JPP JOURNAL OF Pharmacy and Pharmacology
ROYAL PHARMACEUTICAL SOCIETY
JPP Journal of Pharmacy And Pharmacology
Research Paper

Anti-depressant effects of Xiaoyaosan on rat model of chronic unpredictable mild stress: a plasma metabonomics study based on NMR spectroscopy

Xiao-Jie Liu[a,b,*], Yu-Zhi Zhou[a,c,*], Zhong-Feng Li[d], Jie Cui[a,b], Zhen-Yu Li[a,c], Xiao-Xia Gao[a,c], Hai-Feng Sun[a,b], Li-Zeng Zhang[a,c], Guan-Hua Du[e] and Xue-Mei Qin[a,c]

[Corrections added after online publication January 6, 2012: Jie Cui[a,d] and Hai-Feng Sun[a,d] are now written as Jie Cui[a,b] and Hai-Feng Sun[a,b]]

[a]Modern Research Center for Traditional Chinese Medicine of Shanxi University, [b]College of Chemistry and Chemical Engineering of Shanxi University, Taiyuan, [c]Key Laboratory of Chemical Biology and Molecular Engineering of Ministry Education of Shanxi University, [d]Department of Chemistry, Capital Normal University and [e]Institute of Materia Medica, Chinese Academy of Medical Sciences, Beijing, China

Keywords
CUMS; depression; metabonomics; NMR; plasma; Xiaoyaosan

Correspondence
Xue-Mei Qin, Modern Research Center for Traditional Chinese Medicine of Shanxi University, No.92, Wucheng Road, Taiyuan 030006, China.
E-mail: qinxm@sxu.edu.cn

Received May 19, 2011
Accepted October 22, 2011

doi: 10.1111/j.2042-7158.2011.01412.x

*Yu-Zhi Zhou is the co-first author.

Abstract

Objectives To investigate the antidepressant effects of Xiaoyaosan (XYS) in a chronic unpredictable mild stress (CUMS) depression model.

Methods The changes in behaviour and plasma metabolic profiles were investigated after four-week CUMS exposure and treatment. Drugs were administered during the four-week period of CUMS, with the healthy group serving as negative controls, and the fluoxetine and venlafaxine groups serving as positive controls. Plasma samples were collected at 28th day, and the plasma metabolic profiling was measured using NMR, followed by multivariate analysis.

Key findings Exposure to CUMS for four weeks caused depression-like behaviour in rats, as indicated by significant decreases in weight gain, sucrose consumption and locomotor activity. Eleven potential biomarkers, including seven in the Carr–Purcell–Meiboom–Gill spectra, five in the diffusion-edited spectra, and one in both were identified. It was found that trimethylamine-*N*-oxide, alanine, β-hydroxybutyrate, valine, leucine/isoleucine, low-density lipoprotein/very low-density lipoprotein and lipids were lower and phosphatidylcholine, high-density lipoprotein, choline and N-acetyl glycoproteins were higher in CUMS-treated rats, as compared with controls. XYS significantly suppressed behavioural changes and attenuated plasma metabolite changes.

Conclusions XYS produced an obvious antidepressant effect, and the metabonomic approach benefits estimation of the pharmacodynamic action of traditional Chinese medicine prescriptions.

Introduction

Depression is a common mental disorder that presents with depressed mood, loss of interest or pleasure, feelings of guilt or low self-worth and disturbed sleep or appetite.[1] It has been primarily treated by antidepressants that are related to the monoaminergic neuron system.[2] Nowadays, however, with the requirement of greater therapeutic efficacy and less adverse effects, a renewed interest has been generated in herbal medicines from traditional Chinese medicine (TCM). Xiaoyaosan (XYS), a famous traditional Chinese prescription with a long history of clinical use for relieving a wide variety of symptoms caused by *qi* stagnation, is comprised of eight traditional Chinese medicines: *Poria cocos* (Schw.) Wolf, *Paeonia lactiflora* Pall., *Glycyrrhiza uralensis* Fisch., *Bupleurum chinense* DC., *Angelica sinensis* (Oliv.) Diels, *Atractylodes macrocephala* Koidz., *Mentha haplocalyx* Briq. and *Zingiber officinale* Rosc. and has been widely used for the treatment of depressive disorders and manic-depressive illness in China.[3–5]

Chronic unpredictable mild stress (CUMS) is one of the most common antidepressant screening models based on its mimicking of several human depressive symptoms. Many behavioural and biochemical changes induced by CUMS are reversible by antidepressant treatments. Meanwhile, the CUMS model of depression has good face validity, construct validity and predictive validity, which makes it suitable for investigating the pathophysiology of depression and the antidepressant effects of diverse drugs.[6]

As a new member in Systems biology, metabonomic approaches provide a global overview of the integrated response of an organism to a stimulus.[7] Biofluids, such as urine or plasma, are popular in metabonomic studies because they are fairly simple to collect and contain an abundance of metabolic information. Moreover, these biofluids represent the average metabolic status of an organism. Metabonomic technology largely relies on advanced spectroscopic platforms, such as NMR spectroscopy and mass spectrometry (MS), that generate high-density data from biological samples, providing a characteristic 'fingerprint pattern' for a range of biologically important endogenous metabolites that reflect the physiological or pathological status of an organism.[7] The application of metabonomics in studying the pharmacodynamic effects and mechanisms of drugs on diseases has been adopted.[8] These studies indicated drug-induced effects on global metabolites and have yielded valuable results. The complexity of information contained in metabolic profiles has been further explored with multivariate statistical methods to recover key information associated with different phenotypes or pathophysiological conditions.[9] For the identification of discriminating markers between experimental groups, the partial least square discriminant analysis (PLS-DA) model, a chemometric model that reduces a matrix of data to its lowest dimension of the most significant factors, is superior.[10] The combination of high-resolution ^{1}H NMR spectroscopy of biofluids and tissue samples with multivariate statistical analysis has been shown to be useful in pharmacodynamic effect and biomarker discovery, and hence may facilitate development of new diagnostic tools and drug targets.[11]

To our knowledge, metabonomic study of CUMS-induced depression based on NMR has not been reported. Moreover, there is no research about the anti-depression effect of XYS based on plasma metabonomics by NMR. In this study, an integrated NMR-based metabonomic approach was used to screen and identify metabolic perturbations, in Sprague–Dawley rat plasma, associated with depression induced by CUMS treatment. The primary goal of this work was to characterize the plasmatic metabolic changes in CUMS-treated rats, and to evaluate the pharmacological effect of XYS on depression by NMR.

Materials and Methods

Preparation of the decoction of Xiaoyaosan

Traditional Chinese medicines Poria, Radix Paeoniae Alba, Radix Bupleuri, Radix Angelicae Sinensis, Rhizoma Zingiberis Recens., Radix Glycyrrhizae, Herba Menthae, and Rhizoma Atractylodis Macrocephalae, were purchased from the Medicinal Materials Company of Beijing Tongrentang, and authenticated by Professor Xue-Mei Qin, Shanxi University. All the raw materials, 300 g of Poria, 300 g of Radix Paeoniae Alba, 150 g of Radix Glycyrrhizae, 300 g of Radix Bupleuri, 300 g of Radix Angelicae Sinensis, 300 g of Rhizoma Atractylodis Macrocephalae, 100 g of Herba Menthae, and 100 g of Rhizoma Zingiberis Recens, were soaked in water (18.5 l) for 12 h before extraction. They were then extracted three times with boiling water (18.5 l for 2 h, 14.8 l for 1 h, 14.8 l for 1 h) and then the decoction was dried *in vacuo* (70°C) and ground into powder for use. The powder was dissolved in purified water, with the aid of ultrasonication, in three different concentrations for use.

HPLC chromatograms of Xiaoyaosan and its constituent herbs

Reagents and reference compounds

HPLC-grade acetonitrile from Merck (Darmstadt, Germany) was used for HPLC analyses. Other chemicals and solvents purchased from Tianjin Chemical Reagent Co., Inc. (Tianjin, China) were of analytical grade.

The standards of gallic acid, catechin, albiflorin, paeoniflorin, liquiritin apioside, liquiritin and ferulic acid were purchased from the Chinese Institute for the Control of Pharmaceutical and Biological Products (Beijing, China).

Sample preparation

XYS and eight raw material powders were achieved above. An accurately weighed sample of 1.0 g dried powdered sample was introduced with methanol in a 10 ml volumetric flask. A volume of 2 ml of the solution was filtered through a 0.45-μm syringe filter before use. A 20-μl volume of the solution was injected for HPLC analysis.

Chromatography

A Waters HPLC system (Waters, Milford, USA) was equipped with a Model 1525 pumping system, a Model 2487 Dual λ Absorbance Detector, a 20 μl injector, a column oven and Breeze Workstation software. For HPLC fingerprint chromatographic analysis, a reverse-phase column (Diamonsil C18, 250 mm × 4.6 mm, 5 μm) was used. A binary gradient elution system composed of acetonitrile as solvent A and 0.1% H_3PO_4 in water as solvent B was applied for the fingerprint analysis with the gradient elution as follows: 0–8 min, 3% A; 8–20 min, 3–15% A; 20–40 min, 15–30% A; 40–45 min, 30–40% A; 45–47 min, 40–3% A. The flow rate was kept at 1.0 ml/min and the column temperature was maintained at 25°C. The detector wavelength was set at 230 nm.

Animal handling and drug administration

All experiments were approved by the institutional ethics committee and all experimental procedures were in compli-

ance with the National Institutes of Health Guide for Care and Use of Laboratory Animals (Publication no. 85–23, revised 1985). All efforts were made to minimize animal suffering and to use only the number of animals necessary to produce reliable scientific data. Forty-two male Sprague–Dawley rats, 200 ± 20 g, were purchased from the Experimental Animal Center of the Chinese Military Medical Sciences Academy (No. SCXK2005-0004). The rats were housed in propylene cages under standard experimental conditions: room temperature 24 ± 1°C, relative humidity 45 ± 15% and 12-h light–dark cycle (lights on at 8:00 am). Food and tap water were freely available. Rats were allowed to have seven days acclimation before any experimentation.

After acclimation, the 42 rats were randomly divided into seven groups: (1) healthy control group (NS, no stressor and 0.9% NaCl solution); (2) CUMS depression model group (MS, stressor plus 0.9% NaCl solution); (3) high-dose group of XYS (HX, stressor plus XYS at a dose of 92.4 g herb/kg); (4) middle-dose group of XYS (MX, stressor plus XYS at a dose of 46.2 g herb/kg); (5) low-dose group of XYS (LX, stressor plus XYS at a dose of 23.1 g herb/kg); (6) fluoxetine (YB, stressor plus fluoxetine at a dose of 6 mg/kg); and (7) venlafaxine HCl (YW, stressor plus venlafaxine at a dose of 25 mg/kg). The healthy group served as negative controls, while the fluoxetine and venlafaxine groups served as positive controls.

Rats were administered drugs separately via gastric intubation daily for 28 days with a volume of 10 ml/kg (rat body weight). All drugs were given 30 min before the stress exposure.

Chronic unpredictable mild stress procedures

The animals in the CUMS group, XYS group, venlafaxine group and fluoxetine group were individually housed and repeatedly exposed to a set of chronic unpredictable mild stressors as previously described[12] with a slight modification, which is described in Supplementary Information. The healthy control rats were housed together in one cage without disturbance except for necessary procedures such as weighing or cage cleaning.

Behavioural analysis

Open-field test and sucrose preference test were conducted as previously described,[12] and the experimental details are provided in Supplementary Information.

^{1}H NMR experiments

Three hours after the last dosing on day 28, rats were anaesthetized with urethane. Following the onset of surgical anaesthesia, an abdominal incision exposed the abdominal aorta. Blood samples were collected into heparinized tubes and the plasma was collected by centrifugation at 3500 rpm at 4°C for 15 min. A volume of 300 μl of plasma was mixed with 250 μl D_2O, and then centrifuged at 14 000 rpm for 10 min. The supernatants were transferred into in a 5 mm NMR tube. The D_2O provided a field-frequency lock solvent for the NMR spectrometer.

^{1}H NMR spectra of the plasma samples were acquired on a Varian NMRS 600 MHz NMR spectrometer (Varian, Inc., CA, USA) at 25°C by using Carr–Purcell–Meiboom–Gill (CPMG) spin-echo pulse sequence with a total spin–spin relaxation delay ($2n\tau$) of 320 ms to attenuate broad signals from proteins and lipoproteins due to their long transverse relation time. The free induction decays (FIDs) were collected into 64 K data points with a spectral width of 8000 Hz and 64 scans. The FIDs were zero-filled to double size and multiplied by an exponential line-broadening factor of 1.0 Hz before Fourier transformation (FT). In addition, diffusion-edited experiments were also carried out with bipolar pulse pair-longitudinal eddy current delay pulse sequence on plasma samples to obtain the spectra with only signals from lipids. The gradient amplitude was set at 76.5G/cm, with a diffusion delay of 200 ms. A total of 128 transients and 32 K data points were collected with a spectral width of 8000 Hz. A line-broadening factor of 1 Hz was applied to FIDs before Fourier transformation.

All plasma ^{1}H NMR spectra were manually phased and baseline corrected using MestReNova software (Varian, Inc.). For CPMG spectra, each spectrum over the range of δ0.4–4.4 was data reduced into integrated regions of equal width (0.04 ppm). For diffusion-edited spectra, each spectrum over the range of δ 0.1–6.0 was segmented into regions of equal width (0.01 ppm). The regions containing the resonance from residual water (δ 4.6–5.1) were excluded. The integral values of each spectrum were normalized to a constant sum of all integrals in a spectrum to reduce any significant concentration differences between samples.

Multivariate analysis of NMR data

The resulting integral data were imported into SIMCA-P (version 11.00; Umetrics, Umeå, Sweden) for multivariate analysis. All NMR data variables were mean-centered and Pareto-scaled before analysis. Partial least square discriminant analysis (PLS-DA) was used to find differential metabolites between groups. The results were visualized by two-dimensional score plots representing the distribution of samples and the corresponding loading plots providing information on the contribution of each variable to the pattern in the score plots.

Results

Chromatograms of Xiaoyaosan extract and chemical constituents identified

The HPLC chromatograms (supplied in the Supplementary Information) of XYS and eight herbs are shown in Figure S1,

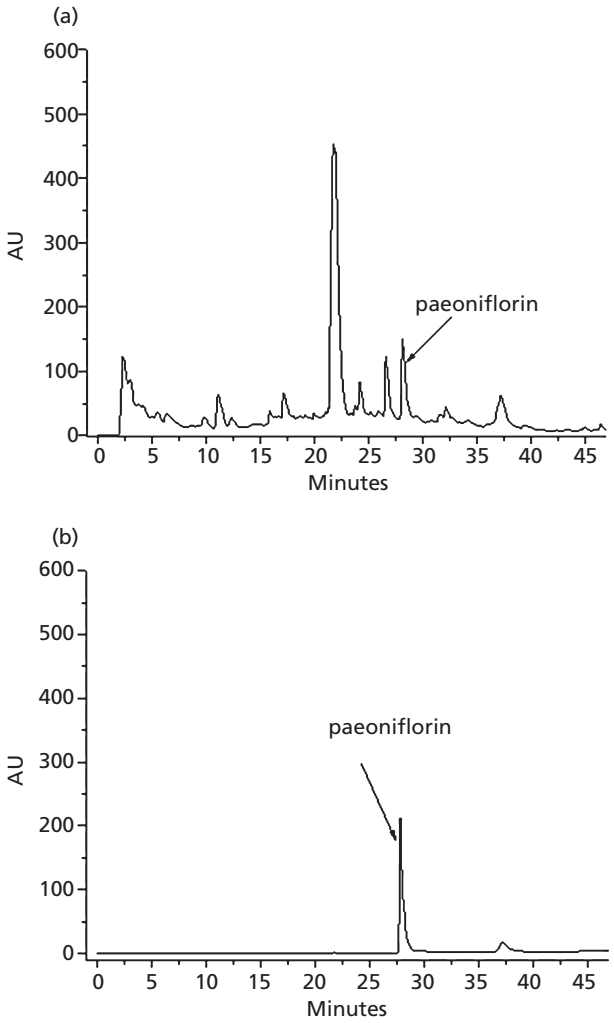

Figure 1 The identified results of paeoniflorin. (a) HPLC chromatogram of Xiaoyaosan. (b) HPLC chromatogram of paeoniflorin.

of which seven characteristic peaks were assigned by comparing their retention time with those of the reference compounds, including gallic acid, catechin, albiflorin, paeoniflorin, liquiritin apioside, liquiritin and ferulic acid. Taking paeoniflorin as an example, the identified results are shown in Figure 1.

Effects on sucrose preference, body weight and open-field activity

The anti-depressant effects of XYS on CUMS-treated rats were analysed according to body weight, sucrose preference test and open-field test. The results (provided in the Supplementary materials, Table S1) showed that all the drug-treated stressed groups had a tendency to return to the normal state, except for the LX group. Twenty-eight days of XYS administration at a dose of 46.2g/kg significantly increased the body weight, sucrose preference and ambulation, and decreased the immobility time of MS rats, compared with the rats receiving CUMS alone. The behaviour results indicated that the depressive status was obviously developed after 28-days CUMS exposure and the XYS prescription 46.2g/kg showed a significant anti-depressant effect.

^{1}H NMR spectra of plasma

Plasma comprises both low-molecular-weight metabolites and high-molecular-weight proteins and lipoproteins.[13] To emphasize the small metabolites in plasma by attenuating the resonances from macromolecules, a CPMG pulse sequence was used to acquire the spectra,[14] The typical CPMG spectrum of plasma samples from the control healthy group is shown in Figure 2. The resonance assignments were made according to published reports and the Metabonomics Toolbox (http://www.hmdb.ca) and the identified metabolites are listed in the Supplementary Information (Table S2). Most parts of the signals from macromolecules were eliminated, leaving only some residual signals from the methyl and methylene groups from lipids of lipoproteins. Based on literature reports, major metabolites in plasma were identified, including amino acids (leucine/isoleucine, valine, alanine and glutamine), organic acids (β-hydroxybutyrate, lactate, acetate, acetoacetate, pyruvate, creatine and creatinine) and glucose (Figure 2). The diffusion-edited NMR spectra of plasma samples,[15] presenting only broad peaks from the lipids of lipoproteins and N-acetyl (NAc) groups of glycoproteins, are shown in Figure 2.

Influence of chronic unpredictable mild stress model on the plasmatic metabolic pattern

To know whether we could distinguish the model group and control group on the basis of the NMR spectra and understand their metabolite differences, PLS-DA was performed on the spectra of the small metabolites and macromolecule metabolites in plasma. The result, which is provided in the Supplementary Information (Figure S2), showed that in all score plots, the model group and control group could be clearly separated from the normal subjects based on plasma samples collected.

The PLS-DA results of the CPMG data of plasma samples from CUMS-treated rats and control healthy rats are shown in Figure S2a (Supplementary Information). The CUMS-treated rats were clearly separated from controls along PC1 with $R^2X = 71.3\%$, $R^2Y = 77.7\%$ and $Q^2 = 77.2\%$ (Figure S2a), and the corresponding loadings plot (Figure S2b) showed an apparent higher level of choline (Cho) (δ 3.20) and N-acetyl methyl groups of glycoproteins (NAc) (δ 2.00) and lower levels of lactate (Lac) (δ 1.32), β-hydroxybutyrate (β-HB) (δ 1.20), alanine (Ala) (δ 1.44)

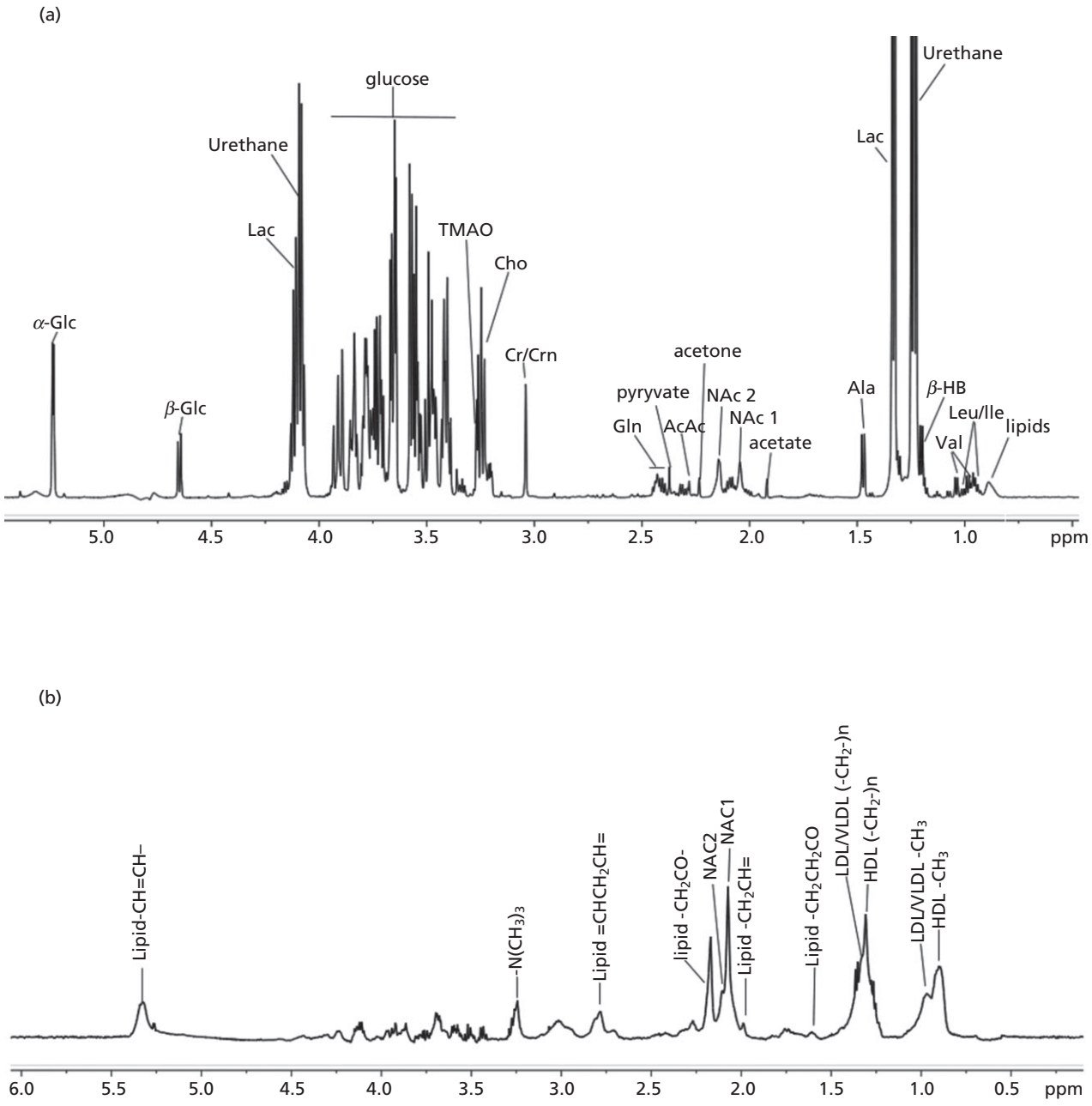

Figure 2 Typical 600 MHz plasma ^{1}H NMR spectra from control healthy group of rats. (a) The CPMG spectrum. (b) The diffusion-edited NMR spectrum. AcAc, acetoacetate; Ala, alanine; Cho, choline; Cr/Crn, creatine/creatinine; Glc, glucose; Gln, glutamine; HDL, high-density lipoprotein; LDL/VLDL, low-density lipoprotein/very low-density lipoprotein; Lac, lactate; Leu/Ile, leucine/isoleucine; β-HB, β-hydroxybutyrate; TMAO, trimethylamine-*N*-oxide; NAc, N-acetyl methyl groups of glycoproteins; -N$(CH_3)_3$, N-methyl groups of phosphatidylcholine (PtdCho).

and trimethylamine-*N*-oxide (TMAO) (δ3.24) in the plasma samples of CUMS-treated rats than in controls. Moreover, slightly lower levels of valine (Val) (δ 1.04) and leucine/isoleucine (Leu/Ile) (δ0.92) were observed in CUMS-treated rats than in healthy controls.

PLS-DA of diffusion-edited NMR spectra of plasma samples resulted in the separation of CUMS and the controls group. The results (Figure S2c, Supplementary Information) presented clear differences in the lipid profiles of plasma between CUMS-treated rats and controls along PC1 ($R^2X = 75.4\%$, $R^2Y = 87.3\%$, $Q^2 = 78.8\%$). Changes in lipid composition were revealed in the corresponding loadings plot (Figure S2d). Lipoproteins can be classified according to their particle sizes into high-density lipoprotein (HDL),

Table 1 Identification results of potential biomarkers

Compound	Chemical shifts ^{1}H CPMG NMR spectra	Changes[a]	Compound	Chemical shifts Diffusion-edited NMR spectra	Changes[a]
TMAO	3.24	↓	PtdCho	3.21, 3.22	↑
Cho	3.20	↑	NAc 1	2.12	↑
NAc	2.00	↑	LDL/VLDL	1.31, 1.30	↓
Ala	1.44	↓	Lipids	2.04	↓
β-HB	1.20	↓	HDL	0.83, 0.84	↑
Val	1.04	↓			
Leu/Ile	0.92	↓			

[a]'↑' and '↓' denote higher or lower amounts relative to the healthy controls. TMAO, trimethylamine-*N*-oxide; Cho, choline; Nac, N-acetyl methyl groups of glycoproteins; Ala, alanine; β-HB, β-hydroxybutyrate; Val, valine; Leu/Ile, leucine/isoleucine; PtdCho, N-methyl groups of phosphatidylcholine; LDL/VLDL, low-density lipoprotein/very low-density lipoprotein; HDL, high-density lipoprotein.

low-density lipoprotein (LDL) and very low-density lipoprotein (VLDL). Despite the severe overlap between signals from these lipoproteins, a difference in chemical shifts of the methyl and methylene groups between HDL, LDL and VLDL could be observed. Compared with controls, the plasma of CUMS-treated rats had a higher level of HDL (~ δ0.85) and lower level of VLDL/LDL (~ δ1.30) (Figure S2d). In addition, negative loadings at δ3.21 and δ3.22 suggested a higher level of phosphatidylcholine (PtdCho) in CUMS rats than in controls. This observation was consistent with the higher level of HDL in CUMS rats since PtdCho is the most predominant lipid in the HDL fraction. However, the signal at δ2.04 arising from the lipids showed a positive loading, indicating a lower level of lipids in the plasma of CUMS-treated rats.

To exhibit the responsibility of each metabolite for these variations more intuitively, loading plots (Figure S2b and S2d) were applied. In PLS-DA loading plots, most of the metabolites were clustered around the origin point. Only a few of them scattered in the margin region, and only these few metabolites contributed to the clustering observed in the score plot and were also regarded as the potential biomarkers. Table 1 lists the identification results; seven potential biomarkers were preliminarily identified from the CPMG spectra and five in the diffusion-edited NMR spectra (NAc in both modes) (Table 1).

Effect of Xiaoyaosan on the plasmatic metabolic profiles induced by chronic unpredictable mild stress model

To determine whether it was possible for XYS prescription to influence the metabolic pattern of the CUMS model subjects and to investigate the best doses of XYS, PLS-DA was used for analysing the NMR spectra data. In the PLS-DA map, each spot represented a sample, and each assembly of samples indicated a particular metabolic pattern of different groups. The PLS-DA score plots from CPMG spectra (Figure 3a) and diffusion-edited spectra (Figure 3b) separated plasma samples into different blocks, and samples subjected to the same treatment were located on the same trajectory, indicating

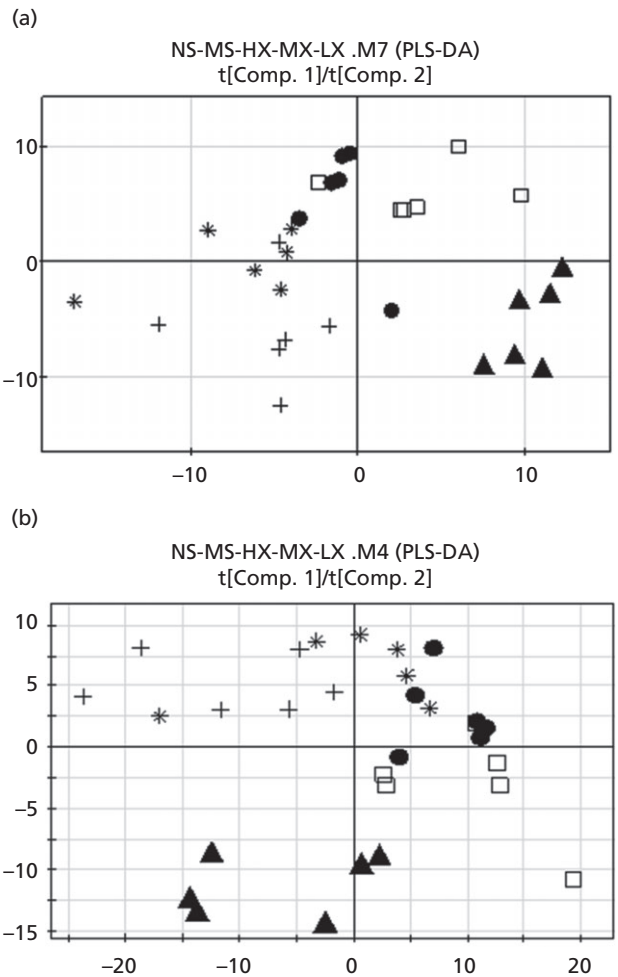

Figure 3 PLS-DA score plots of plasma samples collected from different doses of Xiaoyaosan treatment groups of rats from (a) CPMG spectra and (b) diffusion-edited spectra. □, Healthy control group (NS); ▲, CUMS-model group (MS); ●, Middle-dose group of XYS (MX); *, High-dose group of XYS (HX); +, Low-dose group of XYS (LX).

that XYS treatments had improved the plasma metabolic profiles of rats induced by CUMS model.

The results (Figure 3a and 3b) showed that a separation of the model group and control group was clearly achieved, while the dose group was mainly located between the model group and the control group, exhibiting a tendency of recovery from illness to healthy conditions. Plasma of the XYS at low dose group was located near the model group, indicating they could have similar metabolic profiles. Plasmas of the XYS at high dose and middle dose groups were located near the control group, especially the MX-treated group. Some of the samples in the control and MX groups clustered, suggesting that the MX-treated and control groups could have similar metabolic profiles, and that XYS at middle dose treated could assist restoration of the animals from CUMS treatment.

To assess the antidepressant effect of the XYS prescription, two chemical antidepressants were compared as positive controls, and a PLS-DA model was constructed. The score plots of small metabolites in plasma from CPMG spectra (Figure 4a) and macromolecules from diffusion-edited spectra (Figure 4b) were revealed.

It could be seen from the PLS-DA score plot (Figure 4) that, this change of plasmatic metabolic pattern showed the drug treatment groups are moving toward the control group and the depressive status was being prevented and alleviated, exhibiting a tendency recovering to healthy control group after taking XYS prescription, Fluoxetine and Venlafaxine. Both the small metabolites (Figure 4a) and macromolecules metabolic profiles (Figure 4b) illustrated that the rats in the middle-dose XYS group were moving toward the control healthy rats, showing a significant anti-depression effect.

The PLS-DA analysis of NMR spectra data identified the control and the CUMS-treated rats based on the differences in their metabolic profiles, demonstrating that a depressive model was successfully reproduced. Furthermore, the classic formula of XYS at the middle dose showed significant therapeutic effects in the depressive model, which was consistent with the results of behavioural analysis.

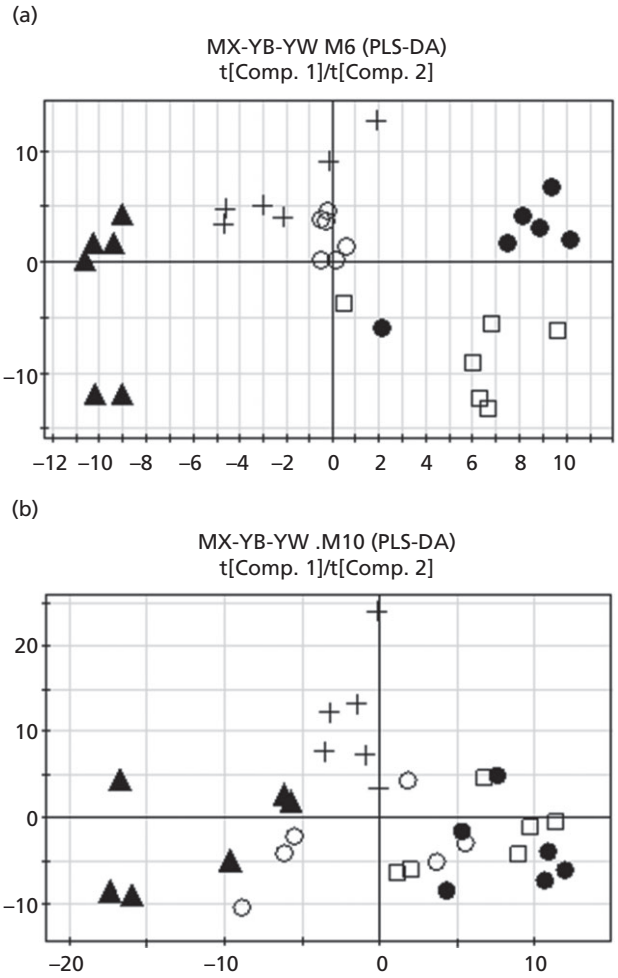

Figure 4 PLS-DA score plots of plasma samples collected from Xiaoyaosan, fluoxetine and venlafaxine treatment groups of rats (a) in CPMG NMR spectra and (b) in diffusion-edited NMR spectra. □, Healthy control group (NS); ▲, CUMS-model group (MS); ●, Middle-dose group of XYS (MX); +, Fluoxetine group (YB); ○, Venlafaxine group (YW).

Discussion

As a traditional Chinese prescription comprising multiple ingredients, HPLC chromatograms of XYS and its consisted herbs have been built. The rough HPLC analysis revealed that the gallic acid, catechin, albiflorin and paeoniflorin were primarily derived from Radix Paeoniae Alba, the ferulic acid was derived from Radix Angelicae Sinensis and liquiritin apioside and liquiritin came from Radix Glycyrrhizae. As the components are very complicated, the analytical work and the evaluation of the disassembled prescription are still ongoing and deserve further research. Meanwhile, another student in our research group has also performed studies focusing on chemical component analysis and quantitative analysis, which will be described in detail in another article. Here we describe, simply, the work in our manuscript.

Since it is believed that long-term exposure to multiple, inescapable stressors can promote clinical depression in humans, an ideal animal model of depression is to employ multiple behavioural tests that approximate the symptoms characterizing human depressive illness.[16] The CUMS procedure is suggested to be an appropriate model to study the onset of antidepressant action in animals.[17] The pharmacological validation of the model is extensive compared with most other animal models of depression. In the CUMS model, rats or mice are exposed sequentially, over a period of weeks, to a variety of mild stressors, and the measure is most commonly used to track the effects of antidepressants.[7] The CUMS procedure mimics the role of chronic stress in

precipitating depression and induces various long-term physical, behavioural, neurochemical and neuroendocrine alterations that resemble those observed in depressed patients.[1]

Animals subjected to the CUMS regimen display several features of clinically depressed humans.[16] In this study, a marked decrease in the amount of sucrose intake, body weight, rearing and grooming and a significant increase in immobility time in an open field test were observed. These behavioural indicators returned to baseline when the model rats were treated with XYS, fluoxetine or venlafaxine. The middle dose of XYS (46.2 g herb/kg) showed the best improvement efficiency among the three doses of XYS. The lower dose (12.75 g herb/kg) had been studied in our previous work,[18] and the results showed that XYS did not show an anti-depression effect at this dose. In this study, we used a higher dose to study the effect of XYS based on reasonable dose setting and as already mentioned, a dose of 46.2 g herb/kg showed the best anti-depressant effects.

The enormous potential of metabonomics as a tool for understanding pharmacodynamic action and the identification of novel biomarkers has been recognized for some time.[19] The identification of biomarkers in mammalian plasma via metabonomic analysis would be particularly valuable.[19] Eleven potential biomarkers that contributed to the presence of depression and the treatment effect of XYS were preliminary identified in the ^{1}H CPMG NMR spectra and diffusion-edited NMR spectra, which maybe related to the metabolic pathways of gut microbiota, ketone body formation, and others.

In the CPMG spectra, TMAO was a responsible metabolite for discrimination. A major route of biosynthesis of TMAO starts with the degradation of choline, which is first metabolized to trimethylamine (TMA) by the enzymes of the gut microflora.[20] The TMA produced is then normally oxidized to TMAO by the flavin monooxygenase system of the liver.[21] It has been reported that the concentration of urinary TMAO gradually increased over time when germ-free rats were introduced into a normal environment, and the stable gut microbiota was eventually established.[22] In our study, CUMS caused a change in the concentration of TMAO, which might be consistent with the evidence of a significant association between major depression and irritable bowel syndrome demonstrated in literature.[23–26]

Choline, a marker of membrane phospholipids, is the precursor of the neurotransmitter acetylcholine, which acts in the cholinergic neurotransmission.[27] The increase of the choline concentration in CUMS-treatment rats observed in this study suggests a lipoprotein-related and phospholipid-related metabolism dysfunction in plasma in rats with CUMS treatment, which is consistent with previous studies about depression or antidepressants.[27–29] Altered choline level might reflect changes occurring in plasma and could be used as an index of physiological abnormalities, including altered neurotransmitter metabolism and recycling/production rates.[30]

A significant decrease in alanine was also observed after CUMS treatment. This decrease may have been due to either decreased synthesis or increased degradation of alanine.[31] It is hypothesized that glutamate is metabolized to α-ketoglutaric acid by alanine aminotransferase.[32] This is accompanied by the production of alanine, and alanine is then changed to pyruvic acid by the same enzyme, accompanied by the production of glutamate.[33] The decrease may also be explained by metabolism of glutamate, an important excitatory neurotransmitter, which was decreased in the depressive rats.[34–36] Thus, as an excitatory neurotransmitter, the metabolism of insufficient glutamate may have reduced the synthesis of alanine.

Previous studies showed that the ketone concentrations among remitted patients were increased after antidepressant treatment, such as with sertraline.[34,37] The effect of CUMS treatment on ketone body formation was reflected in the decrease of β-HB in the plasma. Ketone production has also been shown to positively influence the production of gamma aminobutyric acid,[38] which earlier studies have shown to be reduced in the plasma of depressed patients.

In this study, branched-chain amino acids (BCAAs), including leucine, isoleucine and valine, were decreased in plasma of the CUMS-induced rats as compared with healthy controls. These BCAAs can transport quickly across the blood–brain barrier as major amino group donors for the synthesis of brain glutamate.[35] Recent studies have demonstrated that BCAAs, especially leucine, contribute most to the derivation in astrocytes of glutamate and glutamine so as to maintain the homoeostasis of brain nitrogen.[39] The neurotransmitter 5-hydroxytryptamine (5-HT) is sensitive to the concentration of BCAAs that are transported via the same carrier system.[35] Thus, the decreased concentration of BCAAs in plasma could be an indication of the disturbed release of brain 5-HT that is highly related to central fatigue.[40] Hence, the decreased BCAAs in plasma may suggest the impaired glutamate homoeostasis as well as reflect central fatigue in response to the chronic stress.

In the diffusion-edited NMR spectra, PtdCho, a component of the plasma membrane, was responsible for discrimination. PhtCho is hydrolysed into *cis*-unsaturated free fatty acid and lysophosphatidylcholine by phospholipase A2 (PLA2) or into choline and phosphatidic acid by phospholipase D followed by further hydrolysis of phosphatidic acid into *cis*-unsaturated free fatty acid and lysophosphatidic acid by PLA2.[41] In this study, the increased level of phosphatidylcholine in CUMS-treated rats compared with controls suggested a disorder of phospholipid metabolism. As a basic process of cellular activity, the metabolic disorder may affect the body's other processes of normal-life activity, in particular, non-specific damage, such as lipid over-oxidation.[42]

As membrane transporters, previous research has also demonstrated that glycoproteins could regulate the intracellular levels of glucocorticoid hormones, as well as the function of the glucocorticoid receptors *in vitro*.[43,44] In connection with other components of the stress hormone system, ageglucocorticoids help to terminate stress-induced hypothalamus-pituitary-adrenal (HPA) system activation via negative feedback inhibition at the level of the hypothalamic paraventricular nucleus and the hippocampus.[45,46] Thus, the disorder of intracellular levels of glucocorticoid hormones induced by the increased level of NAc was consistent with the fact that depressed patients display an increased expression of hypothalamic corticotropin-releasing hormone (CRH).[47,48]

In this study, the plasma level of lipids was significant decreased in CUMS-treated rats. Some lipids are components of well-defined membrane domains termed lipid rafts, which have been shown to contain a variety of signalling and transporter proteins and have been investigated for their role in the pathophysiology of multiple central nervous system disorders.[49]Recently, it has been shown that lipid rafts may play an important role in brain serotonin transport[50] and this may have relevance to the pathogenesis of depression.[51] Further studies are needed to elucidate this hypothesis.

All the results demonstrated that chronic unpredictable mild stresses disturbed the metabolic profile of healthy rats, and the eleven potential biomarkers identified may be useful potential biomarkers for the clinical diagnosis of depression. After administration of XYS, fluoxetine and venlafaxine, the metabolic profiles returned towards baseline to different extents, and rats with XYS treated at middle dose of 46.2 g herb/kg had the most similar metabolic profile to that of the control rats. The results indicated the great efficiency of the formula XYS at the metabonomics level, which provides a novel method for evaluating formulas of Chinese medicine.

Conclusions

In this study, a metabonomic approach based on the NMR technique and chemometrics method was employed to demonstrate the plasmatic metabolic characteristics and physiopathological status induced by CUMS. The clear and consistent biochemical changes following CUMS treatment were identified using PLS-DA analysis. At the same time, 11 potential biomarkers for depression induced by CUMS and the protection by XYS were found. The results of the behavioural analysis and the metabonomics techniques suggested that XYS had a protective effect on depression and the best antidepressant effect in rats was shown at a dose of 46.2 g herb/kg. These results also suggested that metabonomics could connect the symptom pathogenesis of Chinese medicine with modern theory of medicine correctly, with potential biomarkers as the proper link. With the development of databases and analytical techniques, the 'material bases' of Chinese medicine symptoms may become more clear, allowing estimation of the pharmacodynamic action and revealing the complex mechanism of drug action. This approach may offer a powerful technique for essential research in Chinese medicine, providing an experimental foundation for the clinical evaluation of depression and a novel approach for the study of Chinese medicine formulas.

Declarations

Conflicts of interest

The Author(s) declare(s) that they have no conflicts of interest to disclose.

Funding

This work was financially supported by National Natural Science Foundation of China (No. 30772759), Programs of International Science and Technology Cooperation of China (No. 2008DFA30430), Program for Science and Technology Development of Shanxi Province (No. 20100312031), and special funds for talent introduction for Shanxi Province.

References

1. Serretti A *et al*. Depressive syndrome in major psychoses: a study on 1351 subjects. *Psychiatry Res* 2004; 127: 85–99.
2. Delgado PL. Depression: the case for a monoamine deficiency. *J Clin Psychiatry* 2000; 61: 7–11.
3. Xiong JY *et al*. Studies on the antidepression effect of Xiaoyao Powder in mice. *Pharm Clin Chinese Mater Med* 2007; 23: 3–5.
4. Xu ZW *et al*. The experimental research on anti-depression effects of xiaoyaosan decoction and danzhi xiaoyaosan decoction. *Acta Chinese Med Pharm* 2003; 31: 16–17.
5. Chen JX *et al*. Effects of the Chinese traditional prescription Xiaoyaosan decoction on chronic immobilization stress-induced changes in behavior and brain BDNF, TrkB, and NT-3 in rats. *Cell Mol Neurobiol* 2008; 28: 745–755.
6. Willner P. Validity, reliability and utility of the chronic mild stress model of depression: a 10-year review and evaluation. *Psychopharmacology* 1997; 134: 319–329.
7. Nicholson JK, Wilson ID. Opinion: understanding 'global' systems biology: metabonomics and the continuum of metabolism. *Nat Rev Drug Discov* 2003; 2: 668–676.
8. Lindon JC *et al*. Metabonomics technologies and their applications in physiological monitoring, drug safety assessment and disease diagnosis. *Biomarkers* 2004; 9: 1–31.

9. Fiehn O. Metabonomics-the link between genotypes and phenotypes. *Plant Mol Biol* 2002; 48: 155–171.
10. Trygg J *et al.* Chemometrics in metabonomics. *J Proteome Res* 2007; 6: 469–479.
11. Nicholson JK *et al.* 'Metabonomics': understanding the metabolic responses of living systems to pathophysiological stimuli via multivariate statistical analysis of biological NMR spectroscopic data. *Xenobiotica* 1999; 29: 1181–1189.
12. Wang D *et al.* Prevention of chronic stress-induced depression-like behavior by inducible nitric oxide inhibitor. *Neurosci Lett* 2008; 433: 59–64.
13. Wu QF *et al.* ^{1}H NMR-based metabonomic study on the metabolic changes in the plasma of patients with functional dyspepsia and the effect of acupuncture. *J Pharmaceut Biomed* 2010; 51: 698–704.
14. Tang HR *et al.* Use of relaxation-edited one- dimensional and two dimensional nuclear magnetic resonance spectroscopy to improve detection of small metabolites in blood plasma. *Anal Biochem* 2004; 325: 260–272.
15. Wu DH *et al.* An improved diffusion-ordered spectroscopy experiment incorporating bipolar-gradient pulses. *J Magn Reson* 1995; 115A: 260–264.
16. Lin YH *et al.* Effect of chronic unpredictable mild stress on brain-pancreas relative protein in rat brain and pancreas. *Behav Brain Res* 2005; 165: 63–71.
17. Grippo AJ *et al.* The effects of chronic Fluoxetine treatment on chronic mild stress-induced cardiovascular changes and anhedonia. *J Biol Psychiatry* 2006; 59: 309–316.
18. Cui J *et al.* Optimal dose for antidepression effect of Xiaoyaosan. *Chinese J Exp Trad Med Formulae* 2010; 16: 194–198.
19. Lindon JC *et al.* Metabonomics in pharmaceutical R&D. *FEBS J* 2007; 274: 1140–1151.
20. Holmes HC *et al.* Choline and L-carnitine as precursors of trimethylamine. *Biochem Soc Trans* 1997; 25: 96S.
21. Lang DH *et al.* Isoform specificity of trimethylamine N-oxygenation by human flavin- containing monooxygenase (FMO) and P450 enzymes: selective catalysis by FMO3. *Biochem Pharmacol* 1998; 56: 1005–1012.
22. Nicholls AW *et al.* NMR spectroscopic-based metabonomic studies of urinary metabolite variation in acclimatizing germ-free rats. *Chem Res Toxicol* 2003; 16: 1395–1404.
23. O'Mahony SM *et al.* Early life stress alters behavior, immunity, and microbiota in rats: implications for irritable bowel syndrome and psychiatric illnesses. *Biol Psychiatry* 2009; 65: 263–267.
24. Ladep NG *et al.* Depression in patients with irritable bowel syndrome in Jos, Nigeria. *World J Gastroenterol* 2006; 12: 7844–7847.
25. Mine K *et al.* The relation between irritable bowel syndrome and a major depression. *Nippon Rinsho* 1992; 50: 2719–2723.
26. Sudo N *et al.* Postnatal microbial colonization programs the hypothalamic-pituitary-adrenal system for stress response in mice. *J Physiol* 2004; 588: 263–275.
27. Huang YY *et al.* Effects of antidepressant treatment on N-acetyl aspartate and choline levels in the hippocampus and thalami of post-stroke depression patients: a study using H-1 magnetic resonance spectroscopy. *Psychiatry Res* 2010; 182: 48–52.
28. Gabbay V *et al.* Lateralized caudate metabolite abnormalities in adolescent major depressive disorder: a proton MR spectroscopy study. *Am J Psychiatry* 2007; 164: 1881–1889.
29. Kaufman MJ *et al.* Selective serotonin reuptake inhibitor discontinuation syndrome is associated with a rostral anterior cingulate choline metabolite decrease: a proton magnetic resonance spectroscopic imaging study. *Biol Psychiatry* 2003; 54: 534–539.
30. Batra NA *et al.* Proton magnetic resonance spectroscopy measurement of brain glutamate levels in premenstrual dysphoric disorder. *Biol Psychiatry* 2008; 63: 1178–1184.
31. Murakami T *et al.* Forced swimming and imipramine modify plasma and brain amino acid concentrations in mice. *Eur J Pharmacol* 2009; 602: 73–77.
32. Waagepetersen HS *et al.* A possible role of alanine for ammonia transfer between astrocytes and glutamatergic neurons. *J Neurochem* 2000; 75: 471–479.
33. Oldendorf WH. Brain uptake of radiolabeled amino acids, amines, and hexoses after arterial injection. *Am J Physiol* 1971; 221: 1629–1639.
34. Paige LA *et al.* Preliminary metabonomic analysis of older adults with and without depression. *Int J Geriatr Psychiatry* 2007; 22: 418–423.
35. Ni Y *et al.* Metabolic profiling reveals disorder of amino acid metabolism in four brain regions from a rat model of chronic unpredictable mild stress. *FEBS Lett* 2008; 582: 2627–2636.
36. Wang XY *et al.* Metabonomics approach to understanding acute and chronic stress in rat models. *J Proteome Res* 2009; 8: 2511–2518.
37. Fitzgerald RL, Herold DA. Improved CEDIA benzodiazepine assay eliminates sertraline crossreactivity. *J Anal Toxicol* 1997; 21: 32–35.
38. Daikhin Y, Yudkoff M. Ketone bodies and brain glutamate and GABA metabolism. *Dev Neurosci* 1998; 20: 358–364.
39. Shimomura Y, Harris RA. Metabolism and physiological function of branched-chain amino acids: discussion of session. *J Nutr* 2006; 136: 232S–233S.
40. Blomstrand E. A role for branched-chain amino acids in reducing central fatigue. *J Nutr* 2006; 136: 544S–547S.
41. Yaguchi T *et al.* 1-Palmitoyl-2-oleoyl-sn-glycero-3-phosphocholine improves cognitive decline by enhancing long-term depression. *Behav Brain Res* 2009; 204: 129–132.
42. Lv ZP. Lipid peroxidation and protective effect of Xiaoyaosan on 'hepatic stagnation' rats. *J Shandong Coll Trad Chinese Med* 1995; 19: 199–201.
43. Pariante CM. Glucocorticoid receptor function in vitro in patients with major depression. *Stress* 2004; 7: 209–219.
44. Pariante CM, Miller AH. Glucocorticoid receptors in major depression: relevance to pathophysiology and

treatment. *Biol Psychiatry* 2001; 49: 391–404.
45. De Kloet ER *et al*. Brain corticosteroid receptor balance in health and disease. *Endocr Rev* 1998; 19: 269–301.
46. Erkut ZA *et al*. Glucocorticoids suppress corticotropin-releasing hormone and vasopressin expression in human hypothalamic neurons. *J Clin Endocrinol Metab* 1998; 83: 2066–2073.
47. Raadsheer FC *et al*. Increased numbers of corticotropin-releasing hormone expressing neurons in the hypothalamic paraventricular nucleus of depressed patients. *Neuroendocrinology* 1994; 60: 436–444.
48. Raadsheer FC *et al*. Corticotropin-releasing hormone mRNA levels in the paraventricular nucleus of patients with Alzheimer's disease and depression. *Am J Psychiatry* 1995; 152: 1372–1376.
49. Korade Z, Kenworthy AK. Lipid rafts, cholesterol, and the brain. *Neuropharmacology* 2008; 55: 1265–1273.
50. Magnani F *et al*. Partitioning of the serotonin transporter into lipid microdomains modulates transport of serotonin. *J Biol Chem* 2004; 279: 38770–38778.
51. Green P *et al*. Arachidonic acid- containing phosphatidylcholine species are increased in selected brain regions of a depressive animal model: implications for pathophysiology. *Prostaglandins Leukot Essent Fatty Acids* 2009; 80: 213–220.

Supporting Information

Additional Supporting Information may be found in the online version of this article:

Figure S1 HPLC Chromatograms of XYS and its consisted herbs. Peaks 1, 4, 6, 7 originated from Radix Paeoniae Alba, peaks 2, 3, 10 originated from Radix Angelicae Sinensis, peaks 5, 8, 9 originated from Radix
Glycyrrhizae. (1) gallic acid, (4) catechin, (6) albiflorin, (7) paeoniflorin, (8) liquiritin apioside, (9) liquiritin, (10) ferulic acid.
Figure S2 PLS-DA analysis of the control and CUMS model groups (A) the score plot from CPMG spectra; (B) the loading plot from CPMG spectra; (C) the score plot from diffusion-edited spectra and (D) the loading plot from diffusion-edited spectra. ■, Healthy control group (NS); •, CUMS-model group (MS).
Table S1 The effects of Xiaoyaosan, Venlafaxine and Fluoxetine on behavior scores
Table S2 Assignments of the metabolites in plasma from Sprague-Dawley rats

Please note: Wiley-Blackwell are not responsible for the content or functionality of any supporting materials supplied by the authors. Any queries (other than missing material) should be directed to the corresponding author for the article.