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AUTOTOXINS SCREENING FROM AQUEOUS EXTRACTS OF *SALVIA MILTIORRHIZA* BGE. BASED ON SPECTRUM-EFFECT RELATIONSHIP BETWEEN HPLC FINGERPRINTS AND AUTOTOXICITY

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Abstract

The spectrum-effect relationships between chromatography fingerprints and efficacy were regarded as a useful key for bioactive compounds screening from complex mixtures. In this study, a new mode for autotoxins exploring based on spectrum-effect relationship between HPLC fingerprints and autotoxicity was established. HPLC method was used to establish five batches fingerprints of Danshen aqueous extracts and eighteen common peaks were picked out by using the similarity evaluation system. Seed germination and seedling growth tests of Danshen were carried out and those observable indicators were comprehensively quantified by principal components analysis to evaluate autotoxicity. Ultimately, grey relational analysis was applied to evaluate the correlation degree of chemical components characterized by common peaks and autotoxicity. According to the magnitude of the correlation degree, ten peaks of the HPLC fingerprints indicating the main active autotoxins chemicals were obtained.This study provides a general model for active components exploring by the combination of chromatography and efficacy.

Key words: Autotoxins screening, Spectrum-Effect Relationship, *Salvia miltiorrhiza* Bge.

Introduction

The dried root and rhizoma of *Salvia miltiorrhiza* Bge., popularly known as Danshen in Chinese, is widely used in traditional Chinese medicine for the treatment of a wide variety of ailments including coronary heart disease, hepatitis, menstrual disorders, menostasis, blood circulation diseases and other cardiovascular diseases (Li *et al*., 2005). Due to its medicinal importance, the past decade has witnessed a particularly strong and rapid upsurge in clinical demand which directly induced tremendous planting area of this herbal medicine (Hu *et al.*, 2005). However, during its consecutively cultivated process, serious monoculture cropping problems that caused a significant yield decline in the successive years under normal planting conditions were found to become a bottleneck for the sustainable development of Danshen medicine.

Autotoxicity, broadly documented as the deleterious allelopathic effect among the individuals of the same species (Ruan *et al*., 2011), is considered as one of the major reasons for monoculture cropping problem and is widely believed to be induced by autotoxins (Huang *et al*., 2010, Hussain *et al*., 2011). At present, autotoxins have been isolated and identified in many different species, such as *Renmannia glutinosa* Libosch (Li *et al*., 2012), cowpea (Huang *et al*., 2010), and so on include others. However, when it comes to *Salvia miltiorrhiza* Bge, little is known up to now about the responsible allelochemicals. It thus is vital to explicit the possible bioactive chemicals. Unfortunately, considering the content of autotoxins in plant is usually traced and the separation method is procedurally tedious, expensive and time-consuming, studies directly addressing this aspect are lacking.

As a widely accepted quality control mode, chromatographic fingerprints, especially High-performance liquid chromatography (HPLC) fingerprints, are blessed with obvious advantages in quality control and chemical analysis of multi-component herbal medicines (Kong *et al*., 2008; Kong *et al*., 2009). Nevertheless, traditional Chinese medicine is a complex mixture and the chemical components characterized by fingerprints cannot fully manifest its multi-therapeutic effects. Only by spectrum-effect relationships analysis methods can we provide regular basis for quality control and efficacy evaluation standard of traditional Chinese medicine. This study was conducted to screen the potential allelochemicals by analyzing the spectrum-effect relationships between HPLC fingerprints and autotoxicity of Danshen aqueous extracts.

Materials and Methods

Materials: The roots and rhizoma of *Salvia miltiorrhiza* Bge. (RSM), collected from Germplasms Nersery of Shandong University of TCM (SDCM), China, (specimens , voucher No. R 201501) in March 2014, were air-dried, finely powdered and used as donor agents. While its seeds were collected as receptor agents. Voucher specimens were deposited in the herbarium of Shandong university of TCM (SDCM). Standards of Rosemary acid and Salvianolic Acid B with purity not less than 98% were obtained from the Yuanye biological technology company (Beijing, China). All chemical reagents were analytical grade and purchased from Tianjin Fu-Yu Chemical Reagent Factory.

HPLC fingerprints chromatographic conditions: Chromatography was performed on Agilent 1260 series HPLC (Agilent Corporation, USA) equipment with a ZORBAXSB-C_{18} column (250mm×4.6mm, 5μm) at 25°C. The mobile phase consisted of acetonitrile (A) and 0.026% aqueous phosphoric acid (B) in a gradient elution mode was as follows: 0-15 min, 17%-23%A; 15-30 min, 23%-25%A; 30-40 min, 25%-90% A; 40-50 min, 90 % (A). The flow rate was 1.0 mL·min^{-1} and the detection wavelength were set at 286nm.

Preparation of standard and sample solutions: An accurately weighed amount of Rosemary acid and Salvianolic Acid B was dissolved in methanol to obtain a final concentration of 15, 160 μg·mL^{-1}, respectively.

The medicinal powder was macerated in water for 48h. After being centrifuged and thermally sterilized, the filtered aqueous extracts was eventually distilled to a serial dilution of 0.02, 0.04, 0.06, 0.08 and 0.1 g·mL^{-1} concentrations, and then filtered through 0.22 μm microporous membrane for HPLC fingerprints.

Validation of methodology: According to the established HPLC programs, method precision and repeatability was evaluated by five successive injections and five replicates of 0.04 g·mL^{-1} concentration solution, respectively. Meanwhile, storage stability of the 0.04 g·mL^{-1} concentration solution in a day (0, 2, 6, 12 and 24 h) was evaluated.

Establishment and Evaluation of fingerprints: The serial dilutions of five solutions were analyzed triplicate to get the fingerprints according to the optimized HPLC method, respectively. The relationship within chromatographic fingerprints could be currently analyzed through similarity comparison. By using the similarity evaluation system for chromatographic fingerprint of Traditional Chinese Medicine (TCM) (Version 2004A), the obtained HPLC fingerprints were automatically matched and the reference chromatogram was formed from the general comparison of the 5 batches of fingerings using the average method. Standards of Rosemary acid and Salvianolic Acid B were confirmed in HPLC fingerprint by exogenous add experiment.

Biological activity determination of autotoxicity

Seed germination and seeding growth test (Souza *et al*., 2010; Barkatullah *et al*., 2010): The solution was bioassayed at each of the five different concentrations (0.02, 0.04, 0.06, 0.08 and 0.1 g·mL^{-1}) on seeds of *S. miltiorrhiza* Bge. with distilled water as control. Each treatment was imposed with five replicates.

Seeds were surface-sterilized with 0.2% potassium permanganate solution, rinsed for three times with distilled water and then evenly sown onto 9 cm diameter Petri dishes (50 seeds/Petri dishes) which filled with two layers of previously autoclaved filter paper. Afterwards, the seeds were moistened with 5 mL of each different concentrations solution according to different treatments respectively, and then incubated in an incubator at 25±1 °C under a 12-hour photoperiod for 9 days. During incubation period, dishes were remoistened with responsible solution when required. The germinated seeds were recorded and removed at intervals of 24 h till 7 DAYS and the germination traits (n=50), including germination rate, vigor, and index were calculated (Li *et al*., 2013; Yadav & Singh, 2013).

Seedlings germinated previously were transferred to plastic pots filled with sterilized quartz sand (20 seedlings/50 g quartz sand). Those pots were sprayed with 10 mL of each of five solutions (distilled water for control group) accordingly and were maintained with natural solar radiation at an average temperature of 25±5°C for 7 days. When required, those pots were sprayed with corresponding solution to keep the sand moist. The growth indexes, such as root and shoots length, total seedling height which was calculated as the sum of root and shoot, fresh and dry seedling weigh were all measured (n=50).

Autotoxicity indexes evaluation: The autotoxicity indexes evaluation was calculated by the formula (Abdelgaleil & Hashinaga, 2007): $IR\ (\%) = [T/C\text{-}1] \times 100\%$, where T is the value of tested item and C is that of blank control group and IR value for different groups was presented as mean±standard deviation (SD) (n=20) and the significant differences among treatment groups were examined by ANOVA and then analyzed using Duncan's multiple range tests at $p<0.05$ level.

Autotoxicity effects evaluation with principal component analysis (PCA): Principal components analysis (PCA) is popularly regarded as a sophisticated tactic for reducing the dimensions of multivariate variations (Kong *et al*., 2009). Therefore, this statistical method was applied to select several main factors which have no correlation to represent all the variables and create variables that are linear combinations of the original variables.

Analysis of spectrum-effect relationship: Grey relational analysis was applied to analyze spectrum-effect relationship between the average peak areas of 18 common peaks (A1 to A18) in HPLC and comprehensive allelopathic effect scores (Z) from PCA analysis by SPSS statistics software (SPSS for Windows 19.0, SPSS Inc., USA).

Results

Validation of methododology and HPLC fingerprints: HPLC methodology results showed that the relative standard deviation (R.S.D.) for method precision and reproducibility, alone with storage stability of sample solutions within 24 h appeared less than 3.00 % both for relative retention time and average peak areas of common peaks (n=5).

The typical HPLC fingerprints of the five batches of aqueous extracts from Radix Salviae Miltiorrhizae were showed in Fig. 1 and the generated reference standard fingerprint was shown in Fig. 2. Eighteen peaks with good separation were picked out as common peaks. The known one, marked peak sixteen, demonstrated the most abundance with satisfactory baseline separations at an average retention time 26.09 min was Salvianolic Acid B and was defined as the reference peak to calculate the average relative retention time (RRT) of other 17 common peaks. The relative retention time and average peak areas of each characteristic peak were shown in Table 1. Similarities between entire chromatographic profiles of 5 sample solutions and the reference fingerprint were 0.930, 0.959, 0.965, 0.944, and 0.998, respectively.

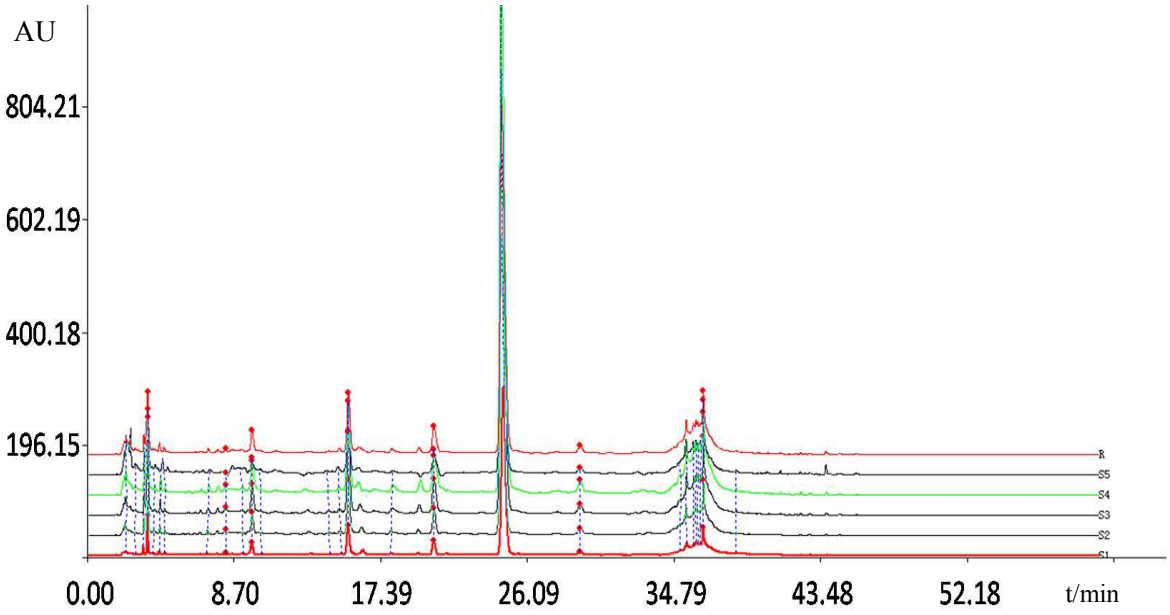

Fig. 1. 5 batches HPLC fingerprints of RSM aqueous extracts and the reference atlas.

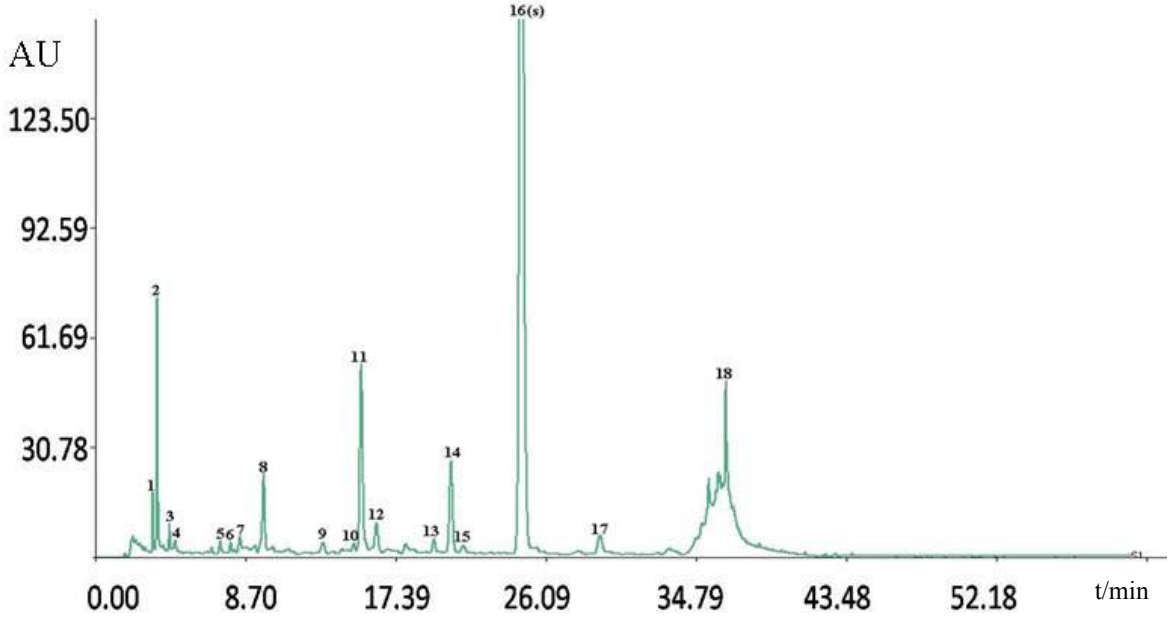

Fig. 2. The reference standard fingerprint from 5 batches aqueous extracts fingerprints of RSM.
Peak 16 was Salvianolic Acid B defined as the reference peak(s) to calculate the average relative retention time (RRT) of other 17 common peaks.

Results of Biological activity determination of allelopathic test: Seed germination and seedling growth test results (Table 2) show that the five batches of aqueous extracts derived from *S. miltiorrhiza* Bge. exhibited significantly different autotoxic effects with each other (p 0.05).The PCA results demonstrated that the first two principal components (Z1, Z2) contained 85.06% of the information of the original eight indexes from the results of "Eigenvalue of the Correlation Matrix", the equations of which were:

$Z1=0.117X_1+0.274X_2+0.223X_3+0.424X_4+0.416X_5+0.420\ X_6+0.407\ X_7+0.408\ X_8$

$Z2=0.601\ X_1+ 0.367X_2+0.584X_3\ \ 0.218X_4\ \ 0.241X_5\ \ 0.238\ X_6\ \ 0.01X_7\ \ 0.02\ X_8$

In this study, X1-X8 were the standardized *IR* value of root length, shoots length, total seedling height, germination rate, index, vigor and *IR* value of fresh and dry weight of seedling for the PCA.

The comprehensive model was established by the formula: $Z=\lambda_1\times Z1/(\lambda_1 \ \lambda_2) +\lambda_2\times Z2/(\lambda_1 \ \lambda_2)$, where λ_1 and λ_1 were the eigenvalues of the first two principal components. Finally, the comprehensive allelopathic effect scores, named Z values for 0.02, 0.04, 0.06, 0.08, 0.1g·mL^{-1} concentrations groups were 30.24,-3.89,-9.79,-6.33,-10.25, respectively.

Correlation analysis results for HPLC fingerprints and autotoxicity effects of aqueous extracts derived from *RAM*: Grey relational analysis was used for the spectrum-effect relationships by calculating the relational degree between area values of 18 common peaks (A1 to A18) in the HPLC and the comprehensive allelopathic effect scores (Z) from PCA analysis. The relational degree (Table 3) between the common peaks and allelopathic effect scores demonstrated that allelopathic effect of aqueous extracts from the RSM had a close correlation with peaks 6, 18, 4, 5, 10, 9, 1, 7, 15 and 12 of the HPLC fingerprints.

Discussion

A range concentration of aqueous extracts (0.001, 0.01, 0.1 g·mL^{-1}) from RSM with distilled water as blank were preliminary performed seed germination and seedling growth tests for optimum concentration selection. Results showed that only 0.10 g·mL^{-1} concentration group has significant difference with the other ones. In view of the viscosity of RSM aqueous extracts, increasing extracts concentration will enhance suction filtration difficulty, so 0.02, 0.04, 0.06, 0.08 g·mL^{-1} concentration gradient was set for spectrum-effect study.

Among hundreds of identified allelochemicals (Peng *et al*., 2004), phenolic acids were most frequently mentioned as putative ones and possess a broad range of phytotoxicities. Although their inhibitory effects still uncertain, Rosemary acid and Salvianolic Acid B have been long recognized two main active phenolic acids in RSM aqueous extracts. In addition, among peaks of HPLC fingerprints, Salvianolic Acid B has the largest peak area and good separation, so it was appropriate to be chosen as standards.

Eight indexes in seed germination and seeding growth tests were carried out to evaluate the autotoxicity effect of RSM aqueous extracts comprehensively. In order to reduce the dimensionality of the original data, PCA statistical method was applied to simplify these indexes. The relationship between comprehensive allelopathic effect scores and corresponding extracts concentrations demonstrated the fact that within a range of concentration allelopathic effect presents low suppression and high promoting trend.

In fact, most efforts on screening potential allelopathic compounds have been done since the 1940s and hundreds of allelochemicals which were classified into 14 categories based on their diversiform chemical structures have been identified (Peng *et al*., 2004). However, how to screen and purify them quickly is still a big challenge. In this study, a new mode for autotoxins exploring was established. With the bond of grey relational analysis, the relationship base on relevancy between HPLC fingerprints and autotoxicity of RSM aqueous extracts were analyzed and potential autotoxins represented by peaks of 6, 18, 4, 5, 10, 9, 1, 7, 15, 12 in HPLC fingerprints were picked out. Simultaneously, the correlations between the remaining peaks area and allelopathic effect were all more than 0.5 indicating the fact that those compounds also devoted certain contribution to autotoxicity of RSM. In the past decades, literatures on allelopathy (Olofsdotter *et al*., 2002) have supported the hypothesis that mixtures of phenolic acids and other organic compounds possess function additivity even though the concentrations of individual compounds are well below their inhibitory levels when comes to phytotoxic affects evaluating. That means autotoxicity was interactions results of involved chemicals groups. Significant knowledge gaps still remained in our understanding on interact action mode of involved chemicals. Future studies may focus on purifying those compounds and deeply discuss interactions mode of those components.

Table 1. The relative retention time and peak area of every common characteristic peak.

Peak No	RRT	Average peak area of common peaks from five batches fingerprints				
		1	2	3	4	5
1	0.14	74.8	153.1	165.8	210.6	231.7
2	0.16	365.0	790.7	1109.4	1530.3	1790.1
3	0.19	57.6	152.6	173.1	266.0	332.5
4	0.20	34.8	39.9	35.1	40.9	42.6
5	0.32	29.0	89.9	153.5	170.2	188.6
6	0.34	23.3	126.1	178.2	213.2	254.1
7	0.36	44.3	109.7	191.5	224.4	262.9
8	0.42	213.7	420.0	675.0	898.6	1196.3
9	0.55	48.0	152.8	187.5	238.2	278.5
10	0.62	36.2	98.6	179.6	202.0	227.2
11	0.65	648.4	1263.2	1919.8	2406.8	3008.5
12	0.68	118.5	216.6	319.9	394.0	486.6
13	0.82	66.0	114.4	283.5	318.8	357.9
14	0.86	39.3	662.2	1089.3	1518.1	2115.7
15	0.88	39.2	86.6	168.5	208.5	244.0
16	1.00	4988.2	10442.6	15646.9	20712.3	27417.5
17	1.20	101.6	219.4	343.0	480.3	672.1
18	1.47	395.6	2396.2	3274.9	4089.9	5107.7

Table 2. Autotoxic effects of five different aqueous extracts from RSM on seed germination and seedling growth.

Treatments ($g \cdot mL^{-1}$)	RI of Seed			RI of Seedling				
	Germination rate	Germination vigor	Germination index	Root lengh	shoots length	Seedling height	Fresh weight	Dry weight
0.02	-0.37±0.101b	-0.67±0.103b	-0.61±0.08b	-0.73±0.09b	-0.03±0.11a	-0.48±0.09b	-0.22±0.03b	-0.22±0.05b
0.04	-0.09±0.04c	-0.92±0.07c	-0.90±0.06c	-0.86±0.06bc	-0.11±0.11a	-0.64±0.07c	-0.41±0.02c	-0.38±0.03c
0.06	-0.05±0.02cd	-0.97±0.01c	-0.95±0.02cd	-0.94±0.02c	-0.72±0.26b	-0.87±0.09d	-0.55±0.02d	-0.48±0.01d
0.08	-0.01±0.01d	-0.99±0.01c	-0.99±0.01d	-0.93±0.01c	-0.81±0.27b	-0.95±0.01d	-0.62±0.06e	-0.51±0.03d
0.10	-0.05±0.35d	-0.99±0.03c	-0.99±0.02d	-0.92±0.01c	-0.82±0.32b	-0.88±0.101d	-0.66±0.02e	-0.53±0.05d
F	126.61	122.38	246.83	150.99	49.75	134.71	254.27	153.62
P	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05	＜0.05

The values denoted by different letters within the same column represent significant difference at 0.05 level as compared with the various concentration treatment groups

Table 3. The correlation relationship between comprehensive allelopathic effect scores (Z) and common characteristic peaks.

Relational order	Peak No	Relational degree	Relational order	Peak No	Relational degree
1	6	0.963	10	12	0.613
2	18	0.889	11	3	0.589
3	4	0.783	12	13	0.588
4	5	0.725	13	17	0.588
5	10	0.690	14	14	0.586
6	9	0.654	15	11	0.584
7	1	0.640	16	2	0.556
8	7	0.637	17	8	0.555
9	15	0.632	18	16	0.552

Conclusion

In short, the importance of spectrum-effect relationships analysis method can provide a novel perspective model for possible bioactive components screening from complex mixtures.

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