Journal of Ethnopharmacology 128 (2010) 482–489

Contents lists available at ScienceDirect

Journal of Ethnopharmacology

journal homepage: www.elsevier.com/locate/jethpharm

Metabolomics study on the anti-depression effect of xiaoyaosan on rat model of chronic unpredictable mild stress

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ARTICLE INFO

Article history:
Received 10 February 2009
Received in revised form 7 December 2009
Accepted 6 January 2010
Available online 14 January 2010

Keywords:
Xiaoyaosan
Anti-depression
Metabolomics
GC–MS
Multivariate analysis

ABSTRACT

Ethnopharmacology: Xiaoyaosan, a famous Chinese prescription, composed of Poria (*Poria cocos* (Schw.) Wolf), Radix Paeoniae Alba (*Paeonia lactiflora* Pall.), Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.), Radix Bupleuri (*Bupleurum chinense* DC.), Radix Angelicae Sinensis (*Angelica sinensis* (Oliv.) Diels), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.), Herba Menthae (*Mentha haplocalyx* Briq.), and Rhizoma Zingiberis Recens (*Zingiber officinale* Rosc.), has been widely used in the clinic for treating mental disorders. Behavior and biochemical analyses indicate xiaoyaosan has obvious anti-depression activity. However, there is no report on the effects of xiaoyaosan using a metabolomics approach.
Aim of the study: A urinary metabolomics method was applied to evaluate the efficacy of xiaoyaosan on rat model of chronic unpredictable mild stress.
Material and methods: Rats were divided into 6 groups and drugs were administered during the 21-day model building period. Urine was measured using GC–MS, processed with XCMS and Microsoft Excel and analyzed by SIMCA-P and SPASS software. Variable importance in projection statistics and loading plot were used to find biomarker ions.
Results: Clear separation between model and each drug group was achieved. High dose group of xiaoyaosan was much closer to control group than middle dose group and amitriptyline group. The time-dependent recovery tendency in high dose group was obtained.
Conclusions: In term of anti-depression effect, high dose xiaoyaosan was the most effective and amitriptyline equaled middle dose xiaoyaosan as shown by metabolomics strategy and behavior tests. Some common and characteristic metabolites on the anti-depression of xiaoyaosan and amitriptyline were obtained. The work showed metabolomics is a valuable tool in studying the efficacy and potential biomarkers of therapeutic effect of complex prescriptions.

1. Introduction

Depression is a serious public mental disease. The mechanism of depression is quite complex. Many synthetic chemical antidepressants were introduced, such as tricyclic antidepressant, selective serotonin reuptake inhibitors and so on. Their therapeutic effects, however, are not satisfying with a variety of side effect such as psychomotor impairment and dependence liability (Sarko, 2000). In search for new therapeutic products for the treatment of depression, medicinal plant research has contributed significantly by demonstrating pharmacological effectiveness of different herbs or their prescriptions (Xu et al., 2003; Zhao et al., 2008a,b).

Xiaoyaosan is a famous traditional Chinese medicine prescription with a long history use in the clinic, containing the following eight herbal medicines: Poria (*Poria cocos* (Schw.) Wolf), Radix Paeoniae Alba (*Paeonia lactiflora* Pall.), Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.), Radix Bupleuri (*Bupleurum chinense* DC.), Radix Angelicae Sinensis (*Angelica sinensis* (Oliv.) Diels), Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.), Herba Menthae (*Mentha haplocalyx* Briq.), and Rhizoma Zingiberis Recens (*Zingiber officinale* Rosc.). From Traditional Chinese medicine (TCM) experience, xiaoyaosan exerts various actions, including soothing the live, improving the circulation of qi to relieve depression. In China, it has been commonly recognized as a safe and effective prescription in the treatment of depressive disorder. Studies have reported that xiaoyaosan showed significant antidepressant effect in decreasing immobility in the tail suspension and forced swimming tests (Xiong et al., 2007).

Until now, a lot of researches have focused on the anti-depression mechanism of xiaoyaosan. Chronic unpredictable mild stress (CUMS), a well-validated animal model, has been used for evaluating antidepressant effects of diverse drugs (Papp et al., 1991; Zhao et al., 2008a,b). Most of the work has been done successfully in individual gene expression, protein structure and

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doi:10.1016/j.jep.2010.01.016

function, as well as biochemical studies on sympathetic nervous system, hypothalamic-pituitary–adrenocortical-axis, noradrenergic and immunological systems, etc. (Grippo et al., 2002; Lucas et al., 2004; Sergeyev, 2005; Bhatnagar et al., 2006). However, existing studies are mainly based on reductionist approaches e.g., receptor binding assays, targets. Little is known about the change of the whole metabolites in an organism treated with xiaoyaosan.

The emerging field of metabolomics provides a promising opportunity to generate novel approaches for addressing the therapeutic effect of drugs, molecular mechanisms, and ultimately towards exploiting new ideal antidepressants. It has been increasingly used as a versatile tool for the discovery of molecular biomarkers in many areas such as diagnosing or prognosing clinical diseases, exploring the potential mechanism of diverse diseases, and assessing therapeutic effects of drugs (Brindle et al., 2002; Nicholson et al., 2002; Kell, 2006; Lindon et al., 2007; Zhao et al., 2008a,b). However, there is no report on the molecular biomarkers for antidepressant effects of xiaoyaosan with a metabolomics approach.

Recent metabolomics technology has successfully applied high-throughput analytical tools to analyze various biological samples and utilized multivariate statistics to extract meaningful biological information from the resultant complex and huge data sets (Lenz and Wilson, 2007; Trygg et al., 2007). Urine has been heavily used in metabolomics studies because it is minimally invasive to the animals or human and primarily reveals an overall metabolic state of the given organism (Beckonert et al., 2007). GC–MS is a robust and unbiased approach to detect unexpected changes (Roessner et al., 2000). Combined with the easily accessible database of NIST (www.nist.gov), GC–MS has gained more application in different fields (Namera et al., 2002; Ni et al., 2007, 2008). In multivariate analysis, principal component analysis (PCA) shows the natural interrelation including possible grouping, clustering and outliers among observations; partial least squares-linear discrimination analysis (PLS-DA) maximizes separation between defined class samples in the data; in some reports, hierarchical cluster analysis (HCA) was also used to estimate linkages between different classes (Parveen et al., 2007). To extract important variables, variable importance in projection (VIP) statistics and loading weights are utilized (Ni et al., 2008). Here we studied the effect of xiaoyaosan in the CUMS rats and explored potential molecular biomarker for the anti-depression effect of xiaoyaosan in comparison with that of a reference antidepressant amitriptyline by metabolomics based on GC–MS.

2. Methodology

2.1. Chemicals

Ethyl chloroformate (ECF), pyridine, anhydrous ethanol, sodium hydroxide, chloroform, and anhydrous sodium sulfate were analytical grade from China National Pharmaceutical Group Corporation (Beijing, China). L-2-chlorophenylalanine (Shanghai Intechem Tech. Co. Ltd., China) was used as an internal quality standard.

Amitriptyline (for many years it has been considered as one of the reference compounds for the pharmacological treatment of depression) was purchased from the pharmacy of Yixintang (Taiyuan, China).

2.2. Preparation of the decoction of xiaoyaosan

The xiaoyaosan was composed of the following eight dried raw materials: 150 g of Poria (*Poria cocos* (Schw.) Wolf), 300 g of Radix Paeoniae Alba (*Paeonia lactiflora* Pall.), 150 g of Radix Glycyrrhizae (*Glycyrrhiza uralensis* Fisch.), 300 g of Radix Bupleuri (*Bupleurum chinense* DC.), 300 g of Radix Angelicae Sinensis (*Angelica sinensis* (Oliv.) Diels), 300 g of Rhizoma Atractylodis Macrocephalae (*Atractylodes macrocephala* Koidz.), 100 g of Herba Menthae (*Mentha haplocalyx* Briq.), and 100 g of Rhizoma Zingiberis Recens (*Zingiber officinale* Rosc.). These eight herbs were purchased from Medicinal Materials Company of Beijing Tongrentang, and authenticated by Prof. Xuemei Qin, School of Pharmacognosy, Department of Chemistry and Chemical Engineering, Shanxi University before preparation. Voucher specimens were deposited in the Herbarium Center of School of Pharmacognosy, Department of Chemistry and Chemical Engineering, Shanxi University.

All the raw materials were extracted by boiling water for three times and then the decoction was dried in vacuo (70 °C) and grinded into powder for use. The yield of the extraction was 27%. The powder was dissolved into water in three different concentrations for use.

2.3. Animals

The study was approved by national legislations of China and local guidelines. A total of 48 male Sprague–Dawley (S.D.) rats (weighing 200 ± 20 g) were commercially obtained from the experimental animal Center of Military Medical Sciences Academy. The rats were maintained under standard laboratory conditions (24 ± 1 °C, 45 ± 15% relative humidity, and 12 h/12 h light/dark cycle) with food and water freely available.

2.4. Chronic unpredictable mild stress procedure and drug administration

After 2 weeks habituation, all the rats were randomly divided into 6 groups ($n = 8$) according to the body weights and behavior scores in open-field experiment: healthy control group (no stress and no drug), CUMS-model group (stress plus pure water), high dose group of xiaoyaosan (stress plus xiaoyaosan at the dose of 13.50 g/kg), middle dose group of xiaoyaosan (stress plus xiaoyaosan at the dose of 6.75 g/kg), low dose group of xiaoyaosan (stress plus xiaoyaosan at the dose of 3.38 g/kg) and amitriptyline group (stress plus amitriptyline 10 mg/kg). Rats in low, middle, and high dose groups were administered the same amount of solution containing different concentrations of xiaoyaosan via gastric intubation, respectively. While the control group was only administered the same amount of distilled water via gastric intubation. The animals, except the control group, were individually housed and repeatedly exposed to a set of chronic unpredictable mild stressors (Harro et al., 1999) as follows: cage tilting and damp sawdust for 24 h (200 ml of water per individual cage, which is enough to make the sawdust bedding wet), noises for 1 h (alternative periods of 60 dBA noise for 10 min and 10 min of silence), swimming in 4 °C cold water for 5 min, exposure to an experimental room at 50 °C for 5 min, 48 h of food deprivation and 24 h of water deprivation, respectively, tail clamp for 1 min, 15 unpredictable shocks (15 MA, one shock/5 s, 10 s duration) and restricted movement for 4 h. One stressor was applied *per* day and the whole stress procedure lasted for 4 weeks with a completely random order. The healthy control rats were housed undisturbed in another experiment room under the same conditions. They had free access to distilled water and food except for the period of water and food deprivation prior to the sucrose preference test.

2.5. Behavior test

2.5.1. Open-field test

The open-field test was performed as previously described (Hallam et al., 2004) and was conducted between 8:00 am to 12:00 am in a quiet room (≤60 dB). The open-field apparatus consisted

Table 1
Effect of xiaoyaosan and amitriptyline on changes of behavior in rats exposed to CUMS on the 21th day.

Groups	C	M	Am	Hx	Mx	Lx
Open-field test						
Rearing	13.5 ± 7.4$^{\#}$	3.28 ± 2.14*	2.8 ± 2.21**	8.14 ± 4.91$^{\#\#}$	8 ± 3.26$^{\#}$	4.14 ± 2.79*
Crossing	75.43 ± 31.84$^{\#\#}$	11.28 ± 5.65**	34.28 ± 17.57$^{*\,\#}$	65 ± 30.34$^{\#\#}$	40.14 ± 26.2*/$^{\#}$	14 ± 7.98**
Dejecta numbers	0.86 ± 0.69$^{\#}$	3.86 ± 2.12*	2.86 ± 2.48	0.57 ± 0.53$^{\#}$	1.28 ± 1.60	3.86 ± 2.54
Body weight change	39.38 ± 5.38$^{\#}$	22.5 ± 11.82*	32.5 ± 10.3	38.5 ± 15.22$^{\#}$	39.88 ± 8.35$^{\#}$	23.12 ± 13.76
Glucose consumption	91.69 ± 2.17$^{\#\#}$	57.86 ± 11.46**	59.17 ± 8.57$^{\#\#}$	79.2 ± 7.46$^{\#\#}$	6.88 ± 12.03*/$^{\#\#}$	81.8 ± 8.96**

Data are expressed as mean ± SD, $n = 8$. C (control group), M (model group), Am (amitriptyline group), Hx (high dose group of xiaoyaosan), Mx (middle dose group of xiaoyaosan), Lx (low dose group of xiaoyaosan).
* $P < 0.05$ versus control group.
** $P < 0.01$ versus control group.
$^{\#}$ $P < 0.05$ versus model group.
$^{\#\#}$ $P < 0.01$ versus model group.

of a square arena 100 cm × 100 cm, with a 40-cm-high side wall, the floor marked with a grid dividing it into 25 equal-size squares. Each animal was tested in the apparatus once. It was placed in the central square and observed for 5 min. Scores were calculated by the amount of time it spent rearing (defined as standing upright on its hind legs), and the number of crossing (grid lines it crossed with at least three paws) and dejecta numbers.

2.5.2. Sucrose preference test

The procedure was performed as described previously (Lin et al., 2005). After a 24-h period of water and food deprivation, each rat was subjected to an individual metabolic cage in which two bottles containing water and 1% sucrose solution were placed. The ratio of the amount of sucrose solution to that of total solution ingested within 1 h represented the parameter of hedonic behavior.

2.5.3. Statistics analysis

Quantitative data were presented as mean ± SE. The significance of variation between groups in data of behavior changes was determined using paired-sample *t*-test by SPSS 11.5 for Windows. The behavior data of each group was compared with that of the model group and the control group, respectively.

2.6. Sample collection and preparation

Urine samples were collected overnight (12 h) in metabolism cages from all the rats. Sodiumazide was added to the collection vessels as an antibacterial agent. After centrifugation at 4000 rmp for 10 min to remove residues, urine samples were immediately stored in aliquots at −70 °C until GC/MS analysis.

The procedure was performed as described previously (Qiu et al., 2007). In brief, each 600 μL aliquot of urine sample was added to a screw-top glass tube, additional 400 μL of anhydrous ethanol, 100 μL of L-2-chlorophenylalanine (internal standard), 100 μL of pyridine, and 50 μL of ECF were added for derivatization. The derivatization procedure was repeated with the addition of 50 μL ECF into the products. The resultant ECF-derivatives was isolated and dried with anhydrous sodium sulfate for following GC/MS analysis.

2.7. GC–MS spectroscopy acquisition

The procedure was performed as described previously (Qiu et al., 2007) but with minor adjustment. The derivatized extracts were analyzed with a trace gas chromatograph coupled with a PolarisQ Ion Trap mass spectrometer (Thermo Fisher Scientific Inc., Waltham, MA, USA). One microlitre of the extract was injected into a DB-5MS capillary column (30 m × 250 μm i.d., 0.25 μm film thickness; 5% diphenyl cross-linked 95% dimethylpolysiloxane; Agilent J&W Scientific, Folsom, CA) in the split mode (5:1). Either the injection temperature or the interface temperature was set to 260 °C; and the ion source temperature was adjusted to 200 °C. Initial GC oven temperature was 80 °C; 2 min after injection, the GC oven temperature was raised to 140 °C with 10 °C min^{-1}, to 240 °C at a rate of 4 °C min^{-1}, to 260 °C with 10 °C min^{-1} again. Helium was the carrier gas with a flow rate set at 1 ml min^{-1}. The measurements were made with electron impact ionization (70 eV) in the full scan mode (m/z 30–550).

2.8. Multivariate statistics and potential biomarkers selection

All the GC–MS raw files were converted to NetCDF format via Xcalibur (Thermo Fisher Scientific Inc., Waltham, MA, USA), and subsequently processed by the XCMS toolbox (http://metlin.scripps.edu/download/) using XCMS's default settings with the following exceptions: xcmsSet (full width at half-maximum: fwhm = 4; S/N cutoff value: snthresh = 8, max = 20), group (bw = 10). The resulting table was exported into Microsoft Excel, where normalization was performed prior to multivariate analyses. The resulting three-dimensional matrix involving peak index (RT–m/z pair), sample names (observations), and normalized peak area percent were introduced into SIMCA-P 11.0 software package (Umetrics AB, Umea, Sweden), which utilizes PCA to display natural separation among the six groups by visual inspection of score plots, PLS-DA to explore the difference between groups by incorporating the known classification and VIP statistics to extract novel potential biomarker ions in the PLS-DA model. Furthermore, hierarchical cluster analysis (HCA) was applied in SPSS 11.5 software package (SPSS Inc., Illinois) to estimate linkages between different classes within the data set. Euclidean distance on the PCs with Ward's linkage methods was used to derive a similarity matrix, which was processed by agglomerative or divisive clustering algorithms to construct a dendrogram.

3. Results and discussion

3.1. Scores on open-field activity, sucrose preference and body weight gain

Open-field test, body weight and sucrose preference test were measured during the experimental period (Table 1). In these tests, there were significant difference ($P < 0.01$) between the rats in high dose group and model group, while no difference between high dose group and control group, suggesting that the metabolite level of rats in high dose group returned to normal level after 21-day treatment of high dose xiaoyaosan in spite of exposure to CUMS, and high dose xiaoyaosan had an obvious effect on stressed rats. Contrary case happened in the low lose group, indicating that low dose xiaoyaosan had little effect on rats exposed to CUMS. Rats in the middle dose group showed a significant increase in

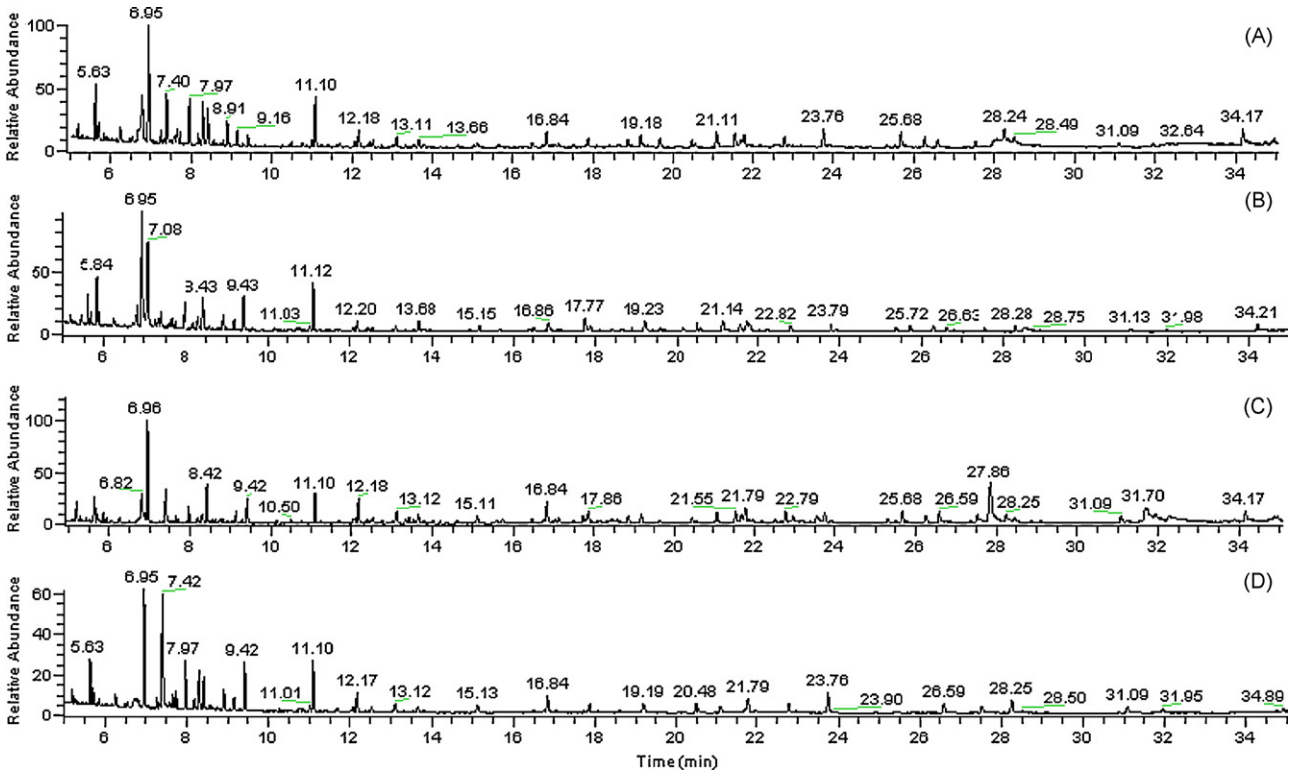

Fig. 1. The GC–MS total ion chromatogram of (A) control group, (B) model group, (C) high dose group of xiaoyaosan and (D) amitriptyline group.

the sucrose preference test ($P < 0.01$), the number of rearing and crossing ($P < 0.05$), and the body weight ($P < 0.05$) compared with model group, but no significant change compared with control group except in the number of crossings. Similar changes were observed in the sucrose preference test ($P < 0.01$) and the number of crossing ($P < 0.05$) in the amitriptyline group. The results indicated that middle dose xiaoyaosan and amitriptyline might have similar antidepressant effect but the antidepressant effect is less than that of high dose xiaoyaosan.

3.2. GC/MS spectra of the six groups

Typical GC/MS chromatograms of urine samples from control group, model group, high dose group and amitriptyline group were illustrated (Fig. 1). Visual inspection of these spectra showed obvious difference among the four groups but the complexity of GC/MS spectra hampered further comparison among groups. Thus, we used XCMS and Microsoft Excel software to pre-treat the GC/MS spectra. The output data set was organized in a three-dimensional matrix encompassing arbitrarily annotated 921 peak indices (RT–m/z pairs), 36 sample names (observations) and peak area percentage (variables).

3.3. Multivariate statistics of the six groups

After processing, all the resulting data sets were subsequently analyzed to examine the clustering of each group by multivariate statistics including PCA and PLS-DA. A ten-component PCA model, accounting for >85.8% of the variance, was initially obtained from the GC–MS data of the six groups. The 2D-PCA scores plot of the second and third principle components (PC2 and PC3) showed that, although there was overlap, except for the low dose group, distinct clustering and clear separation of the 5 groups was obtained. (The low dose group was not discussed in this article because the spots of the low group scattered and one spot located outside of the confidence interval.) The metabolic difference in relation to stress among different drugs and different doses could be reflected. In detail, firstly, a separation of the model group and control group was clearly achieved, suggesting that biochemical perturbation happened in model group. Secondly, high dose group was clearly separated from model group and it was the same case with the middle dose group and amitriptyline group, suggesting that high, middle dose xiaoyaosan and amitriptyline showed apparent anti-depression effect. In additional, high dose group was much closer to control group than both middle dose group and amitriptyline group, showing that the anti-depression effect of high dose xiaoyaosan was the best and that of amitriptyline and middle dose xiaoyaosan was the same but weaker than that of high dose xiaoyaosan.

2D-scores plot from PLS-DA (Fig. 2) showed a better separation than that from PCA resulting from the PLS-DA maximizing the differences between different groups. In 2D-PLS-DA scores plot

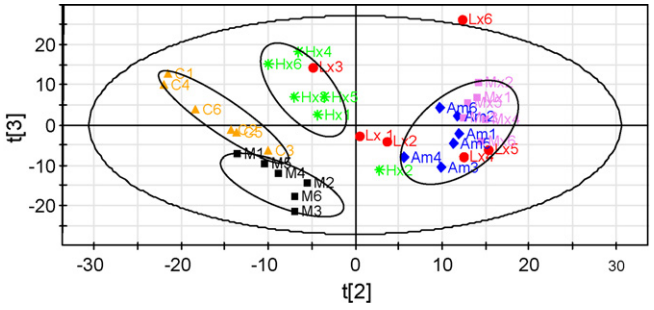

Fig. 2. PLS-DA scores plot derived from GC/MS spectra of the urine from rats in (■ M) CUMS-model group, (▲ C) healthy control group, (□ Am) amitriptyline group, (● Lx) low dose group of xiaoyaosan, (■ Mx) middle dose group of xiaoyaosan and (□ Hx) high dose group of xiaoyaosan on 21th day by SIMCA-P11.0. Distinct clustering and clear separation of model group from control and three drug groups, except the low dose group, was obtained.

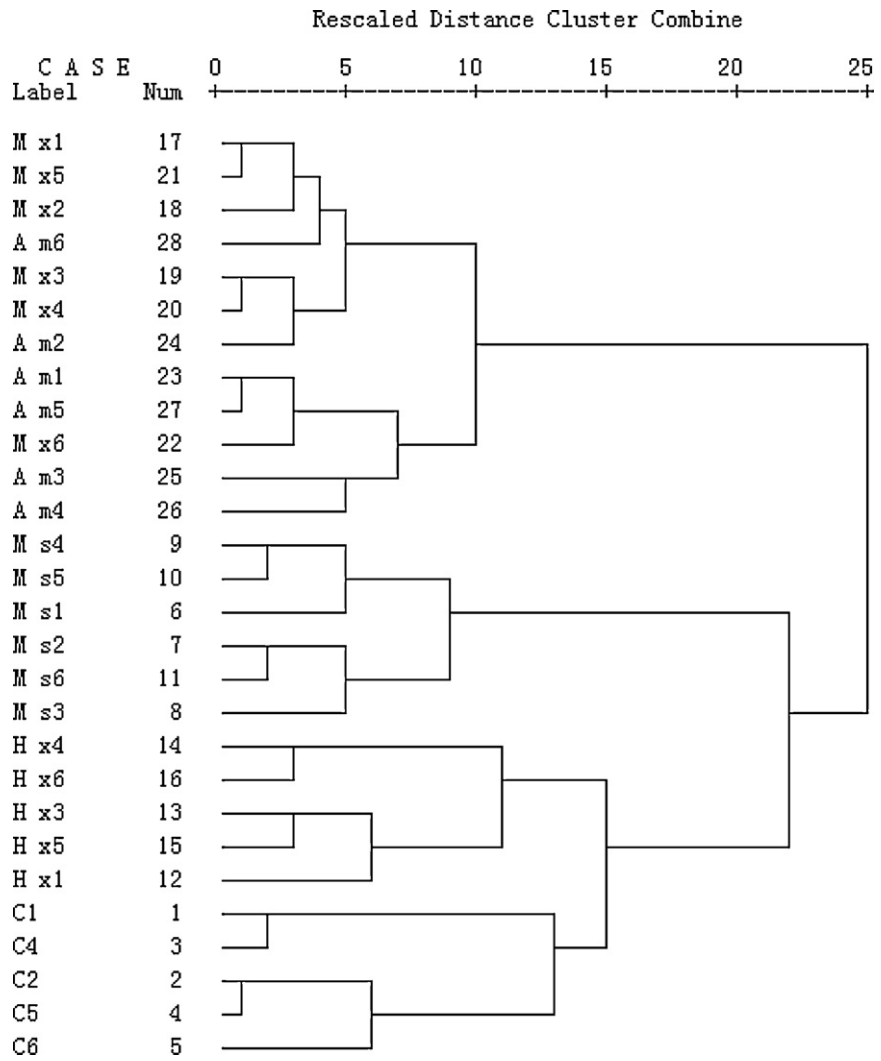

Fig. 3. Dendrogram of hierarchical cluster analysis (HCA) of PC2 and PC3 of CUMS-model group (M), healthy control group (C), amitriptyline group (Am), high dose group of xiayaosan (Hx) and middle dose group of xiaoyaosan (Mx) on 21th day by SPSS 11.5.

all groups clustered and were clearly separated from each other except the low dose group. Similarly, HCA of PC2 and PC3 scores by Euclidean distance separated the samples into two clusters (Fig. 3), with one cluster comprising two clusters admixed with all of the middle dose group and the amitriptyline group. The second cluster was subdivided further into three groups: the high dose group, control group and model group, among which the high dose group and the control group constituted one sub-cluster. All the results from HCA were in agreement with those from PCA and PLS-DA.

In order to gain more insights into drug-related metabolic variations in each group, the initial data set was divided into three subsets according to different groups. The model quality was summarized (Table 2).

3.4. Potential biomarkers responsible for the anti-depression effect of xiaoyaosan

The high and middle dose xiaoyaosan had significant anti-depression effect as shown above. In order to uncover the variables contributing to the anti-depression effect of xiaoyaosan, PCA and PLS-DA were applied to the GC–MS data of high group, middle group and model group. Scores plot from PCA showed obvious separation among the three groups. The PLS-DA showed better discrimination than PCA with a Q^2 value of 0.95 ($R^2X = 0.68$, $R^2Y = 0.98$), suggesting that the model was stable and good to fitness and prediction. The scores plot of the first and second components from PLS-DA (Fig. 4) clearly divided the two xiaoyaosan groups from the model group and a clear separation between the high and middle group was also observed. The difference between model group and xiaoyaosan groups was greater than the individual difference of the rats, showing that the urinary metabolic pattern was significantly changed with the treatment of xiaoyansan. The difference among the three groups revealed that xiaoyaosan had a clear influence on the urinary metabolomes of rat exposure to CUMS and the anti-depression effect of xiaoyaosan was dose dependent.

Table 2
Summary of the parameters for assessing modeling quality.

	PCA model			PLS-DA model			
	No.[a]	R^2X[b]	Q^2	No.[a]	R^2X[b]	R^2Y[b]	Q^2[b]
Hx-M	2	0.663	0.419	3	0.738	0.998	0.981
Mx-M	4	0.779	0.362	2	0.498	0.995	0.923
Am-M	2	0.606	0.371	3	0.678	0.981	0.956

[a] No., the number of components.
[b] R^2X and R^2Y are the cumulative modeled variation in X and Y matrix, respectively, and Q^2 is the cumulative predicted variation in Y matrix. The values of these parameters close to 1.0 indicate a robust mathematical model with a reliable predictive accuracy.

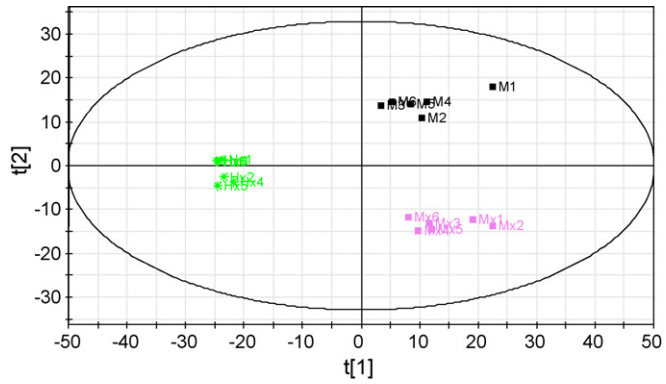

Fig. 4. PLS-DA scores plot derived from GC/MS spectra of the urine obtained from rats in (■ M) CUMS-model group, (□ Hx) high dose group of xiaoyaosan and (■ Mx) middle dose group of xiaoyaosan on 21th day by SIMCA-P11.0. A clearly separation between the model and different doses of xiaoyaosan was obtained, indicating that the urinary metabolic pattern was significantly changed with the treatment of xiaoyaosan.

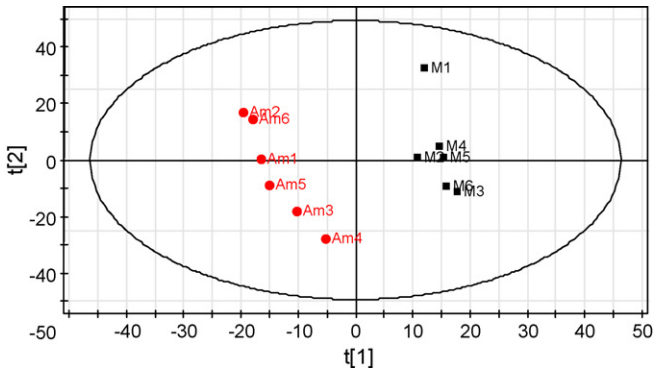

Fig. 5. PLS-DA scores plot derived from GC/MS spectra of the urine obtained from rats in (■ M) CUMS-model group and (● Am) amitriptyline group on 21th day by SIMCA-P11.0.

The important variables accountable for such significant separation could be extracted from loadings plot or VIP statistics of PLS-DA, respectively. The loading plot showed the important variables situating far from the origin but the plot is complex with many variables. VIP ranks the overall contribution of each variable to the generation of the model and was applied to obtain the significant variables. According to the criterion for VIP statistics, variables with VIP value >1.0 are considered as candidate biomarkers (Wold et al., 2001). But, in the VIP plot, some metabolites showed great confidence intervals, suggesting that their contribution to the PLS-DA model might be caused by analytical variation. Such metabolites were excluded from the list. Finally, we generated a list of 13 variables representing individual metabolites as biomarker candidates. Using the commercial databases NIST2005, Huam Metabolome Database (http://www.hmdb.ca) and available literature (Parveen et al., 2007; Qiu et al., 2007), it was possible to identify these biomarker candidates except for four variables, and two amino acids were verified using reference compound. These metabolite ions were shown in Table 3. The table showed that the increased level of five metabolites, oxalic acid, unknown (rt = 23.67 min), hexadecanoic acid, 2-furancarboxylic acid, hexanedioic acid and the decreased level of eight metabolites glycine, hippuric acid, phenylpyruvic acid, unknown (rt = 21.56 min), 8,10-octadecadienoic acid, unknown (rt = 26.28 min), unknown (rt = 27.59 min), tyrosine in the xiaoyaosan groups contributed to the significant differences in metabolomes between model group and xiaoyaosan groups.

Table 3
Potential biomarkers for the anti-depression effect of xiaoyaosan.

	Retention time (min)	Metabolites	Change tendency
1	8.42	Glycine	–
2	17.13	Hippuric acid	–
3	18.86	Phenylpyruvic acid	–
4	19.37	Oxalic acid	+
5	21.56	Unknown	–
6	23.67	Unknown	+
7	23.76	Hexadecanoic acid	+
8	25.68	8,10-Octadecadienoic acid	–
9	26.28	Unknown	–
10	26.60	2-Furancarboxylic acid	+
11	27.59	Unknown	–
12	28.25	Hexanedioic acid	+
13	28.49	Tyrosine	–

+, relatively higher in both high and middle dose groups compared with model group. –, relatively lower in both high and middle dose groups compared with model group.

So these urinary metabolites may be regarded as the potential biomarkers for the anti-depression of xiaoyaosan.

3.5. Common and characteristic potential biomarkers responsible for the anti-depression effect of xiaoyaosan and amitriptyline

PCA and PLS-DA were also applied to the GC–MS data of amitriptyline group and model group. Clear separation between the amitriptyline group and model group was obtained in the scores plots of both PCA and PLS-DA, confirming that the urinary metabolic pattern was significantly changed with the treatment of amitriptyline and amitriptyline had a clear influence on the urinary metabolomes of rat exposure to CUMS. The PLS-DA (Fig. 5) showed a better discrimination than PCA with Q^2 value of 0.908 ($R^2X = 0.60$, $R^2Y = 0.98$), suggesting that the model was stable and good to fitness and prediction. Similar processes were applied to extract the potential biomarkers for the anti-depression effect of amitriptyline. Finally, we generated a list of 13 variables representing individual metabolites as biomarker candidate ions, among which the concentration of four variables increased and nine variables decreased in the amitriptyline group compared with that of model group.

Comparison between the potential biomarkers for the anti-depression effect of amitriptyline and xiaoyaosan was made, resulting in nine common for amitriptyline and xiaoyaosan (Table 4), four characteristic for xiaoyaosan and amitriptyline, respectively (Table 5).

Among the nine common metabolites, there were increased levels of three metabolites including oxalic acid, 2-furancarboxylic acid, hexanedioic acid and decreased levels of six metabolites including glycine, hippuric acid, phenylpyruvic acid, unknown (rt = 21.56 min), 8,10-octadecadienoic acid, unknown (rt = 26.28 min), observed in both the amitriptyline and the xiaosansan groups compared with the model group. These

Table 4
Common potential biomarkers for the anti-depression effect of xiaoyaosan and amitriptyline.

	Retention time (min)	Metabolites	Change tendency
1	8.42	Glycine	–
2	17.13	Hippuric acid	–
3	18.86	Phenylpyruvic acid	–
4	19.37	Oxalic acid	+
5	21.56	Unknown	–
6	25.68	8,10-Octadecadienoic acid	–
7	26.28	Unknown	–
8	26.60	2-Furancarboxylic acid	+
9	28.25	Hexanedioic acid	+

+, relatively higher in amitriptyline group compared with model group. –, relatively lower in amitriptyline group compared with model group.

Table 5
Characteristic potential biomarkers for the anti-depression effect of xiaoyaosan and amitriptyline, respectively.

	Retention time (min)	Metabolites	Change tendency
Xiaoyaosan			
1	23.67	Unknown	+
2	23.76	Hexadecanoic acid	+
3	27.59	Unknown	–
4	28.49	Tyrosine	–
Amitriptyline			
1	8.30	Alanine	+
2	9.63	Benzylcarbinol	–
3	12.18	Unknown	+
4	22.20	Unknown	–

+, relatively higher in xiaoyaosan or amitriptyline group compared with model group. –, relatively lower in xiaoyaosan or amitriptyline group compared with model group.

common metabolites were related to the therapeutic effect of xiaoyaosan and amitriptyline. Moreover, the change tendency of any ion in the xiaoyaosan group and amitriptyline group was consistent compared with the model group.

The four metabolites characteristic for the antidepressant effects of xiaoyaosan included unknown (rt = 23.67 min), hexadecanoic acid, unknown (rt = 27.59 min) and tyrosine. The four metabolites characteristic for the anti-depression effect of amitriptyline was composed of alanine, benzylcarbinol, unknown (rt = 12.18 min) and unknown (rt = 22.20 min). These important metabolites were characteristic for the therapeutic effect of xiaoyaosan and amitriptyline, respectively.

The common and characteristic metabolites revealed that some common and different metabolic pathways might be involved in the therapeutic mechanism of xiaoyaosan and amitriptyline. Exploration of the anti-depression mechanism of xiaoyaosan will be made as a part of our project.

3.6. Time-dependent metabolic trajectory in high dose group of xiaoyaosan

PCA and PLS-DA based on the average peak area percentage in GC–MS spectra of the high dose group were applied to show the time-related trajectory of metabolic pattern with the treatment of high dose xiaoyaosan from day 1 to 21. PLS-DA (Fig. 6) showed a better separation than PCA. In the scores plot of PLS-DA, the spots of day 1 were clearly separated from the other spots from day 7 to 21, suggesting major changes happened in the metabolic network from day 7. These changes in metabolites revealed that the metabolic network during these days might be undergoing a transition period, which is consistent with the fact that a stress reaction state happens in stressed animals at the beginning of the stimuli. Secondly, the spots of day 7–18 gathered near the center of the plot with minor changes along the axis *t*(1) from day 7 to 18, suggesting that minor changes happened during these days with the treatment of both high dose xiaoyaosan and CUMS. It was noted that the spots of day 21 shifted away from the spots of day 7–18 with a tendency back to day 1, which might be an indication of accumulated effect of xiaoyaosan. In all, the plots of day 1–21 showed a contraclockwise change tendency recovering to normal level at the end of the dosing period for the metabolic pattern of high dose group with the treatment of both high dose xiaoyaosan and CUMS. The regression tendency of metabolic network in rats of high group indicated that the anti-depression effect of xiaoyaosan was time dependent.

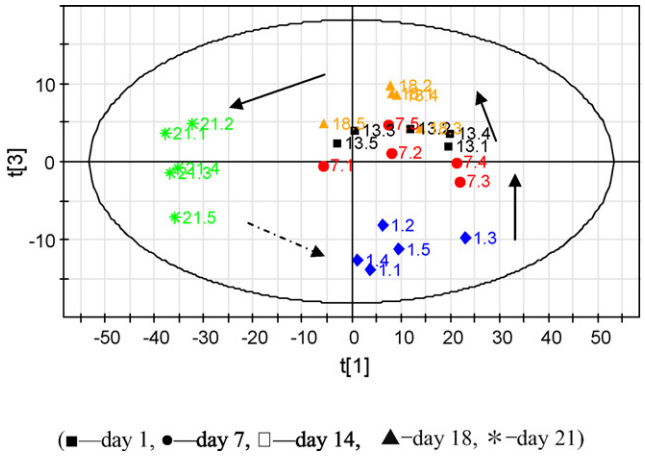

Fig. 6. PLS-DA scores plot derived from GC/MS spectra of the urine obtained from rats in CUMS-model group from day 1 to day 21 by SIMCA-P11.0. The plot showed a time-related trajectory of metabolite patterns at different time points.

4. Conclusions

A metabolomics method based on GC/MS and multivariate statistical techniques has been used to evaluate the efficacy of xiaoyaosan on a depression model of rats induced by CUMS and find the potential biomarkers of the anti-depression effect. Despite a high degree of intersubject variability in the urinary composition, clear biochemical changes with different drugs and different doses was identified using chemometric analysis. Metabolomics analysis with different multivariate statistical techniques and behavior tests reached the same conclusion that the anti-depression effect of high dose xiaoyaosan was the most remarkable and that of the middle dose xiaoyaosan and amitriptyline were the same but weaker than that of the high dose xiaoyaosan. Moreover, the time-dependent regression tendency in high dose xiaoyaosan group from day 1 to 21 was obtained and the effect of xiaoyaosan was time dependent. In urine samples, thirteen metabolites relating to anti-depression effect of xiaoyaosan had been obtained, nine common for xiaoyaosan and amitriptyline, four characteristic for xiaoyaosan and amitriptyline, respectively. Those common and characteristic metabolites suggested that there are some common and specific parts in the anti-depression mechanism of xiaoyaosan and amitripyline. The work demonstrated that metabolomic approach is a potentially powerful tool to evaluate the pharmacological effect and mechanism of complex traditional prescription.

Acknowledgments

The authors wish to thank Qinbao Lin and Jia Wei for assistance with the GC/MS analysis and data analysis. This study was financially supported by National Natural Science Foundation of China (No: 30772759) and International Science and Technology Cooperation Foundation of China (No: 20073726).

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