhibit homocysteine-induced leukocyte rolling and adhesion in mice cerebral venular, thus playing an anti-inflammatory role [26]. Rosarinic acid has vasodilator properties and can prevent hypertrophy, arrhythmia and cardiac dysfunction after myocardial infarction [27]. Lithospermic acid can improve myocardial ischemia-reperfusion injury by promoting the phosphorylation of AMP-activated protein kinase α to activate Nrf2 [28]. Salvianolic acid A can protect cerebrovascular endothelial cells from ischemia and oxygen glucose deprivation (OGD) injury by inhibiting Src signaling pathway [29]. Salvianolic acid C can inhibit the polarization of microglia, promote the formation of endothelial cell tubules, thus playing a role in nerve repair in cerebral ischemia [30]. Rutin is a common flavonoid with significant pharmacological activity. It can inhibit angiotensin converting enzyme (ACE), angiotensin 2 type 1 receptor (ATR1) and mineralocorticoid receptor (MCR) to play a protective and therapeutic role in cardiovascular diseases [31]. Therefore, the above seven components were selected as markers to reflect the pharmacokinetic study of oral *salvia miltiorrhiza* stem-leaf in rats, in order to provide a reference for the absorption, distribution, metabolism and excretion process of *Salvia miltiorrhiza* stem-leaf *in vivo*.

In our previous experiments [21], the plasma and urine of normal rats and rats with microcirculation dysfunction treated with FS and FT by gavage were collected for metabolomics analysis. The results showed that the two effective parts of *Salvia miltiorrhiza* stem-leaf could regulate the metabolic level of rats and restore the biomarkers from abnormal levels to nearly normal levels. Moreover, there was a significant difference in the regulatory ability between the FS and FT groups. This indicates that FS and FT can affect some

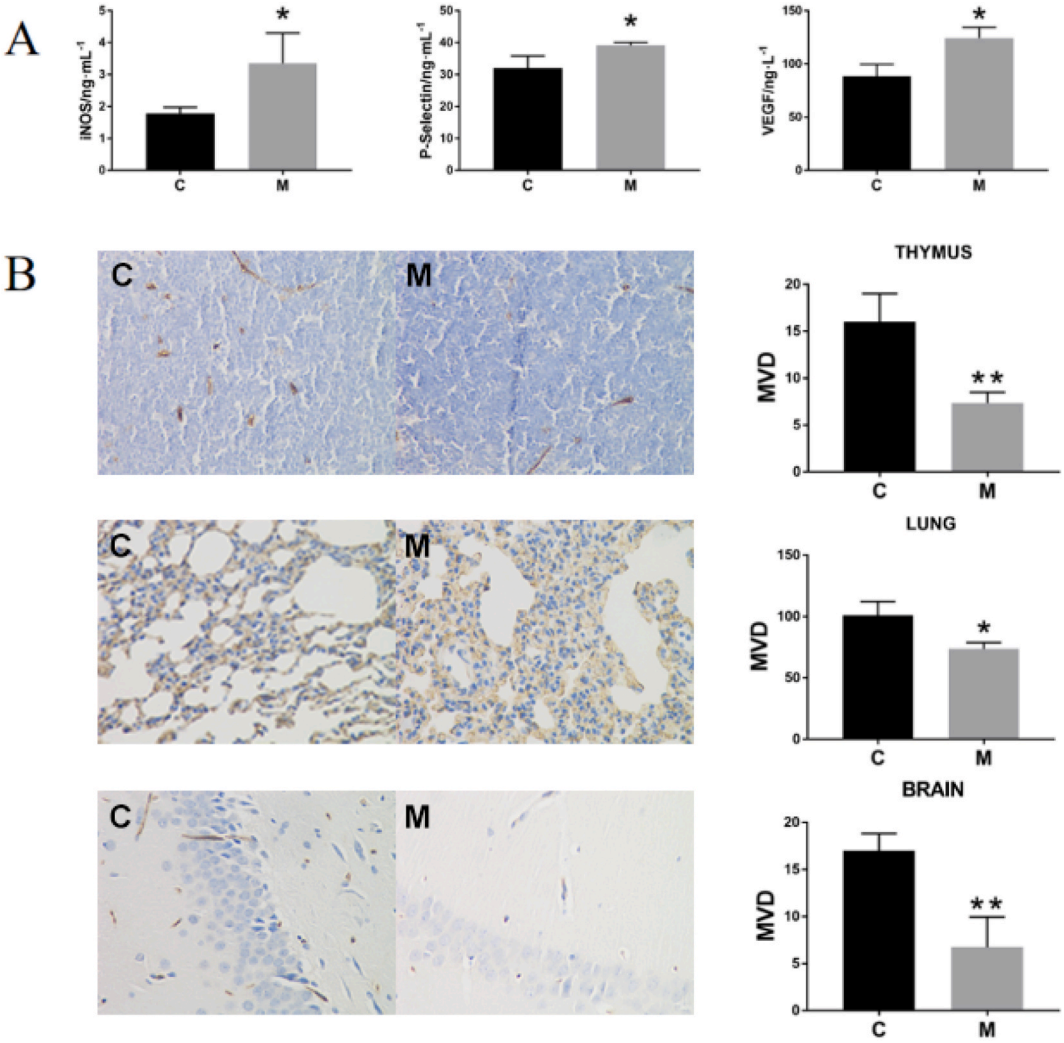

Fig. 2. The expression levels of iNOS, P-Selectin and VEGF in the plasma of blank and model rats (A); MVD of thymus, lung and brain of blank and model rats (B) (C: blank control group; M: model control group) (mean ± SD, n = 6, *p value < 0.05, **p value < 0.01, compared with blank control group).

endogenous metabolites in the body to different degrees. Although both FS and FT parts can improve MCDF, it suggests that the two extracts may play therapeutic effects through different pathways or mechanisms *in vivo*. Based on this, we further studied the pharmacokinetics of FS and FT in normal rats and microcirculation dysfunction rats. In this study, a rapid and sensitive method was established to determine the plasma concentrations of seven active components of FT in rat plasma by UPLC-TQ/MS analysis technology. Then study the pharmacokinetic differences of the main components in the phenolic acid and phenolic acid-flavonoids parts of the stem-leaf of *Salvia miltiorrhiza* in normal and microcirculatory dysfunction model rats. The pharmacokinetic parameters such as T_{max}, C_{max}, $AUC_{0\text{-}t}$, and $AUC_{0\text{-}\infty}$ can reflect the speed and degree of absorption, thus reflecting the exposure characteristics of each component in the drug *in vivo*. The results showed that in the control group, the T_{max} of rosmarinic acid and lithospermic acid was less than 0.39 h, indicating that rosmarinic acid and lithospermic acid could be rapidly absorbed into the blood circulation system after oral administration of FS or FT. Interestingly, the T_{max} of salvianolic acid C after the oral administration of FT was only 0.28 h, while the T_{max} after the oral administration of FS was 0.67 h. This suggests that the presence of rutin, a flavonoid, could somehow improve the absorption rate of salvianolic acid C into the blood. However, it is not clear how these two components interact with each other in the content and results of this experimental study. The T_{max} of danshensu, caffeic acid, rutin, and salvianolic acid A were all greater than 0.47 h, indicating that the absorption rate of these ingredients in the body was relatively slow after oral administration of FS or FT. Compared with the FS group, after oral FT, the C_{max} of caffeic acid, rosmarinic acid, and salvianolic acid C all showed a significant reduction. And the $AUC_{0\text{-}t}$ and $AUC_{0\text{-}\infty}$ of rosmarinic acid were also reduced, and the elimination ratio CLz/F was significantly increased. However, the $MRT_{0\text{-}t}$ of rosmarinic acid was increased both before and after modeling. It suggests that the presence of rutin, a flavonoid component, may reduce the absorption of caffeic acid, rosmarinic acid, and salvianolic acid C in the body, but also prolong

Table 5
Pharmcoknetic parameters of seven compounds of FS and FT in normal and model rats (means ± SD, n = 6).

Chemical compound	Group	C_{max} ($\mu g \cdot L^{-1}$)	CLz/F ($L \cdot h^{-1} \cdot kg^{-1}$)	T_{max} (h)	$T_{1/2}$ (h)	AUC_{0-t} ($\mu g \cdot h \cdot L^{-1}$)	$AUC_{0-\infty}$ ($\mu g \cdot h \cdot L^{-1}$)	MRT_{0-t} (h)	$MRT_{0-\infty}$ (h)
Danshensu	FS-C	88.45 ± 17.93	0.02 ± 0.01	0.67 ± 0.37	5.74 ± 11.19	542.82 ± 141.09	641.77 ± 218.27	6.53 ± 2.11	11.93 ± 15.11
	FS-M	105.53 ± 11.68	0.01 ± 0.00[c]	0.74 ± 1.60	4.47 ± 3.92	880.02 ± 141.73[d]	947.33 ± 140.96[c]	7.53 ± 0.85	9.53 ± 3.95
	FT-C	99.66 ± 15.57	0.02 ± 0.00	0.47 ± 0.07	2.18 ± 2.22	584.67 ± 122.39	588.72 ± 114.83	7.54 ± 0.95	7.81 ± 0.63
	FT-M	165.48 ± 7.83[f]	0.02 ± 0.01	1.00 ± 0.00[f]	6.40 ± 11.48	658.64 ± 172.02	767.88 ± 279.35	5.33 ± 0.98[a,e]	10.76 ± 12.84
Caffeic acid	FS-C	5.78 ± 0.23	0.34 ± 0.11	0.72 ± 0.31	3.62 ± 4.88	37.19 ± 11.53	40.47 ± 18.58	5.78 ± 2.57	7.21 ± 5.95
	FS-M	4.69 ± 0.68[d]	0.39 ± 0.12	0.74 ± 0.42	7.43 ± 6.32	40.79 ± 4.34	55.34 ± 17.59	7.08 ± 0.71	13.24 ± 6.53
	FT-C	4.65 ± 0.38[b]	0.61 ± 0.28	0.61 ± 0.68	6.56 ± 8.00	31.71 ± 11.98	44.40 ± 30.16	5.61 ± 2.50	10.69 ± 10.22
	FT-M	4.90 ± 0.19	0.37 ± 0.18	0.67 ± 0.37	15.91 ± 24.95	45.13 ± 6.76[e]	83.64 ± 78.72	8.58 ± 1.97	25.92 ± 33.62
Rutin	FT-C	5.77 ± 0.62	20.51 ± 6.91	1.06 ± 1.47	7.39 ± 14.34	77.89 ± 11.92	116.30 ± 83.37	7.51 ± 0.81	15.44 ± 18.61
	FT-M	8.02 ± 4.40	16.09 ± 8.21	1.31 ± 1.35	16.88 ± 24.81	85.31 ± 14.60	174.24 ± 135.77	7.32 ± 0.77	28.03 ± 32.94
Rosmarinic acid	FS-C	54.48 ± 5.34	123.40 ± 10.98	0.39 ± 0.09	2.42 ± 2.50	218.23 ± 14.91	222.27 ± 20.65	5.01 ± 0.38	5.48 ± 1.41
	FS-M	75.26 ± 8.79[d]	122.50 ± 10.38	1.00 ± 0.00[d]	2.10 ± 1.73	222.16 ± 16.90	223.68 ± 18.66	4.86 ± 1.22	5.01 ± 1.59
	FT-C	16.43 ± 4.28[b]	205.77 ± 41.89[b]	0.22 ± 0.09[b]	2.91 ± 3.72	104.08 ± 17.24[b]	109.10 ± 28.56[b]	7.95 ± 1.33[b]	8.82 ± 3.43
	FT-M	16.64 ± 5.77	160.41 ± 71.12	0.42 ± 0.33	13.18 ± 20.98	110.31 ± 18.58	178.63 ± 126.72	7.75 ± 2.04[b]	22.36 ± 29.03
Lithospermic acid	FS-C	11.36 ± 1.33	8.98 ± 4.08	0.31 ± 0.16	5.06 ± 8.90	127.72 ± 40.55	164.00 ± 85.05	6.61 ± 1.61	11.11 ± 10.82
	FS-M	22.06 ± 7.33[d]	5.90 ± 1.18	1.92 ± 1.63[c]	3.30 ± 3.49	203.04 ± 33.42[d]	212.18 ± 42.54	7.93 ± 1.52	8.87 ± 3.38
	FT-C	13.65 ± 2.76	17.33 ± 10.21	0.22 ± 0.13	1.03 ± 0.45	118.68 ± 48.84	118.68 ± 48.85	8.67 ± 3.71	8.67 ± 3.71
	FT-M	13.57 ± 2.25	12.55 ± 7.93	1.00 ± 0.82[e]	4.26 ± 7.35	153.31 ± 60.46	188.36 ± 135.49	7.56 ± 2.84	10.72 ± 9.75
Salvianolic acid A	FS-C	13.15 ± 8.35	4.49 ± 3.07	0.63 ± 0.42	9.92 ± 13.65	107.19 ± 46.09	168.39 ± 138.96	7.12 ± 2.26	16.97 ± 17.57
	FS-M	11.51 ± 4.39	2.45 ± 1.18	0.70 ± 0.71	18.73 ± 22.16	131.60 ± 29.33	258.24 ± 212.92	8.55 ± 1.65	29.23 ± 30.59
	FT-C	8.58 ± 1.35	2.22 ± 1.00	0.56 ± 0.36	7.38 ± 9.48	121.73 ± 30.94	162.23 ± 87.32	8.80 ± 1.62	14.98 ± 11.55
	FT-M	10.46 ± 3.59	1.98 ± 0.94	0.83 ± 0.67	9.26 ± 14.00	136.60 ± 37.42	202.37 ± 148.64	7.70 ± 3.21	16.43 ± 18.89
Salvianolic acid C	FS-C	11.01 ± 2.17	63.67 ± 33.45	0.67 ± 0.26	7.30 ± 4.55	30.14 ± 25.77	33.09 ± 32.59	3.65 ± 4.31	4.61 ± 6.46
	FS-M	11.09 ± 3.14	41.35 ± 24.97	0.75 ± 0.27	8.45 ± 8.99	39.25 ± 24.96	55.32 ± 49.60	4.52 ± 4.05	10.25 ± 12.88
	FT-C	7.05 ± 1.64[b]	68.05 ± 46.28	0.28 ± 0.14[b]	3.31 ± 1.82	29.34 ± 21.99	30.07 ± 23.22	4.27 ± 3.46	4.55 ± 3.95
	FT-M	8.95 ± 6.01	63.65 ± 43.57	1.22 ± 0.66[f]	10.67 ± 9.80	29.64 ± 22.98	40.99 ± 46.75	3.90 ± 3.23	8.76 ± 14.72

[a] p value < 0.05.
[b] p value < 0.01, FT vs. FS group.
[c] p value < 0.05.
[d] p value < 0.01, model vs. control group of FS.
[e] p value < 0.05.
[f] p value < 0.01, model vs. control group of FT.

the residence time of rosmarinic acid in the body. The above results indicate that the presence of flavonoids can affect the absorption and metabolism of phenolic acids in the body, and the specific effects need to be further studied on the metabolites and metabolic pathways of the active ingredients of drugs. After modeling, the T_{max} of the seven components increased to varying degrees compared with the control group. After oral administration of FS, the C_{max} of caffeic acid decreased, whereas the C_{max} of rosmarinic acid and lithospermic acid increased. Danshensu showed a decrease in CLz/F, accompanied by an increase in both AUC_{0-t} and $AUC_{0-\infty}$. The AUC_{0-t} of lithospermic acid was also increased. These results indicated that microcirculation disturbance could decrease the absorption

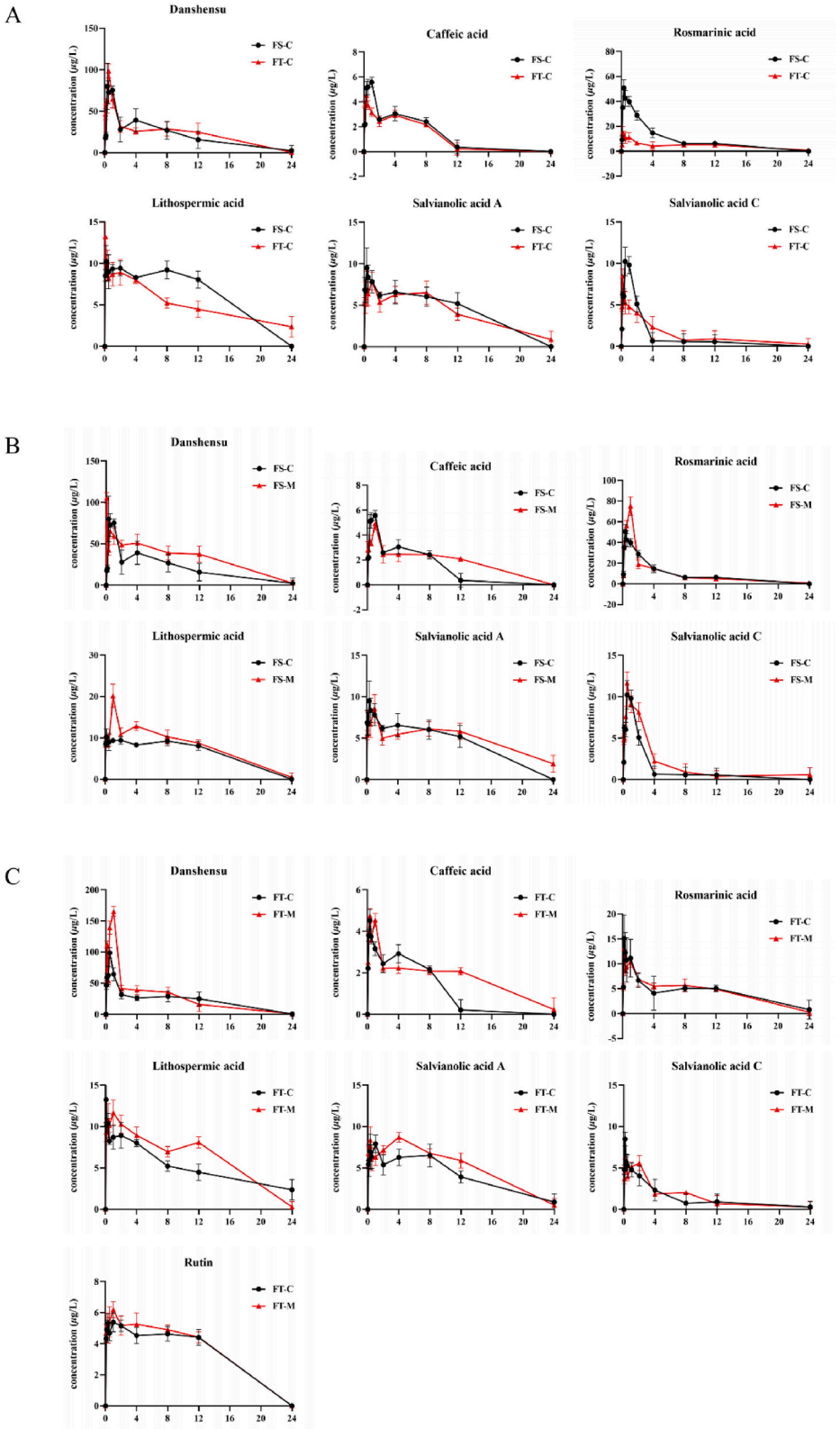

Fig. 3. The mean plasma concentration-time curves of FT and FS in normal rats (A); FS in normal and model rats (B); FT in normal and model rats (C) (n = 6).

of caffeic acid while increasing the absorption of rosmarinic acid and lithospermic acid. At the same time, danshensu exposure increased *in vivo*. What attracted us is that after oral administration of FT, danshensu had a significant increase in C_{max} compared with the control group. The $MRT_{0\text{-}t}$ of danshensu in the model group treated with FT was decreased compared with the control group treated with FT and the model group treated with FS. And the $AUC_{0\text{-}t}$ of caffeic acid increased, while the C_{max} and AUC of the remaining ingredients were not significantly different. These results suggest that the presence of flavonoids may promote the absorption and exposure of danshensu and caffeic acid in the body under the pathological environment of microcirculation disorders. Although C_{max} showed that the absorption of danshonin was enhanced, microcirculation disturbance or flavonoids could shorten its retention time in the body. During the elimination process, the elimination half-life ($t_{1/2}$) of each component was low, indicating that their elimination rate was fast and easy in rat plasma. These results suggest that disturbance of microcirculation may delay the metabolism and elimination of drugs, promote the absorption of drugs into blood, prolong the onset time of drugs. Therefore, the active ingredients can play a better protective role. According to the relevant literature, it may be related to the following mechanisms: Firstly, microcirculation disturbance leads to increased expression of inflammatory factors, such as TNF-α and IL-1β, which inhibits the expression of the ABC drug transporter family. Reduced drug excretion leads to increased *AUC*, indicating that more drug was absorbed into the blood [32,33]. Secondly, the increase of blood viscosity and the decrease of blood flow rate caused by microcirculation disorders can inhibit the activity of liver metabolic enzymes such as CYP450, delay the body's metabolism of drugs, and lead to the prolongation of drug retention time and onset time in the body [34]. Microcirculation disorders can also promote the metabolic elimination of some components, which may be caused by local tissue hypoxia and the accumulation of metabolites. These changes may prompt the body to improve drug utilization by altering the relevant transporters or other mechanisms. At present, there is no evidence that this elimination is detrimental to the efficacy of drugs, so whether it is beneficial to the body needs further research and exploration. Another interesting finding was the apparent bimodal appearance of the drug-time curves for some components. The bimodal phenomenon of danshensu was mainly observed in the control group with oral FS. The bimodal phenomenon of lithospermic acid and salvianolic acid A was observed in both the control and model groups treated with oral FS or FT. However, the results of others [35–39] showed that danshensu, lithospermic acid, and salvianolic acid A did not show a bimodal absorption phenomenon in rats. The bimodal phenomenon of caffeic acid was seen in the control group treated with oral FS or FT. Su's research results [40] showed that the drug time curve of caffeic acid in rats after oral administration of Shaofu Zhuyu decoction showed a bimodal phenomenon. Although the composition of FS and FT is much simpler than that of the Shaofu Zhuyu decoction, there may be interactions between different phenolic acids or between phenolic acids and flavonoids, which lead to the appearance of double peaks. On the other hand, the drug may be absorbed at two different sites of the gastrointestinal tract, either more quickly or more slowly, resulting in a double peak. In addition, it may be caused by the enterohepatic circulation [41].

The interaction between flavonoids and phenolic acids in drug absorption and metabolism needs further study, which can provide a reference for the development and utilization of stem-leaf of *Salvia miltiorrhiza*. This study provides a scientific basis for the development of new products of phenolic acids and phenolic acids-flavonoids part of stem-leaf of *Salvia miltiorrhiza*.

4. Materials and methods

4.1. Animals

Male Wistar rats weighing 200 ± 20 g (license No. scxk (Su) 2017-0007) were purchased from Qinglongshan experimental animal center, Nanjing, China. Rats were reared in the SPF barrier system. The temperature and humidity were controlled at (22 ± 2) °C and (55 ± 10) %, and the light-dark cycle was 12 h. The experiment had been approved by the animal ethics committee of Nanjing University of traditional Chinese medicine with approval number 201810a016.

4.2. Drugs and reagents

Polymer dextran, calcium dobesilate (Nanjing Chunqiu Bioengineering Co., Ltd.), disposable human venous blood sample collection container (EDTA. K2 anticoagulant), disposable human venous blood sample collection container (3.2 % sodium citrate anticoagulant), pentobarbital sodium, and ultrapure water prepared by milli-Q pure water mechanism. ELISA kit for endothelin-1 (ET-1), inducible nitric oxide synthase (iNOS), thromboxane A2 (TXA2), vascular endothelial growth factor (VEGF), platelet selectin, 6-keto-pgf1α, tumor necrosis factor-α (TNF-α), interleukin-1β (IL-1β) (Nanjing Jiancheng Co., Ltd.)

The preparation of phenolic acids-flavonoids effective part from the stem-leaf of *Salvia miltiorrhiza* (FT) is as follows: The stem-leaf of *Salvia miltiorrhiza* was extracted three times by reflux with a 10-fold volume of 60 % ethanol, each extraction for 1.0 h. For every 10 g of dry NKA-2 resin, 2.5 g crude drug was loaded. The solution concentration of drug-loaded was 0.25 g mL^{-1}. Four bed volume (BV) was eluted with 60 % ethanol solution. The average purity of the effective parts of phenolic acids and flavonoids can reach 46.77 % and 4.72 % respectively.

The preparation of phenolic acids effective part from the stem-leaf of *Salvia miltiorrhiza* (FS) is as follows: The phenolic acids-flavonoids extract above was diluted to 0.1 g mL^{-1}, and the pH of the solution was adjusted to 2 with hydrochloric acid. The solution was extracted three times with twice the amount of ethyl acetate. After the ethyl acetate fraction was combined and the ethyl acetate was removed by rotary distillation, the phenolic acids part of the stem-leaf of *Salvia miltiorrhiza* was obtained, in which the purity of phenolic acids was 67.48 %.

4.3. Instruments

Sartorius bt125d electronic analytical balance (selidos, Germany); ultrapure water preparation instrument (milli-Q, millipore, USA); enspire multi-function enzyme labeling instrument (PerkinElmer, USA); kq-250e ultrasonic cleaner (Kunshan Hechuang Ultrasonic Instrument Co., Ltd.); ankegl-16 GII centrifuge (Anting Scientific Instrument Factory, Shanghai); lg-r-80-b computer blood viscosity tester, lg-paber-i platelet aggregation coagulation factor analyzer (Beijing Shidi scientific instrument company); dragonlab-dm0412 centrifuge (Anting Scientific Instrument Factory, Shanghai); ACQUITY UPLC BEH C_{18} column (2.1 × 100 mm, 1.7 μm); Acquity™ Ultra High Performance Liquid Chromatography System equipped with Xevo™ TQ mass spectrometry system and MassLynx4.1 mass spectrometry workstation software (Waters Corporation).

4.4. Animal administration

Wistar rats were adaptively fed for seven days and fasted for 12 h before administration. The blank group and model group were respectively injected with normal saline at a dose of 10 mL (kg d)$^{-1}$ and 10 % polymer dextran 500 (Dextran 500) at a dose of 5 mL (kg d)$^{-1}$ in the tail vein. After three consecutive days, plasma samples were collected from the retroorbital venous plexus. The expression of iNOS, VEGF, and P-Selectin was measured by ELISA kits to confirm the success of the modeling. Each group had six rats. The rats in the blank group were divided into total phenolic acids from the stem-leaf of *Salvia miltiorrhiza* blank group (FS–C) and phenolic acids-flavonoids from the stem-leaf of *Salvia miltiorrhiza* blank group (FT-C). Successfully modeled rats were divided into total phenolic acids from the stem-leaf of *Salvia miltiorrhiza* model group (FS-M) and phenolic acids-flavonoids from the stem-leaf of *Salvia miltiorrhiza* model group (FT-M). On the fourth day, rats in each administration group were given the corresponding drug solution by intragastric administration. The administration groups were all dosed at 100 mg kg^{-1}, and the intragastric dose is 1 mL·100 g^{-1}.

4.5. Sample collection

After 0, 5, 10, 20, 30, 60, 120, 240, 480, 720 and 1440 min of intragastric administration, the rats were anesthetized with ether. The blood from the orbital venous plexus was collected and put into the centrifuge tube added with EDTA-K_2. After mixing and standing for 2 h, plasma samples were centrifuged at 3500 rpm for 10 min and then stored at −80 °C. After the final blood collection, the rats were anesthetized with pentobarbital sodium, Then the thymus, lung, and brain tissues were removed and fixed in formaldehyde. The microvessel density was counted by CD34 staining.

After vortex mixing, 10 μL clarithromycin internal standard solution and 50 μL hydrochloric acid solution were added to every 100 μL plasma thawed to room temperature. After vortexing for 60 s, 500 μL ethyl acetate was added, whirled for 3 min, left standing, and centrifuged at 6000 rpm for 10 min, suck out the supernatant and transfer it to a new centrifuge tube. Then 500 μL ethyl acetate was added to the lower liquid, vortex for 3 min, then stand still, centrifuged at 6000 rpm for 10 min. The twice supernatant was combined, then centrifuged and concentrate to dry. Before UPLC-TQ/MS analysis, 100 μL of 50 % acetonitrile was added into each tube, and the supernatant was taken for analysis after vortexing for 5 min and centrifuging for 15 min at 13000 rpm.

4.6. Preparation of reference substance and internal standard solution

Reference stock solution: accurately weigh the appropriate amount of danshensu, caffeic acid, rutin, rosmarinic acid, lithospermic acid, salvianolic acid A and salvianolic acid C, and dissolve them with methanol to prepare mixed reference substance stock solution with the concentration of 420, 194, 168, 152, 128, 224 and 188 μg mL^{-1} for standby.

Standard curve series concentration reference substance solution: Prepare the reference substance working solution by successively diluting the mixed reference substance stock solution with 90 % methanol, and then add 10 μL of the reference substance working solution to 90 μL of blank plasma according to the method under "4.5".

Preparation of quality control (QC) samples: QC samples with low, medium, and high concentrations were prepared by the same method.

Preparation of internal standard solution: clarithromycin was precisely weighed and dissolved in methanol to prepare the internal standard solution of 10 μg mL^{-1}.

4.7. Mass spectrometry conditions

Electrospray ion source (ESI) adopted positive ion detection mode. The *m/z* acquisition range was 110–1000, and the acquisition time was 0.8–20.8 min. The main mass spectrum parameters are as follows: capillary temperature 350°, evaporator temperature 350°, sheath air 40 kPa, auxiliary air flow 15 kPa, spray voltage 3.5 V, source current 100 A. MS^2 mass spectrometry trigger mode was dynamic data dependence analysis mode,and the previous level peak was selected to carry out collision induced fragmentation CID (scanning), with the collision energy set to 30 %. Sampling cone voltage and collision energy can be seen in Table 6.

4.8. Calculation and statistical analysis of pharmacokinetic parameters in rats

The pharmacokinetic parameters were analyzed and calculated through the non-compartmental intelligent model in the DAS 3.2.8 pharmacokinetic software. The experimental data were expressed as mean ± SD, and the pharmacokinetic parameters of the control

Table 6
Mass spectrometry parameters of salvianolic acids and flavonoids.

Compouds	t_R/min	MW	MRM conversion	Cone voltage/V	collision energy/eV	ESI
Danshensu	3.01	198.05	199.08 > 153.03	10	6	+
caffeic acid	4.48	180.04	181.03 > 88.89	12	26	+
rutin	5.29	610.51	611.29 > 303.10	14	22	+
Rosmarinic acid	6.33	360.31	361.15 > 88.97	10	40	+
Lithospermum acid	6.41	538.46	539.24 > 341.16	10	8	+
Salvianolic acid A	7.64	494.12	493.16 > 295.09	28	18	+
Salvianolic acid C	7.64	492.44	493.22 > 165.01	14	14	+

group and model group were analyzed by SPSS 22.0 software. Student's *T*-test was used to compare the two groups.

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Ethics declarations

This study was reviewed and approved by the animal ethics committee of Nanjing University of Chinese Medicine, with the approval number: 201810a016.

Sample availability

Samples of the compounds and rats plasma are available from the authors.

Data availability statement

The data associated with our study has not been deposited into a publicly available repository, as the data that has been used is confidential.

CRediT authorship contribution statement

Wei Zhou: Writing – original draft, Investigation, Formal analysis, Data curation. **Cheng-jing Sun:** Writing – original draft, Investigation, Formal analysis, Data curation. **Ruo-ying Fan:** Writing – original draft, Investigation, Formal analysis, Data curation. **Zhuo Xu:** Formal analysis, Data curation. **Shu-lan Su:** Writing – review & editing, Validation, Supervision, Project administration, Methodology, Funding acquisition, Conceptualization. **Er-Xin Shang:** Visualization, Software. **Wen Zhang:** Formal analysis, Data curation. **Jin-ao Duan:** Validation, Supervision, Resources, Project administration, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests:Shu-lan Su reports financial support was provided by National Natural Science Foundation of China. Shu-lan Su reports financial support was provided by 333 High-level Talents Training Project Funded by Jiangsu Province (2016). If there are other authors, they declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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