

# Metabolomic profiling of the effects of *Curcumae rhizoma* and *Sparganii rhizome* on stress-led blood stasis

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**Abstract:** Blood stasis (BS) is a complex syndrome with blood flow retardation or cessation. The Traditional Chinese Medicine, *Curcumae rhizome* (CR) and *Sparganii rhizome* (SR), showed promising effects on this disease, and especially effective when used in combination. However, the detailed influence of the TCMs on the BSS disturbed metabolic pathways was still unclear. In this study, a BS model was constructed in SD rat and the TCMs were used individually or in combination to assess the effects. As a result, combination of CR and SR led to the improvement in hemorheology parameters of up to 80% in the BS model. Further analyzing using metabolomics showed several metabolic pathways, including center carbon metabolism, amino acid metabolism, etc., recovered to the normal levels after treatment. Informatively, tyrosine and thymidine exhibited potential importance in the BSS and its treatment process. From these results, the metabolic profiles of BS and the SR-CR treatment were provided, which may helpful for better understanding the BSS mechanism and the development of more effective therapies.

**Keywords:** Blood stasis (BS), Traditional Chinese Medicine (TCM), Metabolomics.

## INTRODUCTION

Blood stasis syndrome (BSS), also widely known as Xueyu in China or Oketsu in Japan, is a combination of several chronic diseases, which results in retardation or cessation of the blood flow. Mental and/or physical stress can cause BBS, which further leads to other serious diseases, including ischemic heart disease and rheumatoid arthritis (Cho *et al.*, 2012). Recently, omics technologies gave new insight into the BBS studies. Specific patterns of metabolites, mRNAs or proteins in BSS were observed, indicating the BSS might be caused by systematic abnormality in several metabolic pathways (Zhao *et al.*, 2012; Zhao *et al.*, 2014). In this regard, the Traditional Chinese Medicine (TCM), which exerts global regulation effects, might be suitable for BSS treatment (Su *et al.*, 2013). However, the detailed influence of TCM on BSS, especially the key metabolic pathway was still not clarified.

TCMs are widely used as the treatment for non-acute diseases and sub-healthy conditions. For BBS treatment, the *Curcumae rhizoma* (CR) and *Sparganii rhizome* (SR) were famous TCMs for their efficacy (Liang *et al.*, 2014; Wang and Yu, 2013). CR and SR were both effective in treating stress-related symptoms, moreover, simultaneous application of the two medicines led to better effect, possibly due to their synthetic effects. However, the complexity of the BSS as well as the TCM itself hindered the understanding of the mechanism in treatment.

Metabolomics study facilitated the study of BBS as well as the treatment effect of TCMs. Attributed to the systematic information provided by metabolomics and the direct relation with physiology performance. To obtain more accurate metabolites information, suitable sampling techniques such as micro dialysis could be applied (Zhou *et al.*, 2017). The micro dialysis was previously use in the metabolomics study in brain for continuous study of metabolic evens. For the chronic abnormal such as BSS, micro dialysis method minimized the stimulation effects, thus may provide more reliable information. In the similar rational, micro dialysis would be preferable in study TCM effects, which were always slow and mild. However, the method was not reported in previous TCM related metabolomics studies.

In this study, the BSS model and effects of two TCMs, CR and SR, were studied in both hemorheology and metabolomics based on microdialysis sampling. To better understand the effects of TCMs, single or mixed treatments were carried out and investigated.

## MATERIALS AND METHODS

### *Plant materials*

*Sparganii rhizome* was purchased from Panan, Zhejiang province of China, authenticated by Prof. Jianwei Chen of Nanjiang University of Traditional Chinese Medicine. *Curcumae rhizome* was purchased from Ruian, Zhejiang province. The dry rhizome was milled and extracted twice using heating reflux method with ratio of 1:15 (rhizome:

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distilled water). The extraction was combined, filtered and concentrated to the concentration of 1 g rhizome/ml.

### Chemicals and reagents

Citric acid was purchased from Nanjing Chemical Reagent Corporation. Glucose were purchased from Sinopharm Chemical Reagent Co., Ltd. (Beijing, China). L-2-chlorophenylalanine was purchased from Shanghai Hengbai Biological Technology Co. Ltd. (Shanghai, China). BSTFA (with 1% v/v TMCS) was purchased from REGIS Technologies. Inc (Morton Grove, Illinois, USA).

### Animal model

To construct the BSS model, 30 male SD rats weighing 300 20 g are supplied by the Animal Center Lab of the Nanjing University of Traditional Chinese Medicine (Nanjing, China. License: SCXK20080033). The animals here received humane care according to the criteria outlined in the "Guide for the care and use of Laboratory Animals" by the National Academy of Sciences and the National Institutes of Health. After 7 days of acclimatization, the rats are divided into 5 groups randomly: control group (Ctr), model group (M), CR-treated group (CR), SR-treated group (SR) and the mixed medicine group (CR-SR). The model and treatment rats received following stimulations regularly for 30 days (twice a day if not specified): Flash, electrical (30 to 35 v, 0.3 seconds with 2 seconds' interval for 5 minutes), tail-clip (about 15 N) for 30 minutes, ice bath (5 minutes) and constrain for 2 hours a day.

After 30 days of stimulation, the Ctr and M groups were fed with saline and the CR, SR and CR-SR groups received different medicines as designed. The treatments were carried out for 7 days.

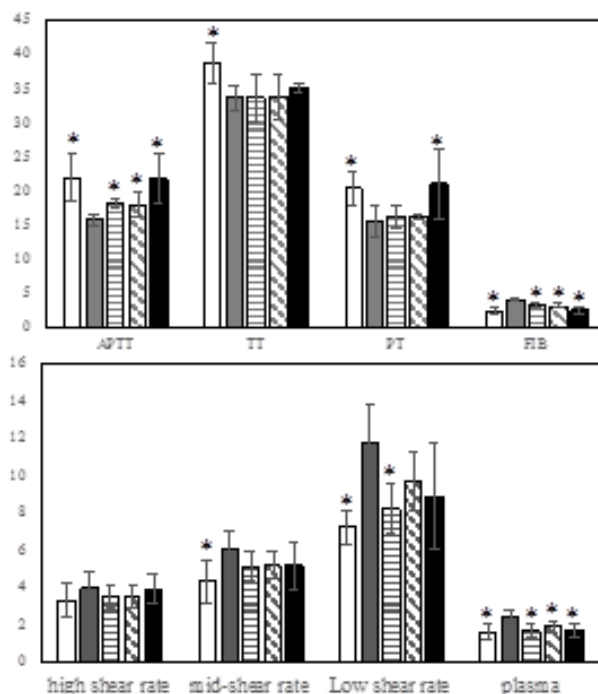
### Sample collection and test indicators

The SD rats were anesthetized by 10% chloral hydrate injection. The rat was laid on the plate and exposed its right jugular hepatic vein (HV). The blood vessel was sliced open and the microdialysis catheter was planted into the vessel with 2 cm inside. The dialysis membrane was placed above the superior vena cava. To avoid the movement, the catheter was then tied with the vessel. The catheters were connected to a micro infusion pump with a flow rate of 2 ml min<sup>-1</sup>. The samples were collected in microvials every second hour, and then frozen and kept at -80°C until analyzed.

### Sample pretreatment

The samples were subjected to gas chromatography-mass spectrometry (GC-MS) analysis to collect and derivatize the metabolites. The analyte was injected splitlessly into an Agilent 7890 GC system (Agilent; Santa Clara, CA, USA) coupled with a Pegasus 4D time-of-flight mass spectrometer (LECO; St. Joseph, MI, USA). Chromatographic separation was performed on a DB-

5MS capillary column (30 m x 250 mm ID, 0.25 um film thickness; J&W Scientific; Folsom, CA, USA). Helium was used as the carrier gas with the front inlet purge flow of 3 ml min<sup>-1</sup> and gas flow rate through the column of 1 ml min<sup>-1</sup> (Du *et al.*, 2016). The initial temperature is kept at 80°C for 0.2 min, and then raised to 180°C at a rate of 10°C min<sup>-1</sup>. Then the temperature was raised to 240°C at a rate of 5°C min<sup>-1</sup>, and finally raised to 290°C at 20°C min<sup>-1</sup>. The temperature of 290°C was kept for 11min. The injection, transferline and ion source temperatures are 280, 245 and 220°C, respectively. The energy is 70 eV. The MS data were acquired in full-scan mode with the m/z range of 20-600 (Liu *et al.*, 2015).



**Fig. 1:** Analysis of the hemorheology in control (Ctr, blank bars), Model (M, grey bars), CR-treated (vertical strips bars), SR-treated (slanted strips bars) and CR-SR-treated groups (black bars). (A) Activated partial thromboplastin time (APTT), thrombin time (TT) and prothrombin time (PT), fibrinogen (Fib); (B) The viscosity in the mid-shear, low-shear rate and the plasma. \*indicates statistical significantly difference compared to the model group.

### Data processing and statistical analysis

Chroma TOF 4.3X software of Leco Corporation and Leco-Fiehn Rtx5 database was used for raw peaks exacting, the data baselines filtering and calibration of the baseline, peak alignment, deconvolution analysis, peak identification and integration of the peak area. The experimental data are presented as mean SD. Statistical significance is assessed by Students' *t*-test. SIMCA 14.1 are used for multivariate analysis.

**Table 1:** Key metabolites changed in BSS model compared to control

Metabolites	VIP	<i>p</i>	Fold change (Con/BSS)
Fructose	5.04	0.01	1.76
Tyrosine	4.41	0.04	2.92
Citric acid	4.20	0.05	2.55
Carnitine	4.07	0.07	1.78
Thymidine	3.66	0.05	0.71
Mannose	4.30	0.05	0.59
Cycloleucine	3.59	0.05	0.72
3-Methylamino-1,2-propanediol	3.68	0.05	0.70
1,2-Cyclohexanedione	3.56	0.06	0.72
Glutamine	3.52	0.10	0.60

**Table 2:** Key metabolites changed in CR-SR treatment (CS/BSS)

Metabolites	VIP	<i>p</i>	Fold change (CR-SR/BSS)
Cycloleucine	5.05	0.00	2.16
D-erythro-sphingosine	4.72	0.02	2.16
Asparagine	4.54	0.01	2.13
Urea	4.27	0.02	1.89
Mucic acid	3.99	0.02	1.78
Tyrosine	3.67	0.04	2.79
2-ketobutyric acid	3.27	0.08	1.61
Cycloleucine	5.05	0.00	2.16
Thymidine	3.36	0.05	0.70
N-(3-aminopropyl)-morpholine	3.36	0.09	0.57
5-Methoxytryptamine	3.27	0.11	0.65
Lactobionic Acid	2.59	0.21	0.58
Leucrose	2.47	0.16	0.65
Acetanilide	2.45	0.16	0.80

## RESULT

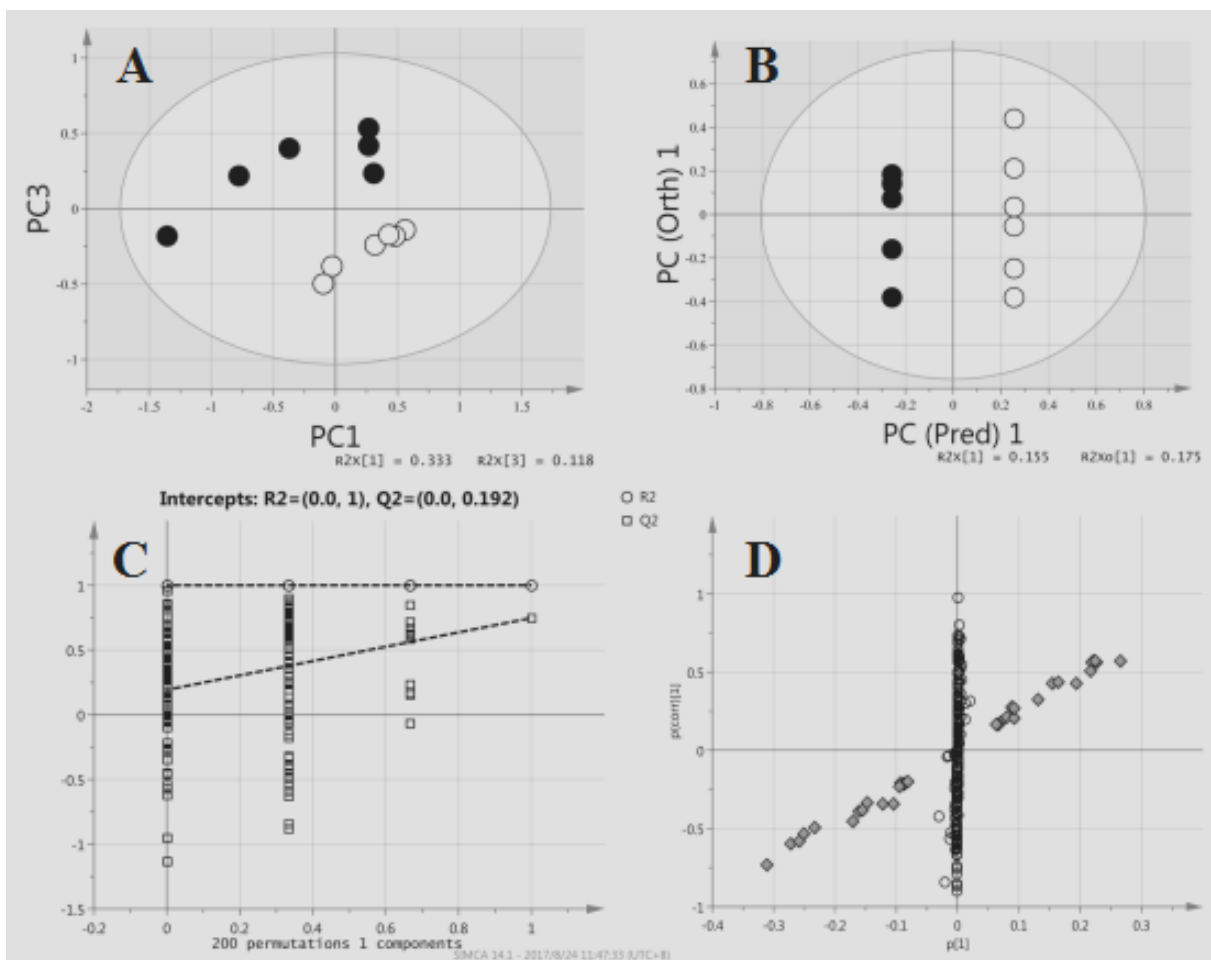
### *Blood stasis syndrome (BSS) resulted from external stresses in rat*

The long-term stress model was constructed by giving regular external stimulations to rats. From the hemorheology data, it was obvious that the stimulations resulted in abnormalities similar to the blood stasis syndrome in the model group. The hemorheology parameters, including activated partial thromboplastin time (APTT) thrombin time (TT) and prothrombin time (PT), showed significant decrease by more than 20% in the model group, while the fibrinogen (Fib) increased by 80% in the model group. On the other hand, the shear rate in the mid-shear, low-shear rate and the plasma all increased significantly by 22 to 65% in the model group compared to control. These phenomena indicating the stresses resulted in detectable vascular abnormalities.

To gain a deeper understanding of this abnormality, micro-dialysis was carried out to extract and collect the metabolites from venous blood. Then the samples were analyzed by GC-MS. Based on the Leco-Fiehn Rtx5 database and related references, the peaks are identified as endogenous metabolites. To illustrate the general trend of

the metabolites information, multivariate data analysis (MVDA) strategy was adopted. First, an unsupervised Primary Component Analysis (PCA) model was constructed for general trends observation (fig. 2). In the PCA score plot, each dot represented one sample, and the distance between dots is an indication of similarity between samples. From the metabolomics-generated PCA score plot, the model and control showed significant difference. Based on this information and to identify the key differentially accumulated metabolites, a supervised OPLS-DA model was constructed and analyzed. A model with good stability and predictive ability was obtained with R<sup>2</sup><sub>Y</sub> of 0.999 and Q<sup>2</sup> of 0.784. The intercept of Q<sup>2</sup> was -0.099 in the 200 times permutation test, also indicating a robust regression model. Then, a cut off of VIP>1 and *p*<0.1 were used to screen the key differential accumulated metabolites.

As a result, 8 metabolites were screened out as key factors distinguished the model and the control groups. Among which, fructose, tyrosine, citric acid and carnitine showed lower levels in BSS model. While thymidine, mannose, cycloleucine and 3-methylamino-1,2-propanediol showed higher levels in BSS model. These results indicated the change of different metabolic pathways, such as sugar metabolism, TCA cycle and amino acid synthesis.



**Fig. 2:** Multivariate analysis of the metabolomic data of control (Ctr, Black dot) and model (M, White dot) groups. (A) The score plot of Principle Component Analysis (PCA) model; (B) The score plot of Orthogonal Partial Least Square Discrimination Analysis (OPLS-DA) model; (C) The permutation test of the OPLS-DA model of Ctr vs. M. (D) The S-plot of the OPLS-DA model. Grey diamonds: the selected differentially accumulated metabolites.

From both the physiology and metabolomics data, a stress-led BSS model was successfully constructed in this study. In consistence with the complexity of BSS, several metabolic pathways were altered in the aspect of metabolites level. Then, the effects of TCMs, CR and SR, on the BSS model were investigated separately or in combination.

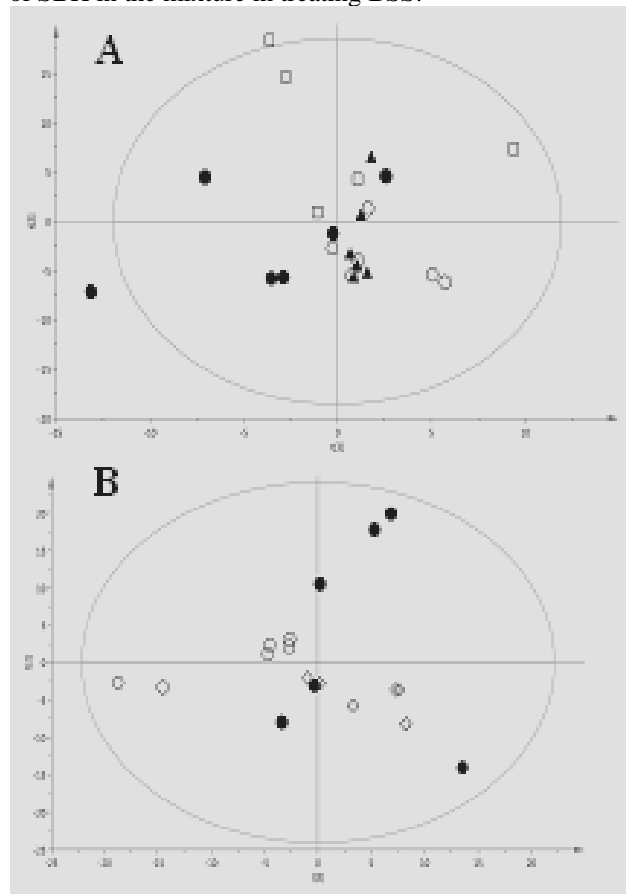
Firstly, the CR and SR extracts were used individually in the BSS rat model. The metabolomic data were analyzed by GC-MS and then the MVDA models were constructed. As shown in fig. 3 A, the SR and model groups could not be clearly separated based on the metabolites data. Comparatively, the metabolites in the CR treated rat were clearly separated from the model group, suggesting the SR treatment influenced the metabolism of the model. However, as shown in the PCA plot, compared to the model group, the CR group showed even greater deviation to control, suggesting its effects on BSS may not be positive. Consisted with this result, the single

medicine showed little improvement in hemorheology. The APTT, TT, PT all showed similar levels in the CR and SR treated group compared to model group. As a conclusion, no obvious positive effect was observed when the two TCMs were used independently. As TCM were traditional used as a mixture of several medicines, thus the combine effect was tested for CR and SR.

#### **The effects of CR-SR mixture**

The CR and SR were combined to treat the BSS in model group. Promisingly, the metabolomics data of the CR-SR group clearly separated from the model group and showed similarities with control in the PCA score plot (fig. 3 B). Then, the OPLS-DA model was constructed to screen the key difference between CR-SR and model. From the selected metabolites, the combination medicine affected several metabolic pathways, including amino acid biosynthesis and terpenoid-quinone biosynthesis. Interestingly, tyrosine and thymidine showed similar levels in CR-SR group compared to the control group.

The metabolites were further projected into the heat-map (fig. 5) to discover the similarities among the two TCMs and the combination group. First, the key different metabolites between control and BSS model were screened from the S-plot (fig. 2 D). Then the relative folds changed compared to mean values were exhibited in the heat-map. Several metabolites, including urea, vinylphenol, mannose, *et al.* showed similar trends in CR-SR and SR group suggested the similar effects of these two treatment. Importantly, tyrosine showed increased levels in CR-SR and SR group, indicating the importance of SBH in the mixture in treating BSS.



**Fig. 3:** The score plot of PCA model of samples from (A) Ctr (black circles), M (white circles), CR (white squares) and SR (black triangles); (B) Ctr (black circles), M (white circles) and CR-SR (white diamonds).

The effects were in coherence to the physiology data, where most of the hemorheology data, including APTT, PT and FIB, returned to normal when mixture was used. Thus, from these results, single medicine showed no clear effects in altering metabolites, but the mixture of these two medicines showed clear positive effects in treating stress-model rats.

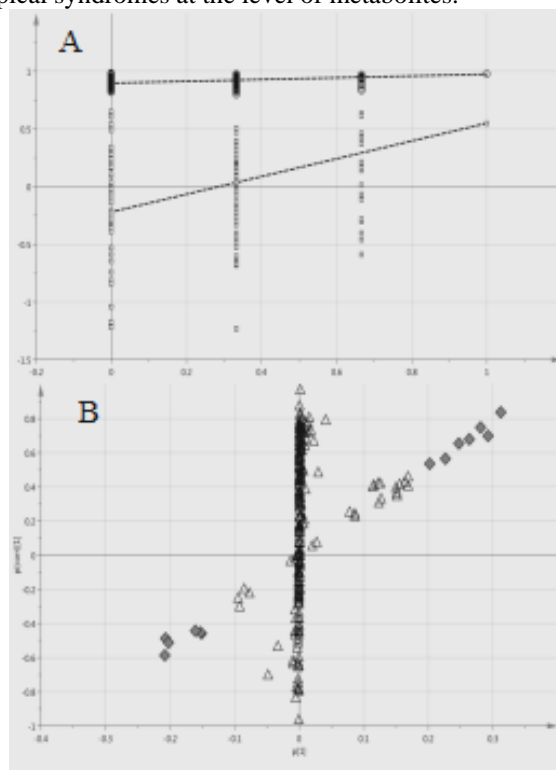
## DISCUSSION

In this study, a GC-MS based metabolomics study was carried out to investigate the effects of TCM on BSS

model. A combination of the two TCM, SR and CR, showed obvious improvement of the rat BSS model both in physiology and metabolomics. From the screened distinctive metabolites in control, model and treated group, the BSS related pathway could be identified and discussed.

### Key metabolites in BSS

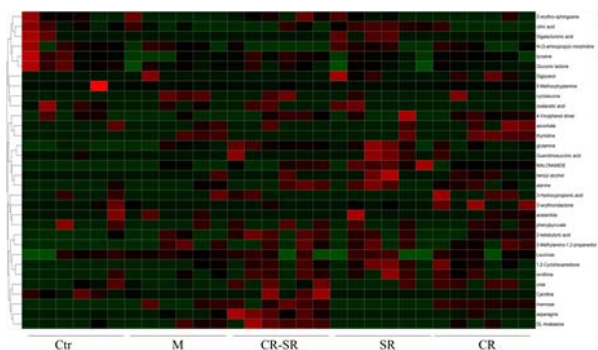
The stress led BSS model were constructed in rat, and the APTT, TT and PT were significantly decreased. Meanwhile the shear rates in BSS model rates were significantly increased. Abnormalities were observed in amino acid metabolism pathways, energy metabolism in the BSS model, which were in consisted with the reported metabolomic profiles in phlegm syndromes and blood-stasis syndrome (Zhao *et al.*, 2014). Noteworthy, reduced tyrosine level was observed in the model group. In previous studies, tyrosine precursor changed significant in rat model of acute blood stasis, and returned to normal when cured (Zhao *et al.*, 2014). Further, tyrosine was screened as a potential biomarker to discriminate phlegm and blood-stasis, and the disturbance in this pathway was closely related to blood-stasis syndrome in coronary heart disease patient (Zhao *et al.*, 2014). In another aspect, from the fructose and citric acid, the center carbon metabolism showed increased levels, which resembled that discovered in swine blood stasis syndrome model. As a conclusion, the rat model constructed in this study represented the typical syndromes at the level of metabolites.



**Fig. 4:** The permutation test (A) and S-plot (B) of Orthogonal Partial Least Square Discrimination Analysis (OPLS-DA) model of CR-SR and model groups.

**Synergistically effects of S and E**

In this study, the effectiveness of SBH-ZT mixture compared to the uncertain effects of the two individual TCMs was highlighted. By using SBH-ZT, the tyrosine level increased significantly compared to the model group. The serum tyrosine reduction was also observed in acute ischemia stroke patients (Ormstad *et al.*, 2013). One possibility is the stress induced the inflammation and led to the depletion of catecholamines. As a feedback response, the tyrosine was metabolized as the precursor for catecholamine synthesis (Ormstad *et al.*, 2013). In this study, the BS model were constructed by continuous stresses, thus possibly triggered the neuro-related regulation and led to the tyrosine reduction. In another aspect, the tyrosine played important role in the CHD and was proposed to be a potential biomarker in patients.



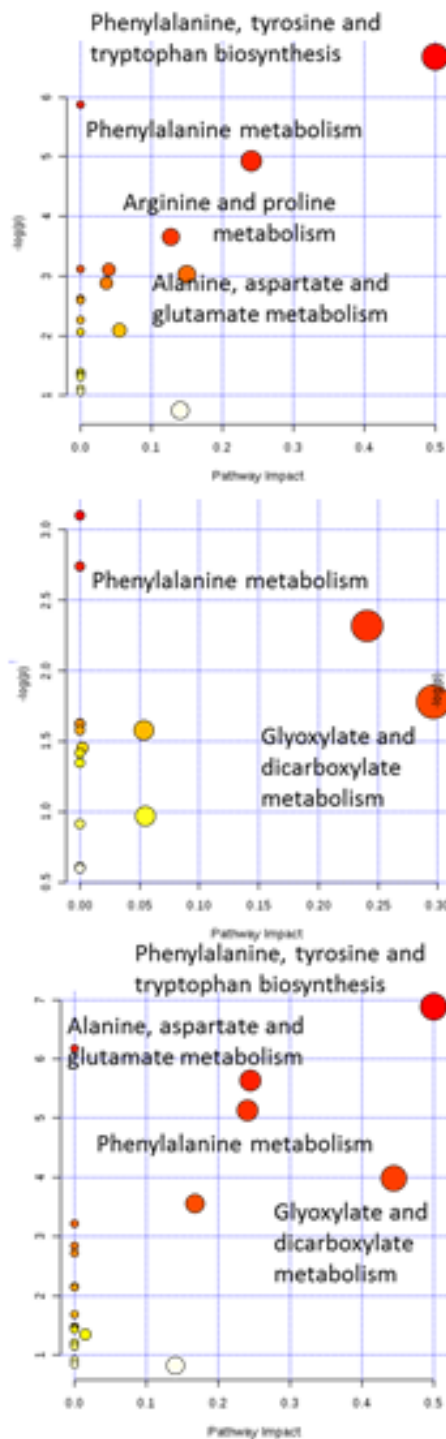
**Fig. 5:** Heat-map analysis of key metabolites in control, model, CR-SR, CR and SR treated groups.

From the heat-map analysis, SR showed a major effect in the treatment, especially, retained the level of tyrosine. In previous report, the bioactive compound, spartolonin B (ssnB), selectively blocked TLR2- and TLR4-mediated inflammatory signaling, thus presented anti-inflammatory in LPS challenged mice (Liang *et al.*, 2011). In this work, the increased level of tyrosine in SR-CR and SR treated groups indicated the alleviation of the inflammatory in BSS model. Also, this result indicated the major role of SBH played in the mixture. This result was further reinforced by the pathway enrichment analysis (fig. 6). It could be observed that the SR-CR influenced the phenylalanine, tyrosine and tryptophan biosynthesis pathway, alanine, aspartate and glutamate metabolism pathway most significantly. These effects were also observed in the SR treated group, but not in the CR treated group. The effects of CR on metabolomics of BSS model was possibly on the phenylalanine metabolism, which were also observed in the CR-SR treated group. These results provided a general understanding of the different roles CR and SR played in the treatment of BSS.

**CONCLUSION**

The findings of this study showed a synergistically effects of SR and CR on the BSS model, where SR might

majorly exert the anti-inflammatory effects and CR improved the amino acid synthesis. Thus the portion of these two TCMs could be optimized in regards of their different roles to further improve the treatment. The information and method proposed in this study may help the understanding of the complex mechanism of TCM in BSS.



**Fig. 6:** Pathway analysis of (A) SR-CR, (B) CR and (C) SR compared to the model group, respectively.

## ACKNOWLEDGEMENT

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