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Research Article

Pharmacological Study of Carbonized Scutellaria Radixin Treating Abnormal Uterine Bleeding by Spectrum-Effect Relationship

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Abstract

Background and Objective: Carbonized Scutellaria Radix (Carbonized Huangqin) is a kind of processed products of *Scutellaria baicalensis*, which was reported to possess the therapeutic effect of gynecological hemorrhage but the material basis is not clear. The purpose of this study was to study the anti-abnormal uterine bleeding (AUB) effect and chemical markers of Carbonized Huangqin.

Materials and Methods: In this study, 15 batches of *Scutellaria baicalensis* collected from different origins were processed into carbonized forms. The HPLC-DAD fingerprint was developed by the optimized chromatographic condition, similarity analysis (SA) and principal components analysis (PCA) were used to evaluate the different samples. Experiment model of abnormal uterine bleeding (AUB) in rats was established to estimate the effect of Carbonized Huangqin. Gray relational analysis (GRA) and orthogonal partial least squares discrimination analysis (OPLS-DA) were applied to analyze the spectrum-effect relationship between HPLC fingerprints and haemostatic effect of Carbonized Huangqin on AUB in rats. **Results:** The similarities of all samples were more than 0.9. Spectrum-effect relationship revealed that peak 13 (wogonin), 14 (chrysin), 15 (oroxylin-A), 5 (baicalein-7-O- β -D-glucoside) and 6 (baicalin) were related to haemostatic effect, they were arranged in the following order: wogonin>chrysin>oroxylin-A>baicalein-7-O- β -D-glucoside>baicalin.

Conclusion: Wogonin, chrysin, oroxylin-A might be the most likely chemical markers of Carbonized Huangqin in the treatment of AUB.

Key words: Carbonized Huangqin, HPLC-DAD, haemostatic effect, spectrum-effect relationship, chemical markers, quality control

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Data Availability: All relevant data are within the paper and its supporting information files.

INTRODUCTION

Scutellaria Radix (Huangqin) is the root of *Scutellaria baicalensis* Georgi and is a frequently-used Chinese medicine belonging to Labiatae and mainly distributed in Shan Xi, Gan Su, NeiMeng, HeBei, in China¹. Carbonized *Scutellaria Radix* (commonly named Carbonized Huangqin) is one kind of processed products of Huangqin. The purpose of processing into carbon is to facilitate the clinical use of gynecological hemorrhage in the guidance of the theory of Traditional Chinese Medicine (TCM)², Carbonized Huangqin usually considered to have the effect of haemostasis and preventing miscarriage³.

Abnormal uterine bleeding (AUB) is a gynecological disorder that usually characterized as a symptom of excessive, prolonged uterine bleeding⁴, which significantly affect the patient's life quality⁵. Previous researches revealed that water extract of Carbonized Huangqin showed a good therapeutic effect on rats of AUB induced by mifepristone and misoprostol⁶. But the material basis of haemostasis of Carbonized Huangqin has not been reported.

High performance liquid chromatography (HPLC) combined with diode array detection (DAD) chromatographic fingerprint has merits such as more convenient, high precision, stability, easy operated and abundant information⁷, Chromatographic fingerprint not only determines the active compounds in processed herbs but also reveals the inherent relationships between multiple compounds⁸. Spectrum-effect relationship can be used to screen active components of TCM based on the results of active study, which has been applied to study the material basis of TCM⁹. The methods of similarity analysis (SA), principal components analysis (PCA), Gray relational analysis (GRA) and orthogonal partial least squares discrimination analyze (OPLS-DA) were commonly used to evaluate the quality of TCM and analyze the chemical differences among different samples^{10,11}. But HPLC-DAD fingerprints were not yet built and the material basis of haemostasis of Carbonized Huangqin has not been reported. So, the model of AUB in rats was established to evaluate the haemostasis effect of Carbonized Huangqin in this study. HPLC-DAD fingerprint and statistical methods include SA, GRA and OPLS-DA used to analyze the active ingredient combined with the haemostasis effect. The results showed that wogonin, chrysin and oroxylin-A are closely related to the haemostatic effect. The aim of this work was to provide a scientific basis for comprehending the material basis of processed products of Carbonized Huangqin.

MATERIALS AND METHODS

The study started in March, 2018 and ended in August, 2019. All the experiments were carried out in Anhui University of Chinese Medicine and Anhui Province Key Laboratory of Traditional Chinese Medicine Decoction Pieces of New Manufacturing technology.

Instruments: The chromatographic determination was performed on an Agilent HPLC-DAD system (Agilent-1260, USA) coupled with an LC-work station. An Agilent HC-C18(2) column (250×4.6 mm, 5 μm) was used to separate the components in the carbonized Huangqin. A rotary evaporator (EYELA, Japan) and an ultrasonic cleaner (Kunshan, China) were also used to extract the components.

Chemicals and plant materials: Standard substances of Baicalein-7-O-β-D-glucoside, Baicalin, Wogonoside, Baicalein, 5,6-hydroxy-7,8,2',6'-tetramethoxy flavone, wogonin, chrysin, oroxylin-A were isolated from the Radix of *Scutellaria baicalensis* in the laboratory of China Academy of Chinese Medical Sciences.

HPLC grade methanol (Merk, German) and analytical grade formic acid were used in the HPLC analysis. Water for HPLC analysis was purchased from Wahaha Company (Hang Zhou, China).

Fifteen batches of the raw *Scutellaria baicalensis* were collected from Neimeng, Hebei, Shanxi and Gansu provinces in China and identified by Professor Huasheng Peng (Resource Center of Traditional Chinese Medicine, Anhui University of Chinese Medicine), the certificated specimens were stored in the college of pharmacy, Anhui University of Chinese Medicine. Sample information was shown in Table 1. All samples of Carbonized Huangqin were processed into carbon under the guidance of Chinese Pharmacopoeia¹². And tabella eclinopod was selected as positive drug and were purchased from Huiyinbi Pharmaceutical Co. (Huoshan, Anhui, China).

Animals: Female (280~300 g) and male (300~320 g) SD (Sprague Dawley) rats (Certificate No. SCXK2018-003) were purchased from Experimental Animal Center of Anhui Medical University (Anhui, China).

All animals were kept at 26±1 °C under 12/12 h dark and light cycles (lights on 6:00 am) with free access to standard laboratory food and water. All animal experiments were approved by the Anhui University of Chinese Medicine on Ethics in the Care and Use of Laboratory Animals (LLSC20160336).

Table 1: Origin information of the carbonized *Scutellaria baicalensis*

Number	Growing area	Number	Growing area
S1	Gansu	S9	Hebei
S2	Gansu	S10	Neimeng
S3	Gansu	S11	Neimeng
S4	Gansu	S12	Shan'xi
S5	Gansu	S13	Shan'xi
S6	Hebei	S14	Shan'xi
S7	Hebei	S15	Shan'xi
S8	Hebei		

Table 2: Gradient elution conditions of mobile phase

Time (min)	Phase A (%)	Phase B (%)
	methanol	0.1% formic acid solution
0.01	25	75
10	35	65
20	45	55
30	50	50
60	55	45
65	80	20

HPLC-DAD fingerprints

Chromatographic conditions: According to the results of pre-experiment, the chromatographic conditions were as follows: The mobile phase consisted of methanol and 0.1% formic acid in water. The optimized gradient elution program was shown in Table 2. All of the components were separated by an Agilent HC-C18 column (250×4.6 mm, 5 μm). The solvent flow rate was 1.0 mL min⁻¹ and the injection volume was 10 μL. The column temperature and detection wavelength were maintained at 30 and 280 nm, respectively. All samples were filtered through a 0.22 μm membrane filter before use.

Preparation of the standard solution: Refer to Li's method¹³, 8 standard substances (Baicalein-7-O-β-D-glucoside, baicalin, wogonoside, baicalein, wogonin, 5,6-hydroxy-7,8,2',6'-tetramethoxy flavone, chrysin, oroxylin-A) were precisely weighed and placed into a volumetric flask and methanol (25 mL) was added into the flask. The concentration of the 8 reference compounds were 0.048, 0.13, 0.24, 0.23, 0.12, 0.013 and 0.11 mg mL⁻¹, respectively.

Preparation of sample solutions: Similarly, refer to Li's method¹³, the sample of Carbonized Huangqin was crushed into a powder using an electric grinder. The obtained powder (0.5 g) was accurately weighed and refluxed extracted twice with 50% ethanol (25 mL) and each extraction time was 1 h. The extract solutions were combined and transferred into a 100 mL flask, 50% ethanol was added to scale. All solutions were filtered with 0.22 μm before use.

Analysis of HPLC fingerprints

Methodological studies: The optimal chromatographic condition was developed by comparing different

chromatographic columns and mobile phase. The sample solution was injected into the HPLC system for 6 times to evaluate the precision of the equipment. Six independent sample solutions from the same batch were prepared and analyzed for repeatability. The stability of the sample solution was also analyzed in triplicate every 4 over 24 h. All values of RSD% were calculated by the peak areas of the eight standard compounds⁹.

SA of fingerprints: Each sample solution of Carbonized Huangqin from different origins was extracted by 50% ethanol and the standard solution were injected into the HPLC system to obtain the HPLC fingerprints. HPLC-DAD fingerprints were manual-matched and established by the similarity evaluation system for chromatographic fingerprint of traditional Chinese medicine recommend by Chinese Pharmacopoeia Committee (Version 2012A) (Beijing, China). Representative reference fingerprint was formed according to the results of the matching results⁹.

Identification of chromatographic peaks: The standard solution and sample solution were injected into the HPLC system, respectively. Partial peaks in the sample solution were identified according to the retention time of each standard substance⁹.

OPLS-DA of different samples: Fifteen peak areas without any preprocessing were selected as input data imported into SIMCA-P 14.0 software. OPLS-DA was used to discriminate the difference of different samples and S-plot was used to predict the potential chemical markers to evaluate the quality of Carbonized Huangqin⁹.

Experiment model of abnormal uterine bleeding in rats: In the part of the animal experiments, Li's and Wang's methods were referred^{14,15}. Female rats in estrus state were selected and copulated with male rats. Female and male rats were coupled in a separate cage overnight and the ratio of female and male rats was 2:1. Vaginal plug or spermatozoid was detected by vaginal smears on the next morning. The presence of either was selected as the evidence of breeding, the day on which was considered as the 1st day of pregnancy. On the 7th day of pregnancy, the rats with AUB were established by intragastric administration of mifepristone (12.5 mg kg⁻¹) and misoprostol (135 μg kg⁻¹) at 8 am and 6 pm, respectively. The model rats were randomly divided into model group, positive control group (0.45 g kg⁻¹), 15 drug experimental groups (1.10 g kg⁻¹). Non-pregnant female rats

were selected as the blank group and were given physiological saline of the same volume as drug groups. There were 8 rats in each group. A weighted cotton ball (85~100 mg) was placed in the vagina of each abortive rat at 8 am and it was replaced by an unused cotton ball at 6 pm every day, the bloody cotton balls were put into an EP tube and stored at 4. The operation was repeated for 7 days. Rats in each group were given extraction of Carbonized Huangqin on the 8th day for 7 days. The normal and the model group were given an equal volume of distilled water for 7 days.

Measurement of bleeding volume (BV): The measurement of the bleeding volume was referred to Zhang's method¹⁶. A 20 μ L venous blood from tail vein was taken from each female rat in each group, then 4 mL of 5% NaOH (V1) was added into the blood. The cotton ball containing uterine bleeding from each female rat was collected and transferred into a beaker. According to the quantity of the bleeding, a suitable amount of 5% NaOH solution (V2) was added to soak and wash the blood. The washed solution was then transferred to another beaker. The processes mentioned above were repeated 2 or 3 times till the washed solution was colorless.

The absorbance of the venous blood solution (20 μ L) and soaked blood solution were both measured using UV-spectrophotometer under the wavelength of 546 nm. The bleeding volume was calculated as the follow formula:

$$\text{Bleeding volume (mL)} = \frac{\text{Venous blood (0.02 mL)} \times \text{the A of washed solution}}{\text{V2 the A of venous blood} \times \text{V1}}$$

Levels of TNF- α , IL-1 β and SOD: The determination of TNF- α , IL-1 β and SOD were referred to Huang's method⁶. Rats in each group were anesthetized and the abdominal aorta blood was collected into the EP tubes and then centrifuged under 2000 rpm min⁻¹ for 10 min to obtain serum. The serum levels of SOD, IL-1 β and TNF- α were assessed using the respective ELISA kits, according to the manufacturer's instructions.

Statistical analysis: The results of bleeding volume and the levels of SOD, IL-1 β and TNF- α in each group were analyzed by SPSS software (22.0, USA). And the result of spectrum-effect relationship was estimated by SIMCA-P software (14.0 Umetrics) with PCA and OPLS-DA. The data were presented as the mean $\pm$ standard deviation. One-way ANOVA was used to perform the statistical analysis, the significance of the difference between each group were determined by LSD tests. The $p < 0.05$ or 0.01 were considered statistically significant.

Analysis of spectrum-effect relationship

GRA: GRA is a commonly used analytical method to reveal the correlation coefficient between various factors. All of the data of peak areas and the pharmacodynamics indexes were normalized by the method of equalization before GRA. The data of 15 common peak (X1-X15) areas were selected as the subsequence $X_i(k)$ and the data pharmacodynamic indexes (BV, TNF- α , IL-1 β , SOD) were defined as parent sequence $Y(k)$. The coefficient of correlation (r) was calculated according¹⁷ to formula 1, 2 and 3:

$$\Delta i(k) = |Y(k) - X_i(k)|$$

$$A_i(k) = \frac{\min \Delta i(k) + \rho \times \max \Delta i(k)}{\Delta i(k) + \rho \times \max \Delta i(k)}$$

Where:

$$\begin{aligned} i &= 1, 2, 3, \dots, n \\ k &= 1, 2, 3, \dots, m \end{aligned}$$

OPLS-DA: The ratio of reduction of bleeding volume, the levels of TNF- α and IL-1 β and the ratio of rise of the level of SOD were selected as Y variable, the peak areas of 15 common peaks were selected as X variable and OPLS-DA was used for assessing the correlation between the value of peak areas (X) and the pharmacodynamics (Y) of the 15 peaks by SIMCA 14.0. The regression coefficient was used to reveal the relative influence of the predictor variables on the dependent variable.

RESULTS

Results of HPLC-DAD fingerprint

Methodological studies: The values of RSD% of the precision, repeatability and stability test were all less than 3%. It indicated that the established HPLC-DAD fingerprint was suitable for Carbonized Huangqin analysis.

HPLC-DAD fingerprints and identification of chromatographic peaks:

15 batches of Carbonized Huangqin samples were determined by the developed chromatography condition as shown in Fig. 1a. The reference fingerprint (Fig. 1b) was generated by Similarity Evaluation System for Chromatographic Fingerprint of Traditional Chinese Medicine and 15 common peaks were identified. Peak 11 with retention time was 39.2 min was selected as the reference peak for its good degree of separation and stability. The retention time and peak areas of 15 peaks were shown in Table 3.

Peak 5, 6, 9, 11, 12, 13, 14 and 15 were identified as Baicalein-7-O- β -D-glucoside, baicalin, wogonoside, baicalein,

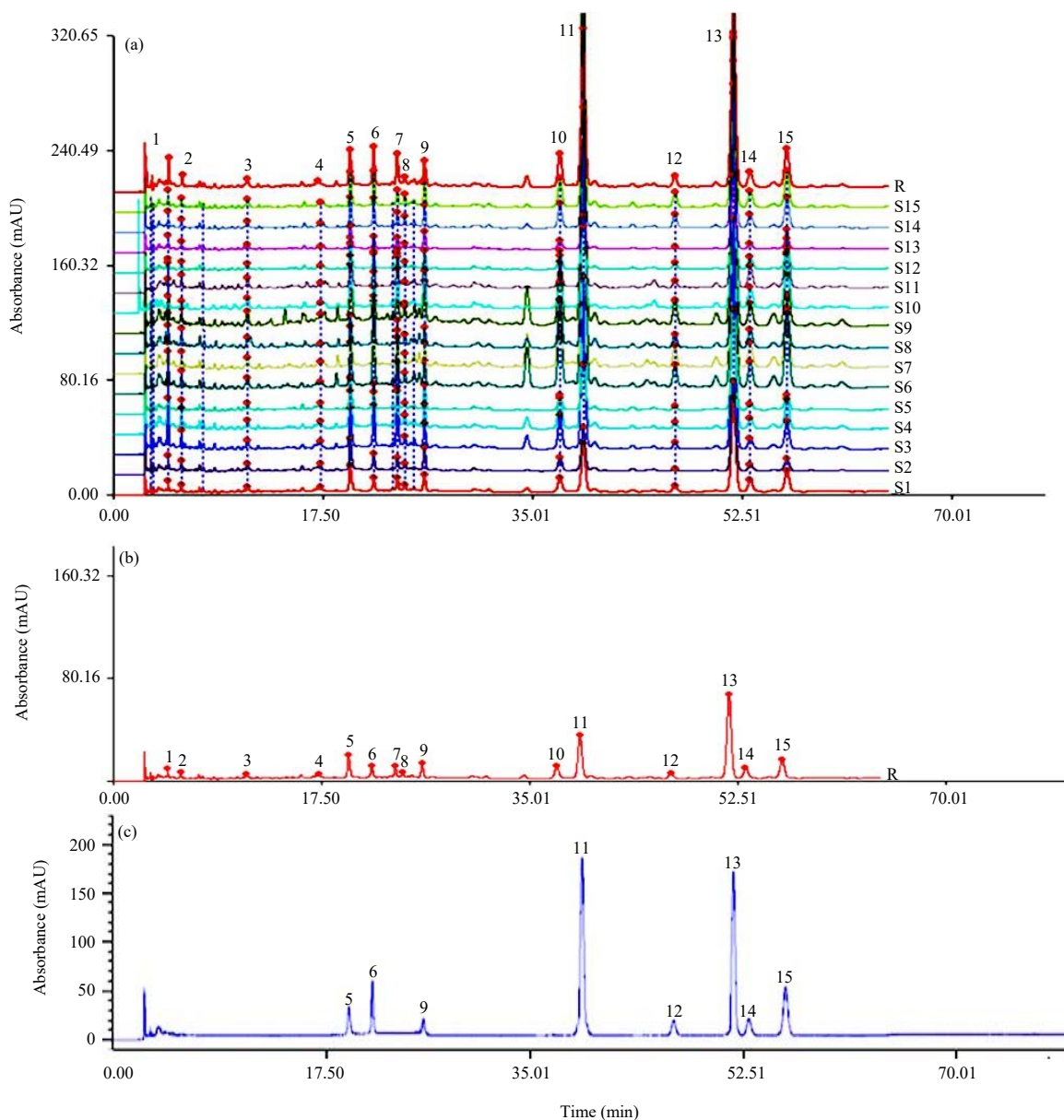


Fig. 1(a-c): HPLC-DAD chromatography, (a) Fingerprint of 15 batches of Carbonized Huangqin, (b) Reference fingerprint of Carbonized Huangqin and (c) Chromatography of 8 mixed reference substances

Numbers 1-15 represent the common peak number, 5: Baicalein-7-O- β -D-glucoside, 6: Baicalin, 9: Wogonoside, 11: Baicalein, 12: Wogonin, 13: 5,6-hydroxy-7,8,2',6'-tetramethoxy flavone, 14: Chrysin, 15: Oroxylin-A

wogonin, 5,6-hydroxy-7,8,2',6'-tetramethoxy flavone, chrysin, oroxylin-A, respectively by comparing with the HPLC chromatogram of the 8 standard substances (Fig.1c).

SA analysis of fingerprints: The similarities between all of the Carbonized Huangqin samples and the reference chromatography were calculated. As shown in Table 4, all of the similarities were more than 0.9, which indicated that the

quality of the Carbonized Huangqin sample was identical and the technology of processing was stable.

OPLS-DA of different samples: According to the results of OPLS-DA, 15 batches of Carbonized Huangqin were artificially divided into 4 categories, mean while these samples were fairly divided into 3 groups (Fig. 2a) by SIMCA-P software.

Table 3: Retention time and peak areas of 15 batches of Carbonized Huangqin

Numbers	Retention time (min)	Peak area (mAU)														
		S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	S13	S14	S15
P1	4.54	40.98	58.39	237.74	281.51	13.59	158.80	151.52	122.21	232.07	27.60	24.40	41.70	49.50	59.08	62.61
P2	5.64	41.16	55.85	92.73	99.31	12.56	86.69	80.96	89.22	87.93	54.66	58.79	45.30	46.46	55.07	56.63
P3	10.37	65.25	21.35	21.61	33.49	30.21	30.99	30.52	33.02	21.99	41.04	38.25	23.43	23.22	18.57	21.11
P4	16.91	14.98	27.30	30.88	29.11	29.79	66.65	59.70	13.61	59.45	37.81	37.19	43.41	21.80	51.74	60.72
P5	19.75	252.52	313.86	412.43	381.41	312.87	492.36	423.77	323.05	630.99	531.47	510.66	253.85	222.83	304.28	349.01
P6	21.71	119.27	149.26	345.57	352.40	152.26	732.91	645.01	436.02	707.98	311.61	309.99	130.47	107.34	251.22	311.27
P7	23.70	105.50	95.88	516.18	549.31	141.63	407.66	365.35	117.49	521.34	294.74	286.64	114.04	87.45	116.03	125.80
P8	24.36	44.82	36.68	79.73	79.00	41.92	90.41	72.66	76.34	97.53	28.92	17.04	42.27	26.55	73.16	85.96
P9	25.97	151.07	214.74	224.99	207.96	226.87	228.01	214.37	177.14	354.58	334.11	330.89	164.92	162.69	200.98	242.36
P10	37.27	238.58	219.93	571.13	578.59	219.51	1152.02	1076.52	847.84	1173.94	428.06	427.81	240.84	157.78	427.15	511.22
P11	39.22	796.18	713.62	2376.94	2495.07	746.06	6729.51	6383.59	3092.31	7529.54	2117.37	2267.26	702.83	548.58	1390.04	1627.47
P12	46.87	108.84	48.14	103.46	105.17	61.92	376.95	349.62	224.96	409.68	114.14	109.43	109.64	38.20	237.12	266.26
P13	51.76	1734.42	1675.10	3687.50	3856.25	1866.09	6437.08	6138.51	3850.55	6787.40	3966.06	4163.57	1760.30	1373.22	2653.84	3159.37
P14	53.16	217.93	129.04	207.25	207.179	123.52	476.53	449.54	356.55	407.25	406.07	409.95	229.31	98.44	258.44	306.97
P15	56.22	392.35	248.38	531.52	555.214	277.34	1438.66	1306.57	800.99	1308.99	1019.78	1077.89	400.88	202.16	584.04	690.27

Table 4: Results of similarity analysis

Numbers	Similarity	Numbers	Similarity	Numbers	Similarity
S1	0.975	S6	0.985	S11	0.985
S2	0.964	S7	0.985	S12	0.963
S3	0.993	S8	0.995	S13	0.962
S4	0.993	S9	0.980	S14	0.985
S5	0.960	S10	0.983	S15	0.985

What's more, peak 13, 11, 15 were far from the diagonal line according to the results of S-plot, it means peak 13, 11, 15 maybe the chemical markers affecting the quality and the effect of samples (Fig. 2b).

Pharmacological investigation

Bleeding volume: As shown in Fig. 3, the volume of bleeding of rats in model group increased significantly compared to blank group. All batches of samples significantly decreased the volume of bleeding and different batches of samples showed different haemostatic effects.

Concentrations of TNF- α , IL-1 β , SOD: According to Fig. 4, in the model group, the concentrations of TNF- α and IL-1 β in the serum of rats increased significantly ($p < 0.01$) and the concentration of SOD decreased significantly ($p < 0.01$) compared with blank group. All of the groups with medication could increase the concentration of SOD and decrease the concentrations of TNF- α and IL-1 β in serum ($p < 0.05$ or $p < 0.01$). It showed that the contentions of inflammatory factors in model rats could be regulated by Carbonized Huangqin, which indicated that the mechanism of haemostatic effect was related to the prevention of inflammation.

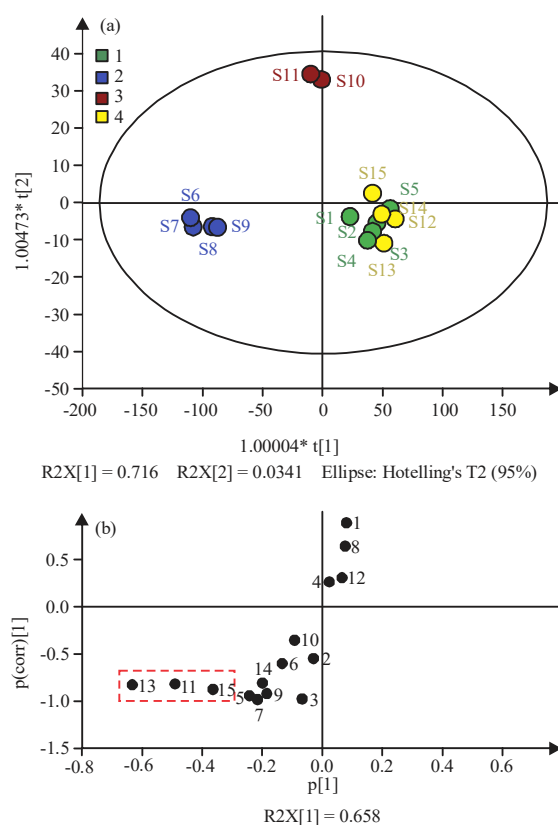


Fig. 2(a-b): OPLS-DA of 15 batches of samples (a) OPLS-DA applied to 15 batches of Carbonized Huangqin and (b) Chart S-plot Score of 15 peaks determined by HPLC)

Spectrum-effect relationship analysis

Gray relational analysis (GRA): According to formula 1-3 mentioned in "2.9.1", the values of correlation coefficient were

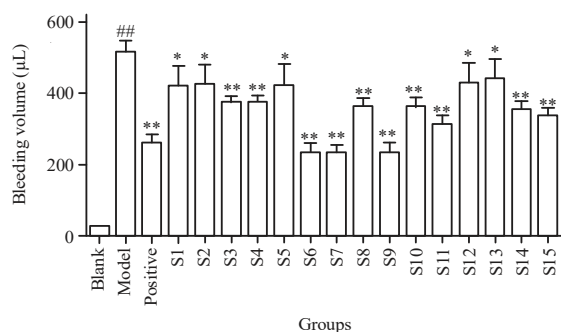


Fig. 3: Effects of 15 batches of Carbonized Huangqin on the bleeding volume in rats
Compared with blank group, ## $p < 0.01$, model group, * $p < 0.05$, ** $p < 0.01$

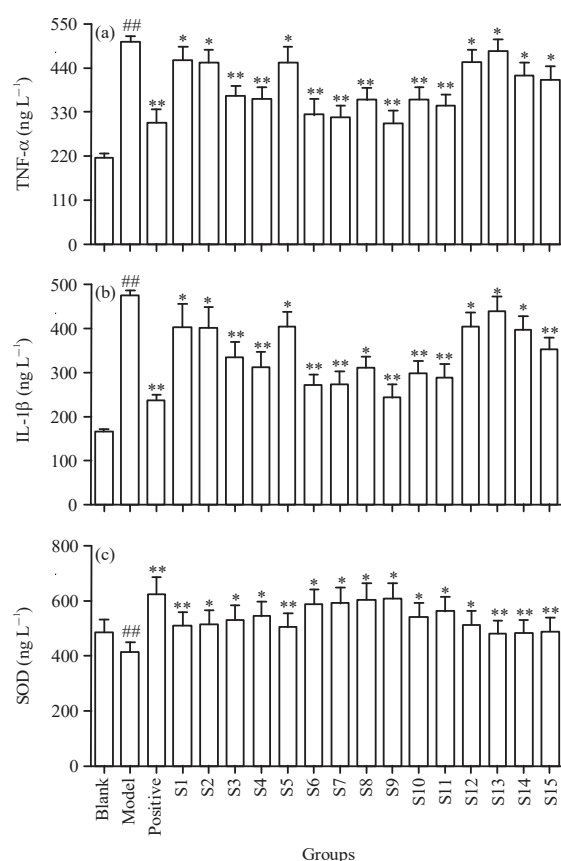


Fig. 4(a-c): Effects of 15 batches of Carbonized Huangqin on the concentrations of (a) TNF- α , (b) IL-1 β and (c) SOD in the serum of rats
Compared with blank group, ## $p < 0.01$, model group * $p < 0.05$, ** $p < 0.01$

calculated and listed in Table 5. The values of correlation coefficient of peak 13, 14, 15, 5 and 6 were more than 0.800, the order was as follows: P13>P14>P15>P5>P6, which showed high influence on inhibiting the uterine bleeding.

Table 5: Gray relational coefficient and gray relational grade of 15 peaks and their order

Numbers	Y1	Y2	Y3	Y4	Average	Order
P1	0.6950	0.7318	0.7299	0.6959	0.7132	14
P2	0.7325	0.7574	0.7505	0.7859	0.7566	9
P3	0.7023	0.7034	0.7244	0.7672	0.7243	12
P4	0.7897	0.7093	0.7089	0.7685	0.7441	11
P5	0.7974	0.7954	0.8250	0.8325	0.8126	3
P6	0.7981	0.8499	0.8109	0.7433	0.8005	5
P7	0.7523	0.7901	0.7719	0.7326	0.7617	7
P8	0.7500	0.7548	0.7654	0.7634	0.7584	8
P9	0.7603	0.7321	0.7545	0.7794	0.7565	10
P10	0.7761	0.8260	0.7909	0.7403	0.7833	6
P11	0.7284	0.7391	0.7098	0.6984	0.7189	13
P12	0.7391	0.7170	0.6940	0.6466	0.6992	15
P13	0.8685	0.8872	0.8721	0.7880	0.8539	1
P14	0.8063	0.8314	0.8324	0.7832	0.8133	2
P15	0.8296	0.8488	0.8438	0.7180	0.8100	4

Y1: Ratio of reduction of bleeding volume, Y2: Ratio of reduction of TNF- α , Y3: Ratio of reduction of IL-1 β , Y4: Ratio of reduction of rise of SOD

wogonin, chrysin, oroxylin-A, baicalein-7-O- β -D-glucoside and baicalin were considered as closely related to pharmacodynamics. What's more, peak 13 (Wogonin) showed the highest average value of correlation coefficient ($r = 0.8539$) among the compared series, which indicated that wogonin might be the most likely chemical marker of Carbonized Huangqin.

Correlation between peak areas and pharmacodynamic indices:

According to Fig. 5 all peaks were positive correlation with the haemostasis effect of Carbonized Huangqin except peak 3, it indicated that the larger peak area of peak 3 was, the worse the haemostatic effect of Carbonized Huangqin was. And, the value of correlation coefficient of peak 13 (Wogonin) was 0.3804, which was the highest. In the other word, the larger peak area of peak 13 was, the better the haemostatic effect of Carbonized Huangqin was moreover, the value of correlation coefficient of peak 15 (Oroxylin-A) was the second. When extracted two principal components $R^2 = 0.929$ (Fig. 5a), which indicated that the model was useful to indicate the strength of each sample on reducing hemostasis rate. As shown in Fig. 5f, peak 4, 8, 12 were negative correlation with the ratio of reduction of IL-1 β and peaks of 1, 2, 3, 5, 6, 7, 9, 10, 11, 13, 14, 15 had positive correlation with the ratio of reduction of IL-1 β , peak 14 (Chrysin) showed the highest value of correlation coefficient among those positive correlation peaks. When extracted two principal components $R^2 = 0.824$, indicated the prediction regression model showed as Fig. 5b is accurate. Figure 5-III revealed that peak 14 (Chrysin) showed the highest value of correlation coefficient (0.787) and the results of the OPLS model (Fig. 5c)

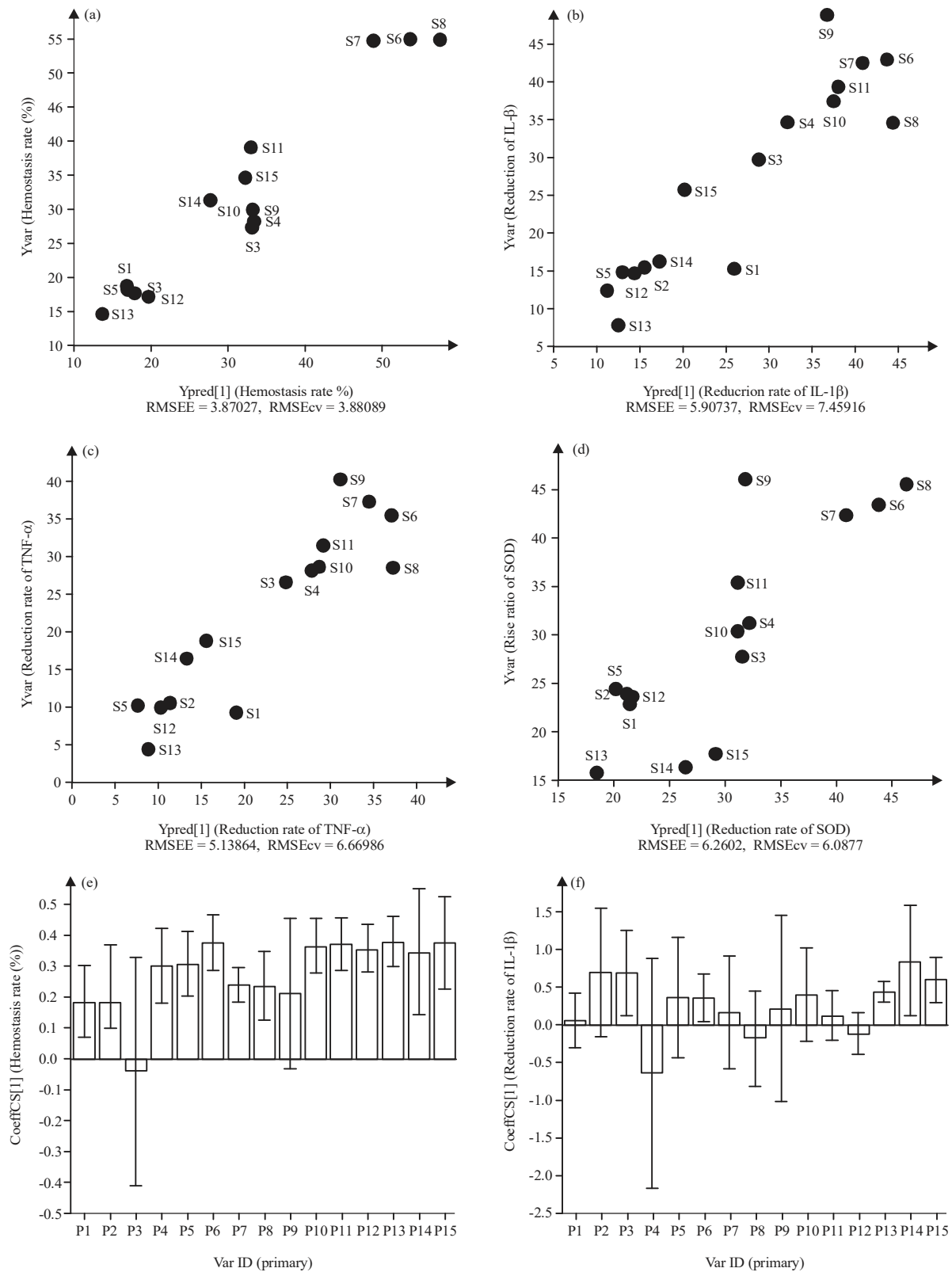


Fig. 5(a-h): Continue

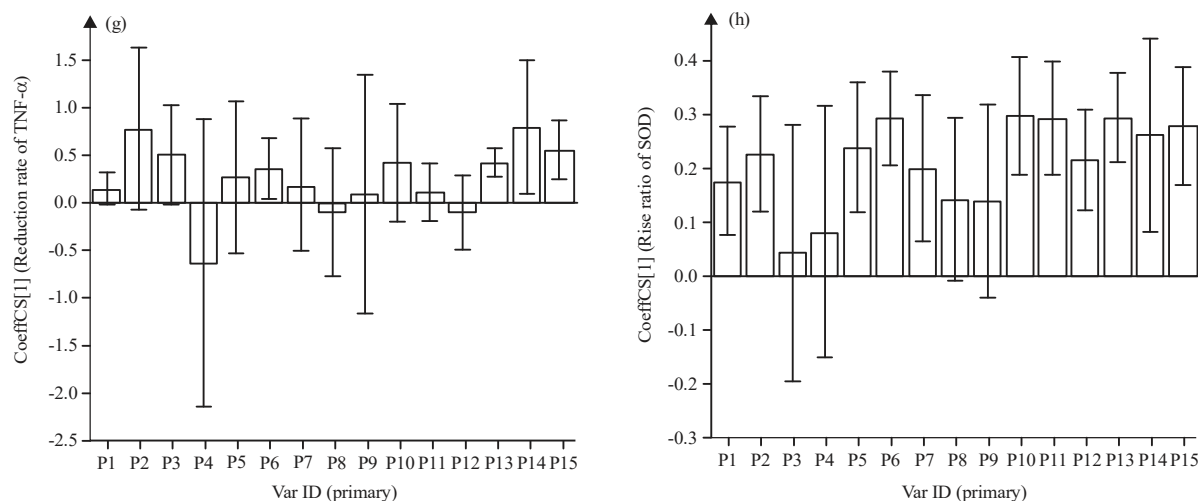


Fig. 5(a-h): Analysis of profile-efficacy by OPLS-DA, the results of (a-d) Linear regression and (e-h) Regression coefficient between 15 peaks and 4 efficacy indexes with haemostatic effect of Carbonized Huangqin

showed good linearly ($R^2 = 0.834$). Figure 5h demonstrated that peak 13 (Wogonin) had the highest value of correlation coefficient to the ratio of rise of SOD and the model of OPLS was also reasonable ($R^2 = 0.847$).

In conclusion, P13 and P14 had the largest correlation with pharmacodynamics, which was in accordance with the results of gray correlation analysis.

DISCUSSION

According to the results of pre-experiment, Agilent HC-C₁₈ column (250×4.6 mm, 5 μm) combined with acetonitrile-water and methanol-0.1% formic acid aqueous solution was selected as the optimized separation condition. The detection wavelength was 280 nm by full wavelength scanning. Moreover, the extraction solvent of Carbonized Huangqin was 50% ethanol, because more components could be extracted by this extraction method. Under the optimized separation condition all of the common peaks in Carbonized Huangqin extracted by 50% ethanol showed good separation and stability. And the HPLC fingerprint of raw Huangqin was established by Zhao *et al.*¹⁸, the components in vivo were also analysis by Tao *et al.*¹⁹. Carbonized Huangqin was one kind of processed products of Huangqin, there were few reports on its quality analysis. So the method of HPLC-DAD fingerprint of Carbonized Huangqin was firstly established in this study.

In normal state of pregnancy, low levels of TNF-α could maintain the homeostasis of the endometrial tissue, while over-expression of TNF-α and IL-1β would be against the

embryo and even caused abortion^{20,21}. Moreover, oxidative stress always existed in pregnancy and it also had many adverse effects on ovary and uterus²². The results of previous study demonstrated that the water extract of Carbonized Huangqin showed good therapeutic effect on rats of AUB and its mechanism might be related to the repairment of the endometrium and inhibition of the inflammatory reaction⁶. However, the haemostatic effect and chemical markers of the 50% ethanol extract of Carbonized Huangqin on rats of AUB had not been reported. So, the volume of uterine bleeding, concentrations of TNF-α, IL-1β and SOD in serum of rats with AUB were detected in this study to explore the therapeutic effect of the 50% ethanol extract of Carbonized Huangqin on rats of AUB. Besides, the study of spectrum-effect relationship was used to analyze the chemical markers of Carbonized Huangqin in the treatment of AUB.

The spectrum-effect relationship is a method to analyze the correlation between the chemical composition in the characteristic fingerprint and pharmacodynamic effect of TCM with software²³. OPLS is widely used in the research of spectrum-effect relationship in recent years. It could provide a regression model and the correlation coefficient between two sets of variables²⁴. According to the results of spectrum-effect relationship in this study, all the samples were fairly divided into 3 groups and the peak areas of 15 peaks chosen as the input date could be used to discriminate the quality of different samples. And wogonin, chrysin, oroxylin-A were considered as the most likely chemical marker of Carbonized Huangqin. Wogonin is a flavonoid aglycone with the highest contention in Carbonized Huangqin¹³, which had been

reported to demonstrate anti-inflammatory effects by obstructing OS and activation of NF- κ B pathway²⁵. Chrysin had the protective effect on suppressing inflammation and apoptotic tissue damage²⁶. Meanwhile, Oroxylin A showed the ability to inhibit the negative effects of TNF- α and activation of NF- κ B through PI3K/AKT pathway²⁷. Whether wogonin, chrysin and oroxylin-A had the effect of anti-AUB, it should be validated in future study.

CONCLUSION

The spectrum-effect relationship between HPLC fingerprint and anti-AUB effective of Carbonized Huangqin was reported for the first time. Wogonin, chrysin and oroxylin-A were considered as the potential chemical markers of Carbonized Huangqin. This method could be used to demonstrate the bioactive constituents of Carbonized Huangqin. Current research provided a new effective method for comprehension of the target bioactive compounds in processed products of TCM.

SIGNIFICANT STATEMENT

This study discovered the potential chemical markers of Carbonized Huangqin in treating AUB, which can be beneficial for the comprehension of the chemical markers of Carbonized Huangqin. This study will help the researcher to uncover the critical areas of the target bioactive compounds in processed products of TCM that many researchers were not able to explore.

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