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Original Research Article

Spectrum-effect relationship between serum HPLC fingerprints and activation of blood circulation and removal of blood stasis by Chuanxiong

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Abstract

Purpose: *To study the active ingredients of Chuanxiong (CX) in promoting blood circulation and removing blood stasis.*
Methods: *Blood-activating effects and serum HPLC fingerprints of CX extracts from different parts of China were studied and the spectrum-effect relationship between them was established by grey correlation analysis.*
Results: *After treatment with CX extracted using different solvents, hemorheology indices were lower than those in the model group, and the effect of dichloromethane was better than that of other solvents ($p < 0.05$ or $p < 0.01$). There were 6 common peaks by fingerprint analysis. Peaks 1 - 5 were identified as senkyunolide I, senkyunolide H, senkyunolide A, coniferyl ferulate and ligustilide, respectively.*
Conclusion: *Analysis of the spectrum–effect relationship indicates the contribution of the five components to the blood-activating effect of CX. The findings lend some scientific justification for using CX to remove blood stasis, and selection of quality evaluation indices for CX.*

Keywords: *Chuanxiong, Blood-activating, Dichloromethane, Serum fingerprint, Senkyunolide I, Senkyunolide H, Senkyunolide A, Coniferyl ferulate, Ligustilide*

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INTRODUCTION

Chuanxiong (CX) is the dried rhizome of *Ligusticum chuanxiong* Hort. CX is a well-known plant used in traditional Chinese medicine (TCM) formulations which activates the blood circulation and relieves pain [1]. Usually, it is employed with *Angelica sinensis* radix and *Salvia miltiorrhiza* for the treatment of irregular menstruation, migraine, coronary heart disease, and angina [2-4]. The main chemical components of CX are volatile oils, alkaloids, organic acids and phenols.

According to TCM theory, if the blood is clear, then pain will be absent. Studies on the pharmacodynamic basis of the effects of CX have focused primarily on chuanxiongzine, ferulic acid, ligustilide and senkyunolide with little or no chuanxiongzine in the original CX or CX extracts. Studies [5-7] have shown that the main components in the plasma of rats after CX

administration are ferulic acid, ligustilide and senkyunolide, but chuanxiongzine is absent. Li and colleagues [8] showed that endoesters were the main active ingredients of CX, and that CX had a good effect on ischemic cardiac disease and cerebrovascular disease. Hence, the identity of the main components of CX that have pharmacodynamic effects is not known.

We investigated the active ingredients of CX that promote blood circulation and remove blood stasis. The aim of the study was to determine the "spectrum–effect relationship" between serum high-performance liquid chromatography (HPLC), as well as the effect of CX on blood circulation and removal of blood stasis by grey correlation analyses.

EXPERIMENTAL

Ethical approval of the study protocol

Animal experiments were undertaken with the approval of the Ethics Committee of Chengdu University of Traditional Chinese Medicine (Chengdu, China). Animal treatments were conducted in strict compliance with the *Guide for the Care and Use of Laboratory Animals* (US National Institutes of Health, Bethesda, MD, USA) [11].

Materials

Twenty-two batches of CX were collected from major producing areas in Sichuan Province, one batch of CX was collected from Fujian Province, and one batch of CX was collected from Shanxi Province, in China, and identified by Professor Yu-Ying Ma and Professor Gui-Hua Jiang (College of Pharmacy, Chengdu University of TCM, Chengdu, China). Information on these 24 batches of CX is shown in Table 1.

HPLC was carried out on a LC-2012 system (Shimadzu, Kyoto, Japan). An automatic hemorrheometer (SA-6000; Succeeder, Beijing, China) was used to assay blood samples. Methanol (HPLC - grade) was obtained from Thermo Fisher Scientific (Waltham, MA, USA). Ethanol, petroleum ether, dichloromethane, ethyl acetate, N-butanol, and picric acid were purchased from Chengdu Kelong Chemical Reagents (Chengdu, China). *Z*-ligustilide, levistilide A, senkyunolide I, senkyunolide A, coniferyl ferulate, and senkyunolide H were obtained from Chroma Biotechnology (Chengdu, China), and their purity was >95%.

Table 1 : Information on chuanxiong batches

Batch number	Place of production	Altitude	Latitude (E)	Longitude (N)	Time of collection
CX1	Meishan, Sichuan	427.0	103º38′10.7″	30º03′25.1″	2017.04
CX2	Meishan, Sichuan	412.0	103º45′05.2″	30º05′11.4″	2017.04
CX3	Meishan, Sichuan	383.0	103º52′15.9″	29º54′44.9″	2017.04
CX4	Meishan, Sichuan	395.0	103º50′30.9″	29º57′05.1″	2017.04
CX5	Meishan, Sichuan	438.0	103º45′19.5″	30º11′46.8″	2017.04
CX6	Meishan, Sichuan	426.0	103º43′49.4″	30º08′20.8″	2017.04
CX7	Meishan, Sichuan	444.0	103º47′18.2″	30º28′43.8″	2017.04
CX8	Meishan, Sichuan	442.0	103º47′57.5″	30º14′55.2″	2017.04
CX9	Meishan, Sichuan	427.0	103º49′22.9″	30º12′36.3″	2017.04
CX10	Shifang, Sichuan	533.0	104°05′55.5″	31°05′36.1″	2017.04
CX11	Shifang, Sichuan	530.0	104°05′54.0″	31°08′27.0″	2017.04
CX12	Pengzhou, Sichuan	674.0	103°54′48.3″	31°05′31.2″	2017.05
CX13	Pengzhou, Sichuan	604.0	103°58′10.3″	31°08′14.4″	2017.05
CX14	Pengzhou, Sichuan	604.0	103°59′53.1″	31°07′56.7″	2017.05
CX15	Pengzhou, Sichuan	599.0	103°59′38.7″	31°06′40.3″	2017.05
CX16	Pengzhou, Sichuan	590.0	103°59′23.3″	31°03′08.5″	2017.05
CX17	Pengzhou, Sichuan	568.0	104°00′57.8″	31°01′03.1″	2017.05
CX18	Xindu, Sichuan	542.0	103°59′46.8″	30°53′59.6″	2017.05
CX19	Dujiangyan, Sichuan	626.0	103°39′14.5″	30°49′48.5″	2017.05
CX20	Dujiangyan, Sichuan	592.0	103°40′21.5″	30°46′38.8″	2017.05
CX21	Dujiangyan, Sichuan	626.0	103°40′45.8″	30°50′45.8″	2017.05
CX22	Chongzhou, Sichuan	573.0	103°39′58.3″	30°43′46.5″	2017.05
CX23	Fuqing, Fujian	122.0	119°23′15.2″	25°44′28.1″	2018.05
CX24	Zhenba, Shanxi	657.0	108°01′39.5″	32°32′45.6″	2018.06

Epinephrine hydrochloride injection was purchased from Grand Pharmaceuticals (Beijing, China), and 0.9% sodium chloride injection was obtained from Sichuan Kelun Pharmaceuticals (Sichuan, China). Compound danshen tablets were obtained from Yunnan Baiyao Group Co., Ltd. (Yunnan, China).

Specific pathogen-free male and female Sprague–Dawley rats (180 – 220 g) were purchased from Chengdu Dashuo Experiment Animals (Chengdu, China). All rats were fed in a temperature-controlled facility with relative humidity of 65% and a 12-h light–dark cycle.

Blood-activating effect of CX extracted using different solvents

Sample preparation

CX samples (250 g/batch) were ground into powder, soaked with 70% ethanol for 10 h, and extracted eight times with 70% ethanol by seepage. The extracts were combined, and concentrated to a flow extract (30 mL) under reduced pressure, then dispersed with the appropriate amount of pure water. Then, they were extracted thrice with petroleum ether, dichloromethane, ethyl acetate or N-butanol, respectively. Extracts of each part were combined and concentrated under reduced pressure, and diluted to 250 mL with 0.5% carboxymethylcellulose sodium (CMC-Na) to obtain samples.

Grouping and treatment of rats

Rats were divided randomly into seven groups: blank; model; compound danshen tablets (CDT); test (four groups). Rats in the blank group did not receive any treatment; rats in the model group only replicated the blood stasis model without gavage drugs; rats in the CDT group and test groups replicated the blood stasis model and were gavaged the corresponding drugs. The specific treatment method was as follows: rats in the blank group and model group were administered pure water at 1 mL/kg/day for 7 days. Rats in the CDT group were given CDTs at 1.8 g/kg/day for 7 days. Rats in different test groups were given the corresponding extract at 1 g/kg/day for 7 days, respectively. All treatments were given by gavage. Eight hours after gavage on day-7, rats in the model group, CDT group, and test groups were administered (s.c.) 0.1% epinephrine hydrochloride injection (0.8 mL/kg) twice at an interval of 4 h, whereas rats in the control group were administered subcutaneously (sc) 0.9 % sodium chloride injection at the same dose as the test groups. Two hours after the first injection of 0.1 % epinephrine hydrochloride, rats in all groups except for the control group were placed in an iced water bath for 5 min. Thirty minutes after gavage on day-8, rats in all groups were anesthetized with 10% chloral hydrate, and blood was collected from the abdominal aorta.

Treatment and assessment of blood samples

First, parameters from whole-blood samples were measured by an automatic hemorrheometer (SA-6000; Succeeder). Then, samples were centrifuged (3000 rpm for 30 min at room temperature), the supernatant separated, and analyzed.

Determination of spectrum–effect relation-ship

S*ample preparation*

The method of sample preparation was identical to that described as sample preparation in blood-activating effect of CX extracted using different solvents.

Grouping and treatment of rats

Rats were divided randomly into 27 groups: blank; model; CDT; CX1–CX24. Rats in the CX1–CX24 groups were given the corresponding extracts at 1 g/kg/day (bodyweight) for 7 days, respectively. Other treatments were identical to those described as grouping and treatment of rats in blood-activating effect of CX extracted using different solvents.

Preparation of serum samples for HPLC

CX powder (100 g) was soaked with 70% ethanol for 10 h, and extracted eight times with 70% ethanol by seepage. The extracts were combined, and concentrated to a flow extract under reduced pressure, and diluted to 20 mL with the appropriate amount of 0.5% CMC-Na to obtain samples for pharmacodynamic studies.

Rats were divided randomly into 25 groups: blank; CX1–CX24. Rats in the blank group were administered 0.5 % CMC-Na (1 mL/kg/day bodyweight) for 3 days. CX1–CX24 groups were administered the corresponding pharmacodynamic sample at 1 mL/kg bodyweight once a day for 3 days, respectively. Thirty minutes after gavage on day-3, rats in all groups were anesthetized with 10% chloral hydrate, and blood from the abdominal aorta was collected. After blood had coagulated, the supernatant was separated after centrifugation (3000 rpm for 10 min at room temperature).

Methanol was added thrice to precipitate proteins. Then, the supernatant was aspirated and filtered through a 0.22-μm membrane to yield serum samples for HPLC.

Preparation of mixed standard solutions

Appropriate amounts of ligustilide, senkyunolide A, senkyunolide I, coniferyl ferulate, and senkyunolide H were accurately weighed, and dissolved in methanol to prepare a series of stock solutions of different concentrations. Appropriate volumes of 5 stock solutions were transferred into the same volumetric flask; methanol was added to obtain mixed reference substance solutions of different concentrations.

HPLC conditions

HPLC was done on a LC-2012 system (Shimadzu) and chromatographic separation was carried on a SP-120-5 C18 column (4.6 mm × 250 mm, 5 μm) operated at 25 ℃. The mobile phase comprised methanol (A) and water (B), with a linear gradient of A: 0–40 min (30–85%) [12]. The injection volume was 20 μL, and the flow rate was 1.0 mL/min. The detection wavelength of the HPLC fingerprint was 280 nm.

Validation of HPLC fingerprint method

Precision, stability within 16 h, and repeatability were used to assess the performance of instrument, stability of the target, and operational consistency, respectively [12].

Similarity evaluation of fingerprints

The HPLC chromatograms of 24 samples of serum from rats administered CX were imported into Similarity Evaluation System for Chromatographic Fingerprint of TCM (SESCF, Version 2004A) (Beijing, China), and generated reference standard fingerprint (R), including 6 characteristic peaks (common peaks) by multipoint correction and peak matching, and the similarity values between the chromatogram of each serum from rats administered CX and the reference fingerprint were evaluated by this software.

Statistical analysis

Data from pharmacodynamic test are presented as mean ± standard deviation (SD). Differences among different groups were analyzed by one-way ANOVA on SPSS 21.0 (IBM, Armonk, NY, USA). Bivariate correlation analyses between different hemorheology indices of CX and common peak areas were done using the grey correlation coefficient.

RESULTS

Selected extraction solvent for blood-activating effect of CX

Compared with the model group, almost all hemorheology indices in the test groups were reduced. After treatment with CX extracted using different solvents (petroleum ether, dichloromethane, ethyl acetate, N-butanol), the hemorheology indices decreased compared with those in the model group. The effect of dichloromethane solvent was better than that of other solvents (Table 2).

Blood-circulation effects of CX extracts

Compared with the blank group, the hemorheology indices we assessed in the test groups were increased, which suggested that the iced water-induced coagulation model had been created successfully. After treatment with different CX extracts, the hemorheology indices changed compared with those in the model group (Table 3).

Table 2: Effect of CX extracted (using different solvents) on blood parameters

Group	Viscosity of whole blood (mean ± SD)			Plasma viscosity (mean ± SD)	Fibrinogen (mean ± SD)	Index of erythrocyte aggregation (mean ± SD)
	1 (s^{-1})	*50 (s^{-1})*	*200 (s^{-1})*			
Control	78.37 ± 5.45	10.10 ± 0.57	7.38 ± 0.35**	3.52 ± 0.11**	7.75 ± 0.23**	10.34 ± 0.57
Model	87.65 ± 3.08	11.38 ± 0.45	9.46 ± 0.39	4.96 ± 0.21	10.92 ± 0.47	11.17 ± 1.33
Compound danshen tablet	84.13 ± 2.33	10.34 ± 0.64	7.73 ± 0.40**	3.73 ± 0.28**	8.20 ± 0.62**	10.60 ± 0.25
Petroleum ether	87.78 ± 14.49	11.29 ± 0.34	8.23 ± 0.28*	4.66 ± 0.10	10.26 ± 0.30	10.82 ± 0.18
Dichloromethane	87.29 ± 2.26	10.44 ± 0.41	7.79 ± 0.24**	4.16 ± 0.19**	9.15 ± 0.42**	10.67 ± 0.36
Ethyl acetate	92.71 ± 3.35	10.73 ± 0.31	8.61 ± 0.34	4.67 ± 0.18	10.28 ± 0.30	10.98 ± 0.50
N-butanol	102.06± 3.74*	10.84 ± 0.53	8.30 ± 0.35*	4.71 ± 0.13	10.37 ± 0.35	11.67 ± 0.56

*$P < 0.05$, **$p < 0.01$, compared with the model group

Table 3: Effect of CX extracts on blood parameters

Group	Viscosity of whole blood (mean ± SD)			Plasma viscosity (mean ± SD)	Fibrinogen (mean ± SD)	Index of erythrocyte aggregation (mean ± SD)
	1 (s^{-1})	*50 (s^{-1})*	*200 (s^{-1})*			
Blank	24.16 ± 1.31**	4.88 ± 0.49**	3.79 ± 0.37**	1.31 ± 0.25**	2.97 ± 0.26**	5.58 ± 0.50**
Model	40.80 ± 8.66	6.27 ± 1.99	4.73 ± 1.34	1.79 ± 0.15	3.92 ± 0.33	7.81 ± 0.40
Compound danshen tablets	26.64 ± 3.91**	5.00 ± 0.37**	4.04 ± 0.35**	1.40 ± 0.11**	2.97 ± 0.29**	6.10 ± 0.46**
CX1	32.59 ± 4.97**	5.48 ± 0.60**	4.26 ± 0.43*	1.52 ± 0.28**	3.34 ± 0.60**	7.63 ± 0.63
CX2	30.19 ± 3.36**	5.49 ± 0.33**	4.34 ± 0.25	1.40 ± 0.14**	3.32 ± 0.41**	6.96 ± 0.68**
CX3	31.62 ± 3.86**	5.60 ± 0.55*	4.49 ± 0.41	1.44 ± 0.19**	3.21 ± 0.40**	6.98 ± 0.67**
CX4	29.42 ± 3.16**	5.44 ± 0.37**	4.32 ± 0.28	1.46 ± 0.13**	3.21 ± 0.29**	6.81 ± 0.55**
CX5	29.45 ± 2.44**	5.89 ± 0.59	4.76 ± 0.55	1.54 ± 0.09*	3.22 ± 0.32**	6.99 ± 0.93**
CX6	27.84 ± 2.38**	5.25 ± 0.40**	4.19 ± 0.36**	1.61 ± 0.52	3.24 ± 0.36**	6.14 ± 0.68**
CX7	29.36 ± 2.29**	5.62 ± 0.47*	4.50 ± 0.42	1.44 ± 0.15**	3.17 ± 0.33**	6.56 ± 0.62**
CX8	28.22 ± 2.82**	5.56 ± 0.20*	4.57 ± 0.25	1.50 ± 0.22**	3.20 ± 0.18**	5.85 ± 0.51**
CX9	31.62 ± 3.00**	5.62 ± 0.42*	4.42 ± 0.31	1.51 ± 0.18**	3.17 ± 0.71**	7.15 ± 0.30*
CX10	31.41 ± 6.03**	5.48 ± 0.77**	4.30 ± 0.57	1.68 ± 0.11	3.74 ± 0.25	7.18 ± 0.52*
CX11	27.71 ± 4.35**	5.16 ± 0.4**	4.10 ± 0.37**	1.43 ± 0.22**	3.17 ± 0.47**	6.75 ± 0.72 **
CX12	35.34 ± 3.93**	5.89 ± 0.44	4.57 ± 0.32	1.46 ± 0.12**	3.21 ± 0.27**	7.72 ± 0.44
CX13	31.51 ± 6.26**	5.39 ± 0.76**	4.21 ± 0.55*	1.35 ± 0.13**	3.09 ± 0.30**	7.45 ± 0.77
CX14	30.66 ± 3.35**	5.39 ± 0.42**	4.22 ± 0.31*	1.51 ± 0.23**	3.14 ± 0.17**	7.24 ± 0.39*
CX15	30.35 ± 5.77**	5.15 ± 0.88**	4.01 ± 0.67**	1.40 ± 0.16**	3.08 ± 0.35**	7.54 ± 0.29
CX16	35.77 ± 4.19*	6.03 ± 0.57	4.69 ± 0.44	1.58 ± 0.21*	3.46 ± 0.46*	7.53 ± 0.47
CX17	28.07 ± 4.81**	5.42 ± 0.32**	4.35 ± 0.22	1.52 ± 0.13**	3.36 ± 0.32**	6.15 ± 0.51**
CX18	36.28 ± 4.80*	5.99 ± 0.60	4.63 ± 0.43	1.43 ± 0.09**	3.15 ± 0.20**	6.99 ± 0.61**
CX19	32.35 ± 3.96**	5.29 ± 0.28**	4.51 ± 0.67	1.44 ± 0.12**	3.16 ± 0.26**	6.79 ± 0.69**
CX20	31.53 ± 4.77**	5.77 ± 0.53	4.59 ± 0.41	1.34 ± 0.12**	3.09 ± 0.24**	6.90 ± 0.80 **
CX21	30.42 ± 6.37**	5.43 ± 0.81**	4.28 ± 0.60	1.64 ± 0.36	3.40 ± 1.00**	7.07 ± 0.76**
CX22	32.15 ± 2.89**	5.67 ± 0.61*	4.46 ± 0.51	1.60 ± 0.32	3.25 ± 0.77**	7.44 ± 0.46
CX23	27.64 ± 4.26**	5.14 ± 0.90**	4.08 ± 0.76**	1.48 ± 0.26**	3.07 ± 0.23**	6.83 ± 0.70**
CX24	29.12 ± 3.93**	5.60 ± 0.47*	4.49 ± 0.37	1.56 ± 0.17*	3.34 ± 0.18**	6.50 ± 0.72**

* $P < 0.05$, ** $p < 0.01$, compared with the model group

Validation of the HPLC-fingerprint method

Testing of precision, stability within 16 h, and repeatability indicated that the relative standard deviation (RSD, n = 6) of retention time and peak area value of 6 common peaks were <5% (Table 4, Table 5 and Table 6, Table 7, Table 8, Table 9) [12]. Hence, our method was feasible for analyzing the HPLC fingerprints of CX extracts.

HPLC fingerprints

The HPLC fingerprints and the reference fingerprint of 24 batches rat serum were shown in Figure 1. And similarities among the HPLC chromatograms of these 24 samples of rat serum and reference standard fingerprints were analyzed. The RSD of the six common peak areas in the 24 samples of rat serum were >35%, indicating that the areas of each common peak in different serum samples varied widely.

The similarities in the HPLC chromatograms of 24 samples of rat serum were in the range 0.012–1.000. Similarities between the HPLC chromatograms of 24 samples and reference standard fingerprints were in range 0.174–0.98.

Identification of common peaks

The common peaks were identified as senkyunolide I, senkyunolide H, senkyunolide A,

Table 4: Relative retention time in precision test

Common peak	1st	2nd	3rd	4th	5th	6th	RSD/%
1	0.568	0.570	0.568	0.567	0.570	0.563	0.46
2	0.620	0.621	0.617	0.619	0.613	0.612	0.58
3	1.000	1.000	1.000	1.000	1.000	1.000	0.00
4	1.029	1.029	1.029	1.030	1.029	1.028	0.07
5	1.161	1.160	1.158	1.165	1.162	1.155	0.30
6	1.521	1.520	1.515	1.529	1.524	1.503	0.59

Table 5: Relative peak area in precision test

Common peak	1st	2nd	3rd	4th	5th	6th	RSD/%
1	8.211	8.375	8.463	8.552	8.705	8.724	2.33
2	2.143	2.157	2.068	2.043	2.041	2.060	2.47
3	1.000	1.000	1.000	1.000	1.000	1.000	0.00
4	0.256	0.256	0.251	0.247	0.252	0.270	3.07
5	3.828	3.823	3.908	3.966	3.979	3.943	1.75
6	1.862	1.708	1.774	1.764	1.901	1.688	4.72

Table 6: Result of relative retention time in stability test

Common peak	0h	2h	4h	6h	8h	16h	RSD/%
1	0.770	0.770	0.770	0.771	0.770	0.769	0.06
2	0.816	0.816	0.817	0.817	0.817	0.816	0.07
3	1.000	1.000	1.000	1.000	1.000	1.000	0.00
4	1.313	1.314	1.314	1.314	1.314	1.316	0.06
5	1.517	1.518	1.518	1.519	1.518	1.522	0.10
6	1.977	1.977	1.977	1.978	1.978	1.984	0.14

Table 7: Relative peak area in stability test

Common peak	0h	2h	4h	6h	8h	16h	RSD/%
1	20.480	20.290	20.997	20.233	19.716	19.950	2.19
2	5.809	6.047	5.799	6.076	5.582	5.657	3.43
3	1.000	1.000	1.000	1.000	1.000	1.000	0.00
4	2.057	1.976	2.140	2.052	1.919	1.969	3.93
5	5.627	5.590	5.918	5.790	5.740	5.501	2.65
6	5.601	5.900	5.935	5.733	5.430	5.531	3.57

Table 8: Result of relative retention time in repeatability test

Common peak	1st	2nd	3rd	4th	5th	6th	RSD/%
1	0.556	0.557	0.549	0.561	0.556	0.566	0.99
2	0.592	0.594	0.585	0.597	0.593	0.602	0.94
3	1.000	1.000	1.000	1.000	1.000	1.000	0.00
4	1.030	1.029	1.028	1.029	1.028	1.030	0.08
5	1.163	1.158	1.158	1.161	1.155	1.165	0.30
6	1.527	1.513	1.512	1.521	1.509	1.529	0.56

Table 9: Relative peak area in repeatability test

Common peak	1st	2nd	3rd	4th	5th	6th	RSD/%
1	6.427	6.852	7.024	6.356	6.677	6.288	4.47
2	1.870	1.836	1.834	1.934	1.921	1.828	2.51
3	1.000	1.000	1.000	1.000	1.000	1.000	0.00
4	0.283	0.280	0.293	0.286	0.286	0.271	2.59
5	4.711	4.854	4.710	4.409	4.650	4.665	3.11
6	1.653	1.587	1.576	1.606	1.570	1.672	2.64

coniferyl ferulate, and ligustilide, respectively, by comparing the retention time and ultraviolet-absorption curves of target peaks with those of standards (Figure 2).

Bivariate correlation analyses

Bivariate correlation analyses (Table 7) showed that the contribution of the six common peaks to the hemorheology indices of CX were in the order: peak 1 (senkyunolide I) > peak 5 (ligustilide) > peak 3 (senkyunolide A) > peak 4 (coniferyl ferulate) > peak 2 (senkyunolide H) > peak 6.

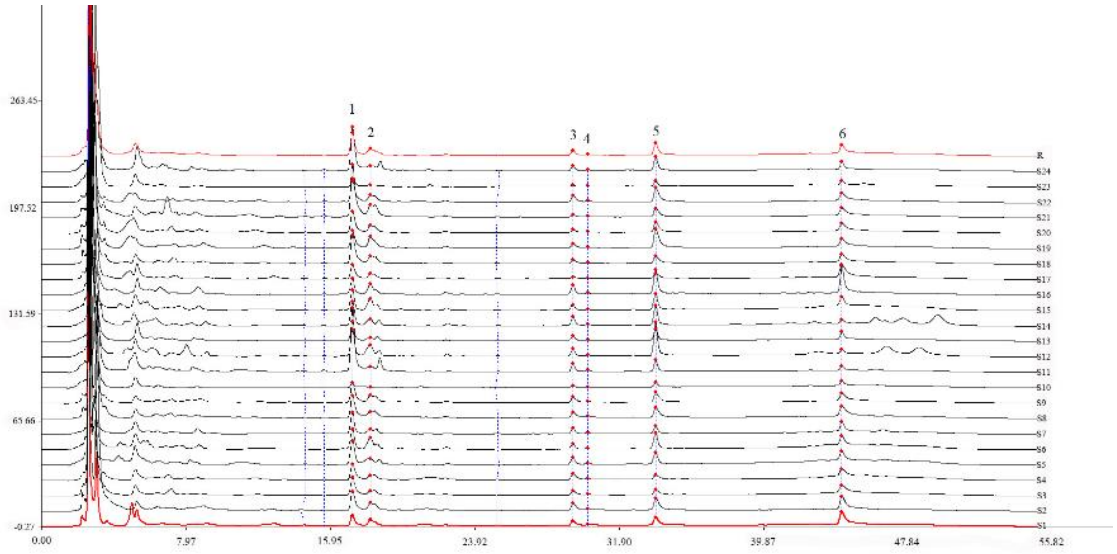

Figure 1: HPLC chromatograms of 24 samples of serum from rats administered CX1–CX24 extracts and the reference standard fingerprint including 6 common peaks (peak 1 - 6)

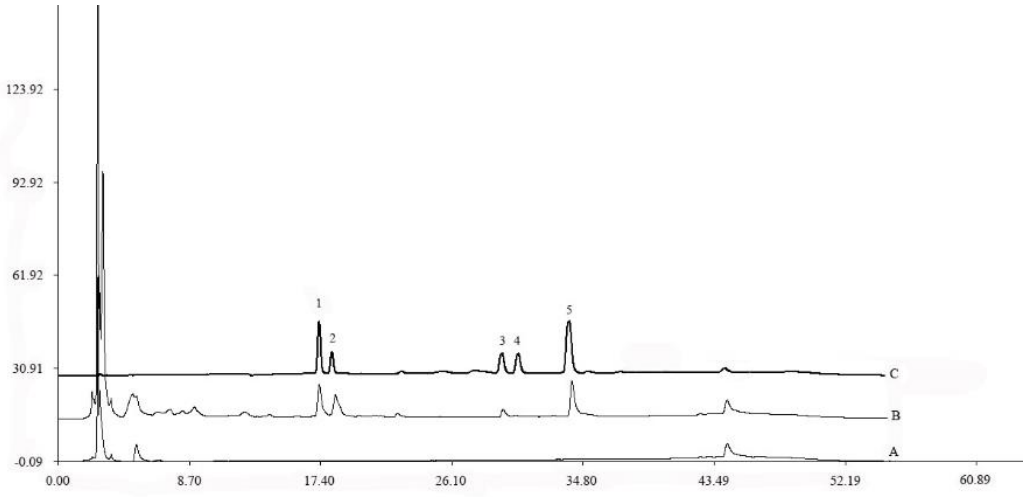

Figure 2: HPLC chromatograms of blank (A), serum sample (B) and mixed standards (C). Peak 1: senkyunolide I; peak 2: senkyunolide H; peak 3: senkyunolide A; peak 4: coniferyl ferulate; peak 5: ligustilide

Table 7: Correlation coefficient between different hemorheology indices of CX and six common peak areas

Number of common peaks	Correlation coefficient						Average of correlation coefficient
	Viscosity of whole blood			*Plasma viscosity*	*Fibrinogen*	*Index of erythrocyte aggregation*	
	1 (s^{-1})	*50 (s^{-1})*	*200 (s^{-1})*				
1	0.7637	0.7699	0.7694	0.7511	0.7563	0.7582	0.7614
2	0.6945	0.6999	0.7023	0.6992	0.7066	0.7072	0.7016
3	0.7168	0.7316	0.7314	0.7189	0.7226	0.7213	0.7238
4	0.7140	0.7291	0.7296	0.6962	0.7066	0.7175	0.7155
5	0.7365	0.7370	0.7385	0.7209	0.7239	0.7288	0.7309
6	0.6692	0.6792	0.6859	0.6626	0.6719	0.6678	0.6727

DISCUSSION

Clinical studies have shown that cardiovascular diseases and cerebrovascular diseases are related mostly to blood stasis, so improving blood stasis would be a rational approach. Reports [13,14] have shown that ligustilide, senkyunolide

I, senkyunolide H, senkyunolide A, ferulic acid and ligustrazine can improve blood stasis, respectively. However, not all of these compounds have been reported to be in CX or in the body fluids of animals. Therefore, there was insufficient evidence in the previous studies to demonstrate that these components are the active ingredients of CX.

Subcutaneous injection of epinephrine and an iced water bath is the most commonly used method to replicate acute blood stasis [15-17]. Water temperature, the injection site, and the time blood is collected have a considerable influence on experimental results. Epinephrine is a vasoactive drug, which causes the death of rats by increasing the blood pressure suddenly when injected close to the head or injected into a blood vessel by mistake. Therefore, injecting epinephrine accurately and mastering the time to start blood collection are the key to successful modeling.

Thirty minutes after the last gavage was selected as the time to start blood collection because there were no more peaks after 30 min. In this work, methanol was selected as the organic mobile phase by comparing the shape and separation of the peak.

The spectrum – effect relationship can be used to determine the main medicinal ingredients of a target drug based on the contribution of its different components. In this study, we studied the active part of CX for blood - activating, and compared the blood - activating effects and serum HPLC fingerprints of CX from different origins, then evaluated the spectrum - effect relationship of them by grey correlation coefficient. The greater the correlation coefficient, the greater was the contribution of the corresponding peak to the blood-circulation activity of CX extracts [18]. We revealed that the contributions of six common peaks to the blood-circulation activity of CX extracts were in the order: peak 1 (senkyunolide I) > peak 5 (ligustilide) > peak 3 (senkyunolide A) > peak 4 (coniferyl ferulate) > peak 2 (senkyunolide H) > peak 6. Compared with the HPLC fingerprints of medicinal materials, serum HPLC fingerprints can better explain the effect of different ingredients on drug efficacy. This study revealed the blood promoting effect of 5 active ingredients through systematic research. Combined with the previous reports, senkyunolide I, ligustilide, senkyunolide A, coniferyl ferulate and senkyunolide H may be the medicinal material basis of CX for blood - activating.

CONCLUSION

In this study, we investigated the spectrum–effect relationship between the blood-circulation activity and serum HPLC fingerprints of CX extracts for the first time. Our study suggested that ligustilide, senkyunolide A, coniferyl ferulate, senkyunolide H and senkyunolide I helped to improve blood circulation in rats. Our study provides scientific justification for using CX to remove blood stasis.

DECLARATIONS

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Data availability

The data used to support the findings of this study are available from the corresponding author upon request.

Conflict of interest

No conflict of interest is associated with this work.

Contribution of authors

We declare that this work was done by the authors named in this article and all liabilities pertaining to claims relating to the content of this article will be borne by the authors.

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