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ORIGINAL ARTICLE

Screening and Validating of 1, 1-Diphenyl-2-Trinitrophenylhydrazine Scavengers from Danshen-Honghua Herbal Pair

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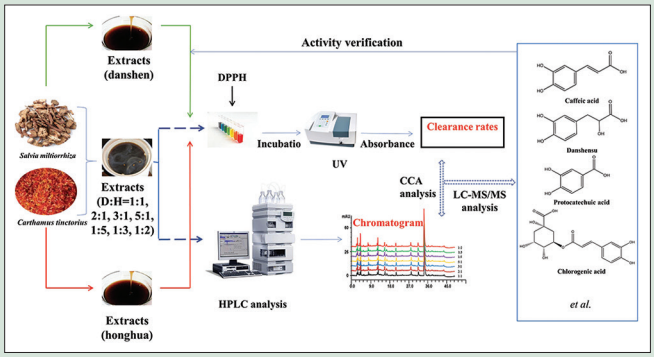
ABSTRACT

Background: *Salvia miltiorrhiza* (Danshen [DS]) and *Carthamus tinctorius* (Honghua [HH]) are commonly used traditional Chinese medicines for activating blood and removing stasis, and they were usually used as DS-HH (Danshen-Honghua [DH]) herbal pair in clinical applications. Characterizing the antioxidant active ingredients in DH herbal pair may be helpful for understanding their curative effect on cardiovascular diseases. **Objective:** The objective of the study is to screen the 1, 1-diphenyl-2-trinitrophenylhydrazine (DPPH) scavenging active compounds in DH herbal pair by spectrum-effect relationship analysis. **Materials and Methods:** First, the water extracts of DH herbal pair with different ratios (DS: HH = 1:0, 0:1, 1:1, 2:1, 3:1, 5:1, 1:5, 1:3, and 1:2) were prepared. Then, the clearance effects of DH herbal pair and single drugs on DPPH were compared, meanwhile, high performance liquid chromatography was applied for chemical analysis of DH extracts. Finally, DPPH scavengers in DH herbal pair were predicted and identified by spectrum-effect relationship analysis and liquid chromatography-mass spectrometry (LC-MS) analysis. **Results:** Compared with single drugs, the extracts of herbal pairs had higher clearance rate to DPPH. Eighteen potential active compounds (peaks) in the extract of DH herbal pair were predicted, and 13 of them were tentatively identified by LC-MS analysis. Furthermore, antioxidant activities of eight pure compounds from the DH herbal pair were validated by DPPH radical scavenging assay with Vitamin C as positive control drug. Among them, six compounds including danshensu, protocatechuic acid, coffee acid, chlorogenic acid, rutin, and salvianolic acid A were found to have high antioxidant activity. **Conclusion:** DH herbal pair showed strong clearance effect on DPPH, and danshensu, protocatechuic acid, coffee acid, chlorogenic acid, rutin and salvianolic acid A are the active components.

Key words: 1, 1-diphenyl-2-trinitrophenylhydrazine scavenger, active ingredients, antioxidation effect, Danshen-Honghua, spectrum-effect relationship analysis

SUMMARY

- A 1, 1-diphenyl-2-trinitrophenylhydrazine scavenger screening method was established and applied in the study of potential active components in *Salvia miltiorrhiza-Carthamus tinctorius* extracts
- Combining the results of spectrum-effect analysis, liquid chromatography-mass spectrometry analysis and antioxidant activity assay, six active compounds in Danshen-Honghua were discovered as scavengers targeting 1, 1-diphenyl-2-trinitrophenylhydrazine.



Abbreviations Used: DS: Danshen, HH: Honghua, DH: Danshen-Honghua, TCMs: Traditional Chinese medicines, LC-MS: Liquid chromatography-mass spectrometry, DPPH: 1, 1-diphenyl-2-trinitrophenylhydrazine, VC: Vitamin C, CCA: Canonical correlation analysis.

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INTRODUCTION

Salvia miltiorrhiza Radix et Rhizoma (Danshen [DS] in Chinese) is the dried root or rhizome of *S. miltiorrhiza* Bunge, in which the main components are phenolic acids and diterpenes.[1] Carthami Flos (Honghua [HH] in Chinese), the dried flower of *Carthamus tinctorius* L., generally composes of flavonoids, fatty acids, volatile oils, and polysaccharides.[2] DS, HH, and their herbal pair have the function of activating blood and removing stasis, which were used to treat coronary heart diseases, chronic heart failure, hypertension, menstrual disorders, cerebrovascular diseases, and other cardiovascular diseases.[3] Studies had shown that antioxidant active ingredients can act on the target of activating blood and removing stasis.[4-6] Ren *et al*. explored the relationship between antioxidant capacity and coagulation-fibrinolytic system in cardiovascular disease, the results showed that the activity of fibrinolysis became weaker when the antioxidant enzyme activity was decreased.[7] Khullar *et al.* reported a significant decrease in antioxidant substances such as glutathione in hypertensive patients.[8] There are also increasing evidence that excess reactive oxygen species or decreased antioxidant capacity can induce vascular wall inflammatory responses, leading to cardiovascular disease.[9] On the other hand, recent studies had shown that atherosclerosis is a process of vascular remodeling associated with oxidation-antioxidant imbalance, the body's antioxidant capacity decrease can promote vascular smooth muscle cells proliferation and induce oxidative stress, so as to accelerate the formation of atherosclerosis.[10,11] Herein, the antioxidant target has a close correlation with the cardiovascular diseases. Hence, the finding of antioxidant active

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ingredients in DS, HH, and their herbal pair may be meaningful for understanding their treatment of cardiovascular diseases.

On the other hand, antioxidant is an important step in preventing aging because free radicals or oxidants can decompose cells and tissues, affect metabolic functions, and cause different health problems.[12] Traditional chemically synthesized antioxidants such as Dibutylhydroxytoluene, Butyl hydroxyanisole, Propyl gallate, and tert-butyl hydroquinone have significant antioxidant effects.[13] However, traditional synthetic origin antioxidants have some drawbacks, such as long-term contact can lead to nephrotoxicity, potential mutagenesis, teratogenicity, and other diseases.[14] At present, natural origin antioxidants have attracted the attention of more and more researchers due to the advantages of fewer side effects. For example, it is reported that the polyphenols and bioflavonoids isolated from natural plants have significant scavenging free radical effects and less potential side effects.[4,15] Fan *et al*. explored the antioxidant activity of natural polyphenolic compounds, and the results showed that rutin, resveratrol, caffeic acid, and phloretin has a strong antioxidant effect.[16]

The spectrum-effect relationship analysis combines the efficacy and chemical compositions of the fingerprint of natural products and was originally used to develop control markers that can truly reflect the inherent quality of products.[17] Furthermore, spectrum-effect analysis was also used to screen the active components from natural products.[18] Therefore, in this study, a spectrum-effect analysis method was developed to screen the active constituents for scavenging free radical in Danshen-Honghua (DH) herbal pair. First, the clearance effect of DH herbal pair and single drugs on 1, 1-diphenyl-2-trinitrophenylhydrazine (DPPH) was compared. Then, the components in the DH herbal pair were analyzed by high performance liquid chromatography (HPLC). Furthermore, the potential DPPH scavengers in DH herbal pair were predicted by spectrum-effect analysis, and their structures were identified by liquid chromatography-mass spectrometry (LC-MS) analysis. Finally, the antioxidant activity of the predicted compounds was verified *in vitro*.

MATERIALS AND METHODS

Samples

Crude drugs of DS and HH were both purchased from Chongqing Heping Pharmacy Co., Ltd (Chongqing, China), in December 2017. The voucher specimens of *S. miltiorrhiza* Bunge (No. SM2017090101) and *C. tinctorius* L.(No. CF2017090101) were deposited at the Pharmaceutical Engineering Laboratory in School of Chemistry and Chemical Engineering, Chongqing University, Chongqing, China.

Chemicals and reagents

Vitamin C (VC) (≥98%) and 1,1-diphenyl-2-trinitrophenylhydrazine, (DPPH, ≥98%) were purchased from Beijing Solarbio Science and Technology Co., Ltd (Beijing, China). The reference compounds danshensu, protocatechuic acid, coffee acid, chlorogenic acid, hydroxysafflor yellow A, tanshinone IIA, rutin, and salvianolic acid A (≥98%, determined by HPLC) were obtained from PUSH Bio-technology Co., Ltd.(Chengdu, China). HPLC-grade acetonitrile, methanol and formic acid were obtained from Beijing InnoChem Science and Technology Co., Ltd (Beijing, China). All of the experimental water was purified by water purification system.

Instruments

A rotary evaporator (ZFQ 85A, Shanghai Medical Instrument Special Factory, Shanghai, China), a freezing dryer system (DZF-6050, Shanghai Jing Hong Laboratory Instrument Co., Ltd., Shanghai, China), a water purification system (ATSelem 1820A, Antesheng Environmental Protection Equipment Co., LTD., Chongqing, China), an ultraviolet-visible spectroscopy (UV-Vis) spectrophotometry (Mettler Toledo, Inc., Shanghai), and an Agilent 1260 Series liquid chromatograph system (Agilent Technologies, Palo Alto, California, USA) were used in this study.

Preparation of Danshen-Honghua extracts

All the dried raw DS and HH were pulverized and griddled through 50 mesh sieves (about 0.29 mm) before extraction. Seven different proportions of the herbs were prepared with ratios of 1:1, 2:1, 3:1, 5:1, 1:5, 1:3, and 1:2 (g/g) DS to HH, respectively. Moreover, 20 g of DS and HH mixed powder was extracted with 200 mL water in a glass-stoppered conical flask at 75°C for 1.5 h. After extraction, the mixture was filtered through gauze, and the residue was collected and repeated the extraction twice. The two filtrates were combined and evaporated in a rotary evaporator at 55°C under reducing pressure to remove the solvent. The extracts were further dried by lyophilization with freezing dryer system to obtain the DH extracts with a yield of about 25.2% (w/w, dried extract/crude herb). All pre- and post-dilution solutions were stored at 4°C. Before HPLC analysis, the sample solutions were filtered through a 0.22 μm nylon membrane filter (Shanghai Titan Scientific Co., Ltd., Shanghai, China).

Preparation of reference standard solutions

Reference substance solutions of danshensu, protocatechuic acid, coffee acid, chlorogenic acid, hydroxysafflor yellow A, tanshinone IIA, rutin and salvianolic acid A, and positive control (VC) were all prepared by dissolving the respective substance in methanol, and were diluted to the required concentrations for scavenging free radical assay, respectively. All the solutions were stored at 4°C in the dark before use.

Antioxidant activity assay

The methods of measuring *in vitro* antioxidant capacity include DPPH, FRAP, ABTS, ORAC, and PCL. Studies have shown that the DPPH method has the advantages of rapidness, sensitivity, and direct feasibility.[19] Furthermore, VC and vitamin E are the most commonly used free radical scavengers, which often serve as positive control drugs.[12,20]

The antioxidant assay was performed in cuvette (Thermofisher, USA.), 1.5 mL test solution and 1.5 mL DPPH (200 μmol/L) were mixed and incubated for 10 min at room temperature (about 25°C). The absorbance was monitored at 517 nm by a UV-Vis spectrophotometry. Methanol was used as blank control, and VC was used as positive control. Free radical scavenging activity was expressed as the clearance percentage of DPPH:

$$\text{Clearance rate } (\%) = \frac{A_0 - A_s}{A_0} \times 100\%$$

Where A_0 and A_s are the absorbance of the blank and sample group, respectively. All trials were performed independently in triplicate and the results were shown with mean value of the triplicate observations.

HPLC analysis

HPLC analysis was performed on an Agilent 1260 Series liquid chromatography system, which was equipped with a vacuum degasser, a binary pump, an autosampler, and a diode array detector, controlled by an Agilent ChemStation software. An Agilent Zorbax SB-Aq column (250 mm × 4.6 mm, 5 μm) preceded by a Zorbax SB-C_{18} guard column (12.5 mm × 4.6 mm, 5 μm) was adopted for the analysis. The mobile phase consisted of solvent A (0.1% formic acid aqueous solution) and solvent B (acetonitrile) using a gradient elution program as follows: 5% B at 0–2 min, 5%–15% B at 2–10 min, 15%–22% B at 10–24 min, 22%–29% B at 24–35 min, 29%–37% B at 35–42 min, 37%–5% B at 42–47 min. The flow rate of mobile phase was set at 1.0 mL/min with

10 μL per sample injection. The UV detection wavelength was set at 265 nm, and the column temperature was conditioned at 37°C.

Liquid chromatography-mass spectrometry analysis

Shimadzu LC/MS-MS 8060 electrospray ionization mass spectrometer (ESI-MS), consisting of a Triple Quadrupole Detector as the mass detector (Shimadzu, Kyoto, Japan) and coupling with HPLC was used for LC-MS identification. The LC conditions were the same as described previously. The ESI-MS conditions were as follows: The ESI was used in both positive and negative mode; nitrogen gas was used for desolvation at a flow rate of 3 L/min at 250°C; the temperature and flow rate of drying gas were 400°C and 10 L/min, respectively; the cone voltage was (+) 20 and (−-) 20 V; MS data were recorded in the full scan mode (*m*/z 50–1500) and MS^2 data were recorded in the range of m/z 50–1200.

Spectrum-effect relationship analysis

The spectrum-effect analysis was performed by transferring DH fingerprint peak area and free radical scavenging activity test results into SPSS software for canonical correlation analysis (CCA). The optimized HPLC fingerprints of seven ratios DH samples were calculated and generated by professional software named similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine (Version 2012) (Chinese Pharmacopoeia Committee, Beijing, China). CCA was used to assess the spectrum-effect relationships between the areas of 87 peaks in fingerprint and the free radical clearance ratios.

The main processes of spectrum-effect relationship study on TCMs include the following three steps: The first step is to get the reliable chromatographic fingerprint with a clear concentration distribution of chemical components (spectrum) and the pharmacological activities of TCMs (effect). The second step is to build up the spectrum-effect relationship model, that is to link the peaks in the chromatographic fingerprint to the pharmacological effects of TCMs, and the potential active compounds can be screened out by "spectrum" and "effect" relationship analysis. The final step is to identify the structures of predicted active components and used as quality control markers, which can accurately reflect the therapeutic effect and inner quality of TCMs.[21-24]

Statistical analysis

All data are presented as mean ± standard deviations of at least three different experiments. The statistical analysis was performed with SPSS (version 24, SPSS, Inc., Chicago, IL, USA).

RESULTS AND DISCUSSION

Clearance effects of Danshen-Honghua extract on 1, 1-diphenyl-2-trinitrophenylhydrazine

The scavenging effect of DH extracts on DPPH was shown in Table 1. The results indicated that all of the DH extracts (1 mg/mL) had strong DPPH scavenging activity as compared to the positive control VC. Some of the DH extracts (1:5 and 1:2) showed a weaker antioxidant effect than single herbal extracts when the concentrations of DPPH and sample were kept constant. However, others DH herbal pair extracts (1:1, 2:1, 3:1, 5:1, and 1:3) especially for DH 5:1 displayed stronger antioxidant effect than single herbal extracts, which indicated synergistic effect of the herbal pair may occur on the clearance of DPPH.

In order to obtain the best screening performance for active compounds in the complex matrix, the scavenging effects of DH extracts with different concentrations on DPPH were investigated. As shown in Figure 1, among the high (1 mg/mL) and low (0.0625 mg/mL) concentrations of all DH herbal pairs, DH 5:1 exhibited the strongest scavenging effect, indicating that DS may play a leading role in the antioxidant activity of DH herbal pairs. On the other hand, the incubation time, DPPH concentration, and sample concentration were optimized (data not shown). After investigation, a sufficient incubation time (10 min) and a sufficient DPPH concentration (25 μg/mL) were used in this study.

Spectrum-effect relationship analysis

The optimized HPLC fingerprints of DH samples with seven ratios were shown in Figure 2. A total of 87 peaks involved were detected in the calculation of spectrum-effect relationship. CCA was used to assess the spectrum-effect relationship between the areas of 87 peaks and the clearance rate, and the results were shown in Table 2. As suggested by the correlation coefficients, the highly relevant peaks were 4, 7, 9, 14, 16, 17, 18, 23, 24, 28, 31, 33, 41, 43, 45, 53, 66, and 82 with Pearson relational grade more than 0.8. In other words, these 18 peaks might be the main active components of the herbal pair for antioxidant effect.

Identification of the potential 1, 1-diphenyl-2-trinitrophenylhydrazine scavengers in Danshen-Honghua extract by liquid chromatography-mass spectrometry analysis

HPLC-MS/MS analysis was used to identify the chemical structures

Table 1: Different ratios of Danshen-Honghua extracts and Vitamin C (1 mg/mL) on 1, 1-diphenyl-2-trinitrophenylhydrazine clearance effect (*n*=9)

Sample	Clearance rate (%)
Vitamin C	96.8±0.7
DH 1:0	89.7±0.5
DH 0:1	80.4±0.8
DH 1:1	86.4±0.1
DH 2:1	87.1±0.2
DH 3:1	86.7±2.0
DH 5:1	90.4±0.3
DH 1:5	79.7±0.7
DH 1:3	85.4±1.0
DH 1:2	77.2±1.2

DH: Danshen-Honghua; VC: Vitamin C

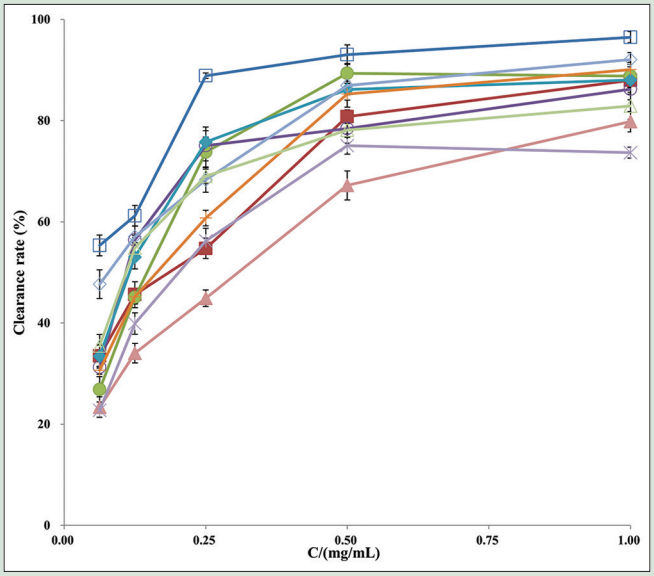

Figure 1: Clearance rates to DPPH of Danshen-Honghua extracts at different concentrations. VC (□), DH 1:0 (■), DH 0:1 (●), DH 1:1 (○), DH 2:1 (◆), DH 3:1 (+), DH5:1 (◇), DH1:5 (▲), DH1:3 (△), DH1:2 (×)

of compounds in DH extracts. Based on the fragmentation behaviors, retention time and MS data [Table 3] of the peaks in the test samples, 13 compounds (danshensu, protocatechuic acid, tanshinone IIA, caffeic acid, 4,5-dihydroxy brass-6,7-dioxopyranoside, chlorogenic acid, hydroxysafflor yellow A, isorhamnetin-3,4'-diglucoside, rutin, 6-hydroxykaempferol 3-O-rutinoside-6-O-glucoside, kaempferol-3-O-rutinoside, salvianolic acid A, and cirsilineol) were tentatively identified, and the structures of these compounds were shown in Figure 3. Eight pure reference compounds including danshensu, protocatechuic acid, tanshinone IIA, caffeic acid, chlorogenic acid, hydroxysafflor yellow A, rutin and salvianolic acid A were then obtained for further *in vitro* activity tests.

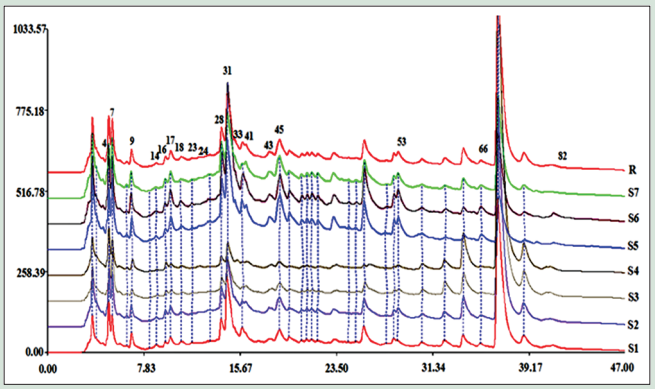

Figure 2: High performance liquid chromatography chromatograms of Danshen-Honghua extracts: DH 1:1 (S1), DH 2:1 (S2), DH 3:1 (S3), DH 5:1 (S4), DH 1:5 (S5), DH 1:3 (S6), DH 1:2 (S7) and control map (R)

In vitro activity tests for the predicted compounds

To confirm the ability of the hit compounds with scavenging free radical, *in vitro* scavenging free radical assays were performed. Five concentrations of each compound were tested, and the results were shown in Figure 4. As a well-known antioxidant, VC showed strong antioxidant effect. From the results shown in Figure 4, among the eight identified compounds, danshensu, protocatechuic acid, caffeic acid, chlorogenic acid, rutin, and salvianolic acid A processed strong DPPH scavenging effects in a dose-dependent manner. However, tanshinone IIA had no clearance effect on DPPH, possibly because tanshinone IIA do not destroy the activity of the oxidase or did

Table 2: Correlation coefficients between chromatogram peaks and clearance rates

Peak number	1	2	3	4	5	6	7	8	9	10	11	12	13
Antioxidant rate	−0.536	−0.196	−0.773	−0.902*	−0.705	−0.780	−0.827*	−0.490	−0.817*	−0.455	−0.774	0.063	0.543
Peak number	14	15	16	17	18	19	20	21	22	23	24	25	26
Antioxidant rate	0.811*	−0.731	0.858*	−0.925*	−0.872*	0.589	−0.521	−0.704	0.458	−0.835*	−0.813*	−0.670	−0.656
Peak number	27	28	29	30	31	32	33	34	35	36	37	38	39
Antioxidant rate	−0.248	−0.860*	−0.652	−0.688	−0.887*	−0.414	0.845*	−0.662	0.165	−0.366	−0.524	−0.218	−0.778
Peak number	40	41	42	43	44	45	46	47	48	49	50	51	52
Antioxidant rate	−0.516	0.868*	−0.300	−0.818*	−0.425	0.846*	−0.394	−0.724	−0.612	−0.698	0.291	−0.250	−0.603
Peak number	53	54	55	56	57	58	59	60	61	62	63	64	65
Antioxidant rate	0.900*	−0.446	−0.344	−0.635	−0.372	−0.552	−0.357	0.581	−0.219	−0.475	0.610	−0.292	−0.664
Peak number	66	67	68	69	70	71	72	73	74	75	76	77	78
Antioxidant rate	0.866*	−0.363	0.645	0.680	0.441	0.334	−0.169	0.569	−0.583	−0.207	−0.119	0.762	−0.228
Peak number	79	80	81	82	83	84	85	86	87				
									0.426				
Antioxidant rate	0.614	0.601	−0.109	0.819*	−0.463	0.137	0.176	−0.093					

Pearson correlation, "*r*" represents the relevant strength; *0.8 ≤| r |≤ 1 means very significant correlation

Table 3: High performance liquid chromatography- mass spectrometry/mass spectrometry data of 18 predicted active compounds from Danshen-Honghua herbal pair

Peak number	t_R (min)	MW	MS^1 (m/z)	MS^2 (m/z)	Formula	Structural identification
4	3.373	198	197.05	178	$C_9H_{10}O_5$	Danshensu
7	4.172	129	130.00	114; 94; 82	-	Unknown
9	4.886	-	-	-	-	Unknown
14	7.043	154	153.01	109	$C_7H_6O_4$	Protocatechuic acid
16	8.125	294	295.15	277; 249	$C_{19}H_{18}O_3$	Tanshinone IIA
17	8.996	180	179.10	135	$C_9H_8O_4$	Caffeic acid
18	9.545	774	773.45	301	-	Unknown
23	11.394	652	653.35	434; 387	-	Unknown
24	11.779	612	611.40	449; 286	$C_{27}H_{32}O_{16}$	4, 5-dihydroxyflavone-6, 7-dioxopyranoside
28	12.034	354	353	191; 161	$C_{16}H_{18}O_9$	Chlorogenic acid
31	12.683	612	611.40	491; 473; 403; 353; 325; 283; 205	$C_{27}H_{32}O_{16}$	Hydroxysafflor yellow A
33	14.114	640	639.30	476	$C_{27}H_{30}O_{17}$	Isorhamnetin-3, 4'-diglucoside
41	17.105	772	773.35	695; 672; 303; 187; 112	$C_{33}H_{40}O_{21}$	6-hydroxykaempferol 3-*O*-rutinoside-6-*O*-glucoside
43	20.054	610	611.25	498; 432; 420; 399; 321; 251; 206; 163	-	Unknown
45	20.324	610	611.25	303	$C_{27}H_{30}O_{16}$	Rutin
53	25.988	594	593.30	284	$C_{27}H_{30}O_{15}$	Kaempferol-3-*O*-rutinoside
66	33.605	494	493.20	295	$C_{26}H_{22}O_{10}$	Salvianolic acid A
82	44.435	330	329.35	287; 245	$C_{17}H_{14}O_7$	Cirsilineol

MS: Mass spectrometry; MW: Molecular weight

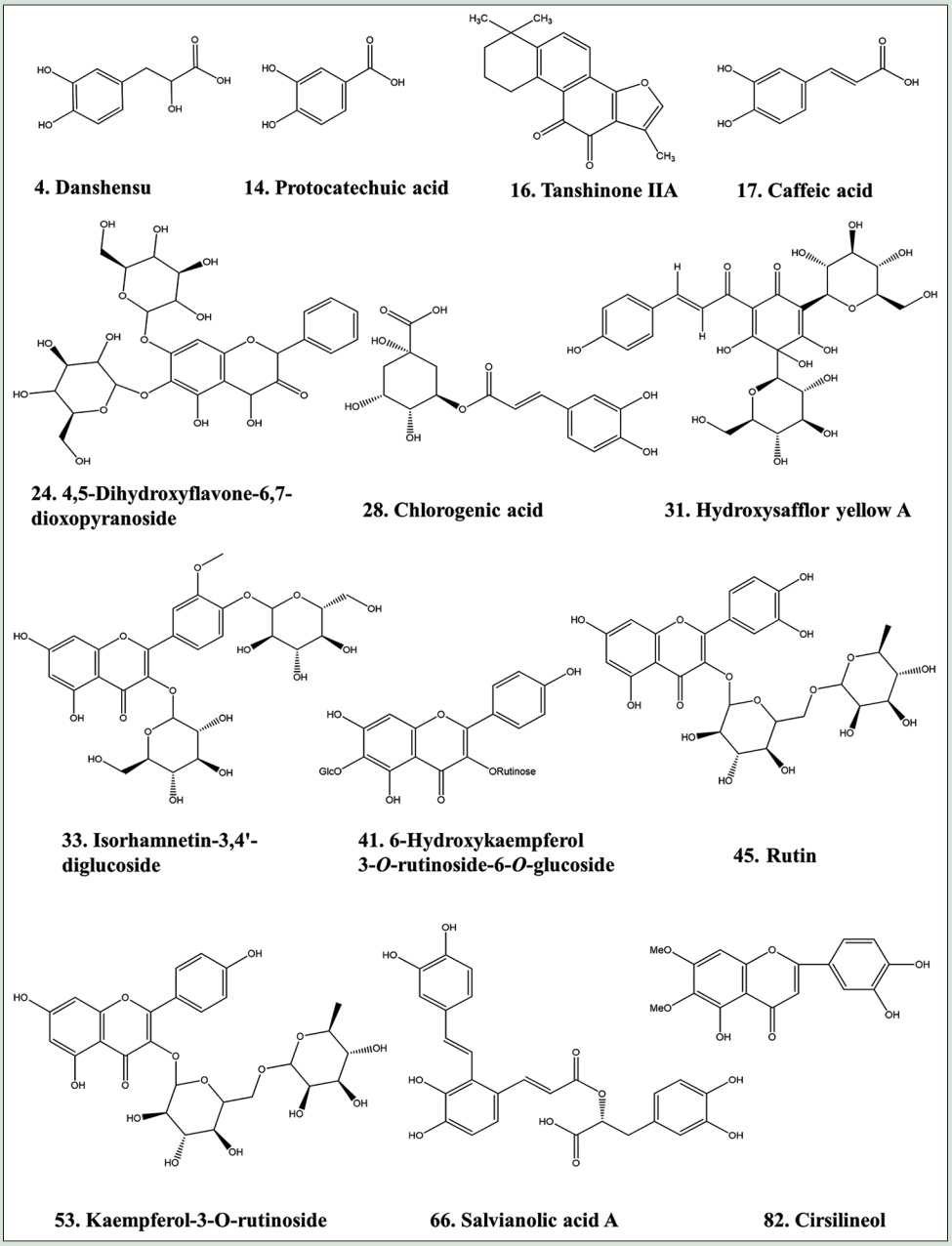

Figure 3: Chemical structures of compounds identified in Danshen-Honghua herbal pair. The numbers of compounds are the same as peaks numbers in Figure 2

not combine with the substance that caused the oxidation reaction (such as metal ions). In addition, hydroxysafflor yellow A did not show significant activity at a relatively high concentration (2 mmol), with IC_{50} value of 875 μmol. In general, the validation results of these active compounds were in consistent with the result of DH extracts, which phenolic acids in DS played a leading role in the antioxidant effect of DH herbal pair.

From the chemical structures of compounds shown in Figure 3, the phenolic hydroxyls may be the key moiety for these compounds to exert an antioxidant effect. Therefore, tanshinone IIA has no DPPH scavenging effect may be related to its lack of phenolic hydroxyl, while hydroxysafflor yellow A has a weaker DPPH scavenging effect as it contains only one phenolic hydroxyl. In addition, some of the identified compounds such as cirsilineol, isorhamnetin-3, 4'-diglucoside, kaempferol-3-*O*-rutinoside, and 6-hydroxykaempferol-3-*O*-rutinoside-6-*O*-glucoside also contain such phenolic hydroxyls in their structures and might be potential antioxidant active ingredients. However, pure substances of these compounds are required for the further activity tests.

CONCLUSION

In this study, combining the results of the spectrum-effect analysis, LC-MS analysis and antioxidant activity assay, six active compounds including danshensu, protocatechuic acid, caffeic acid, chlorogenic acid, rutin and salvianolic acid A were discovered as DPPH scavengers in DH herbal pair. In addition, other potential antioxidant active ingredients such as cirsilineol, isorhamnetin-3,4'-diglucoside, kaempferol-3-O-rutinoside, and 6-hydroxykaempferol-3-O-rutinoside-6-O-glucoside, which gained

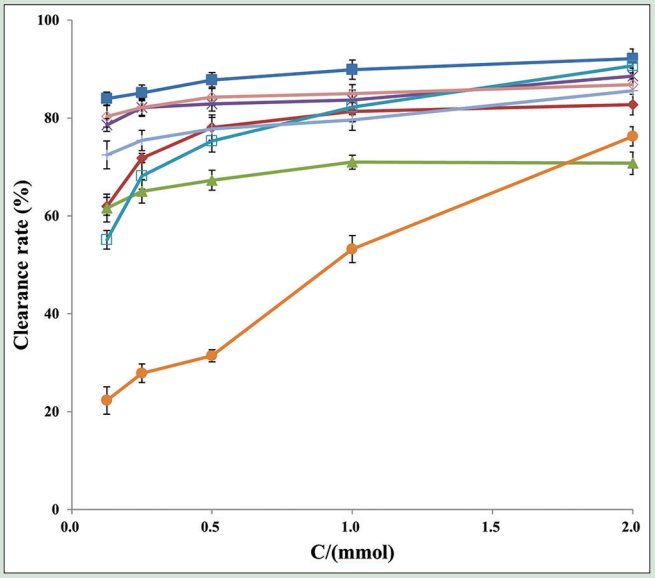

Figure 4: Clearance rates to DPPH of predicted active components at different concentrations. VC (■), Danshensu (◆), Protocatechuic acid (▲), Coffee acid (×), Chlorogenic acid (□), Hydroxysafflor yellow A (●), Rutin (+), Salvianolic acid A (◇)

similar structures with the hit compounds, might also be identified. These results proved that the proposed method could effectively screen antioxidant active ingredients from TCMs such as DH herbal pair.

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Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Qu C, Pu ZJ, Zhou GS, Wang J, Zhu ZH, Yue SJ, *et al.* Comparative analysis of main bio-active components in the herb pair Danshen-Honghua and its single herbs by ultra-high performance liquid chromatography coupled to triple quadrupole tandem mass spectrometry. J Sep Sci 2017;40:3392-401.
2. Gao LN, Cui YL, Yan K, Qiu C. Advances in studies on compatibility of *Salvia miltiorrhiza* radix et rhizoma and carthamii flos. Chin Tradit Herbal Drugs 2016;47:671-9.
3. Wang Z, Ma S, Zhang Q, He S, Li Q, Hu J, *et al.* Matrix solid-phase dispersion coupled with high-performance liquid chromatography diode array detection for simultaneous determination of four lipophilic constituents from *Salvia miltiorrhiza* bunge. J Chromatogr Sci 2017;55:316-26.
4. Pan K. Research progress on antioxidation of Chinese herbs for promoting blood circulation and removing blood stasis. Guide Chin Med 2013;11:328-9.
5. Sun GX, Gao YN, Sun JS. Correlation between quantified HPLC fingerprint and its anti-oxidation activity of *Salvia miltiorrhiza* radix et rhizoma. Cent S Pharm 2013;11:535-8.
6. Wang JM, Yao C, Li CQ, Kang WY. Spectrum-effect relation of antioxidant activity of Flos Carthami based on DPPH, ABTS and FRAP assay. Chin Pharm J 2017;52:825-31.
7. Ren YS, Jia GL, Chen SL, Zhang YS, Zhang DX, Wu ZG. Relationship between antioxidant enzyme parameters and coagulation fibrinolysis system in unstable angina patients. J Cardiovascul Pulmon Dis 2002;21:88-9.
8. Khullar M, Relan V, Sehrawat BS. Antioxidant activities and oxidative stress byproducts in human hypertension. Hypertension 2004;43:e7-8.
9. Cao J, Wang HY. Total antioxidant capacity and cardiovascular disease. Geriatr Health Care 2011;17:58-60.
10. Yokoyama M. Oxidant stress and atherosclerosis. Curr Opin Pharmacol 2004;4:110-5.
11. Noma K, Goto C, Nishioka K, Jitsuiki D, Umemura T, Ueda K, *et al.* Roles of rho-associated kinase and oxidative stress in the pathogenesis of aortic stiffness. J Am Coll Cardiol 2007;49:698-705.
12. Zhao X, Song JL, Yi R, Li G, Sun P, Park KY, *et al.* Comparison of antioxidative effects of insect tea and its raw tea (Kuding tea) polyphenols in kunming mice. Molecules 2018;23. pii: E204.
13. Chen HY, Zhu WL, Li MH, Huang QY, Qiu QD, Li C, *et al.* Stability properties of common antioxidants of PG, TBHQ, BHA and BHT. Cereal Oil Proc 2014;7:44-46, 49.
14. Xu JJ, Liu B. Spectrum-effect relation in antioxidant activity of menthae haplocalycis herba based on DPPH and FRAP assay. J Beijing Univ TCM 2015;38:405-10.
15. Zhou X, Tang L, Xu Y, Zhou G, Wang Z. Towards a better understanding of medicinal uses of *Carthamus tinctorius* L. in traditional Chinese medicine: A phytochemical and pharmacological review. J Ethnopharmacol 2014;151:27-43.
16. Fan JB, Cai QT, Feng XQ, Hou Y, Yu XF, Jiang M. Comparison of antioxidant activity of five natural polyphenolic compounds. Food Ferment India 2015;40:77-83.
17. Zhu CS, Lin ZJ, Xiao ML, Niu HJ, Zhang B. The spectrum-effect relationship-a rational approach to screening effective compounds, reflecting the internal quality of Chinese herbal medicine. Chin J Nat Med 2016;14:177-84.
18. Hou Y, Cao X, Dong L, Wang L, Cheng B, Shi Q, *et al.* Bioactivity-based liquid chromatography-coupled electrospray ionization tandem ion trap/time of flight mass spectrometry for β_2AR agonist identification in alkaloidal extract of *Alstonia scholaris*. J Chromatogr A 2012;1227:203-9.
19. Zhang XJ. A study on the Correlation Between Alizarin Fingerprint and Antioxidant Activity. Harbin: Heilongjiang University; 2014.
20. Duan XJ. Study on Quality Evaluation and Spectral-Effect Relationship of *Salvia miltiorrhiza* var. Alba by Chemometrics. Jinan: ShanDong University; 2014.
21. Luo GA, Wang YM, Cao J, Yang XD. Studies on establishment of modern quality standards system of traditional Chinese medicine in China. World Sci Technol Mod TCM 2002;4:5-11.
22. Xu GL, Xie M, Yang XY, Song Y, Yan C, Yang Y, *et al.* Spectrum-effect relationships as a systematic approach to traditional Chinese medicine research: Current status and future perspectives. Molecules 2014;19:17897-925.
23. Liu L, Li W, Ding JX. Researches on pharmacodynamic material basis of traditional Chinese medicine based on "spectrum-activity" correlation analysis. Natl Prod Res Dev 2013;25:410-5.
24. Xiao S. A method for research of antibacterial constituent recognition of traditional Chinese medicine (*Acalypha australis* Linn.) by spectrum-effect relationship. Beijing: Chin Acad Agric Sci 2013.