Metabolic profiles revealed synergistically antidepressant effects of lilies and Rhizoma Anemarrhena in a rat model of depression

Hongli Du1,2* | Hongxia Zhao1* | Xueli Lai3 | Qishan Lin4 | Zhenyu Zhu1 | Yifeng Chai1 | Ziyang Lou1

1 School of Pharmacy, Second Military Medical University, Shanghai, China
2 Department of Pharmacy, Eastern Hepatobiliary Surgery Hospital, Shanghai, China
3 Changhai Hospital, Second Military Medical University, Shanghai, China
4 Proteomics/Mass Spec Facility, Center for Functional Genomics, State University of New York at Albany, New York, USA

Correspondence
Zhenyu Zhu, Yifeng Chai and Ziyang Lou, School of Pharmacy, Second Military Medical University, Shanghai, China.
Email: zzyzyzhuzhu@163.com; yfchai2003c@163.com; ziyanglou@163.com

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Abstract
Depression is a major cause of illness and disability. We applied untargeted metabolomics using mass spectrometry to identify metabolic signatures associated with depression in serum and explored the antidepressant effects of lilies and Rhizoma Anemarrhena on an experimental model of chronic unpredictable mild stress (CUMS). Meanwhile metabolomics based on UHPLC-Q-TOF/MS was used to study the change in metabolites in CUMS rat serum and to evaluate the effects of Rhizoma Anemarrhena and lilies (alone and in combination). Partial least squares-discriminant analysis identified 30 metabolites as decisive marker compounds that discriminated the CUMS rats and the control rats. The majority of these metabolites were involved in amino acid metabolism, the tricarboxylic acid cycle, and phosphoglyceride metabolism. The reliability of the metabolites was evaluated by the administration of lilies, Rhizoma Anemarrhena, fluoxetine and the combination of lilies and Rhizoma Anemarrhena to the CUMS rats. Behavior studies demonstrated that treatment with the combination of lilies and Rhizoma Anemarrhena resulted in optimal antidepressant effects. The combination treatment was almost as effective as fluoxetine. Our results suggest that lilies and Rhizoma Anemarrhena demonstrate synergistically antidepressant effects in CUMS via the regulation of multiple metabolic pathways. These findings provide insight into the pathophysiological mechanisms underlying CUMS and suggest innovative and effective treatments for this disorder.

KEYWORDS
antidepressant, chronic unpredictable mild stress (CUMS), lilies, metabolomic, Rhizoma Anemarrhena

1 | INTRODUCTION
Depression is a common mental disorder. The clinical features of depression include a depressed mood and slow thought-processing. Depression may be accompanied by loss of appetite, loss of interest, weight loss, fatigue and/or insomnia. Patients may lack confidence, have low self-esteem, and even experience suicidal ideation and behaviors (Barber, 2011; Dhingra and Bhandker, 2014; Mizuki et al., 2014). Depression interferes with the daily life and occupational activities of patients, resulting in a considerable burden to the family and community of depressed individuals (Müller et al., 2011). The pathogenesis of depression is complex and the cause of depression remains unclear and involves many factors (Brigitta, 2002; Duman and Voleti, 2012). Synthetic chemical antidepressants can alleviate depressive behaviors.

Abbreviations used: ACTH, adrenocorticotropic hormone; ANOVA, analysis of variance; BH, Bai He – lilies; CORT, corticosterone; CUMS, chronic unpredictable mild stress; EIC, extracted ion chromatogram; ESI, electrospray ionization; Flu, fluoxetine; FST, forced swimming test; HPA, hypothalamic–pituitary–adrenal; OFT, open-field test; TCA, tricarboxylic acid cycle; UHPLC-Q-TOF/MS, ultra-performance liquid chromatography coupled with quadrupole–time-of-flight mass spectrometry; VIP, variable importance; ZB, Zhimu Baihe – combination of lilies and Rhehizoma Anemarrhena; ZM, Zhi Mu – Rhehizoma Anemarrhena.
symptoms. However, high toxicity and side effects such as somnolence, hypertensive crisis and hepatotoxicity are common (Desanty and Amabile, 2007; Khurana and Baudendistel, 2003). Additionally, the currently available antidepressants only provide relief for approximately two-thirds of patients (Deng et al., 2015). Therefore, the exploration of novel, effective and reliable antidepressant drugs is necessary.

Healing with herbs has a long history, and herbs and their extracts play an important role in preventing and treating diseases. Many herbs are associated with treating mood disorders such as depression. Zhimu Baihe Decoction (BZT) is a traditional Chinese medicine that first appeared in JinGuiYaoLue written by Zhang Zhongjing (Han Dynasty). BZT is primarily composed of Rhizoma Anemarrhenae (ZhiMu, ZM) and lily (BaiHe, BH). BH is used for yin deficiency, moisturizing the lungs and the treatment of insomnia and dreaminess, while ZM is used to clear away heat-evil, nourish yin and moisten dryness (Pharmacopoeia Commission of PRC, 2010). In combination, these two herbs are employed to stop dry coughing because of yin deficiency, clear away heat-evil, calm the nerves and contribute to sleep (Li et al., 2015). BZT is also used to treat ‘Baihe disease’. The main symptoms of this disease are imbalance of spirit, diet, sleep, behavior, language and sensory functioning similar to that described in depression (Zheng, 2007). Previous research has demonstrated that BH, ZM and combination of them, Zhimu Baihe (ZB), have certain antidepressant activities (Ren et al., 2006; Guo et al., 2010). The active ingredients are saponin compounds.

Depression is a primary cause of disability and contributes substantially to the burden of metabolic disorders (Cecile et al., 2013). Metabonomics enables the simultaneous quantitative measurement of a large number of low molecular weight molecules in a sample (Peng et al., 2012). Global metabolite profiling of biofluids has been applied to drug discovery and the assessment of the therapeutic or toxic effects of drugs. It has also been used to explore the effects of natural products and traditional Chinese medicines on diseases and their underlying mechanisms (Wu et al., 2014; Ma et al., 2013). We applied a metabolomics approach using ultra-performance liquid chromatography coupled with quadrupole-time-of-flight mass spectrometry (UHPLC-Q-TOF-MS) to identify metabolic signatures associated with depression. These results were used to understand the antidepressant effects of ZM and BH in a chronic unpredictable mild stress rat model of depression (CUMS). The primary objective of the study is to elucidate the antidepressant effects of ZB on metabolic profiles and to examine the underlying mechanisms of depression.

2 | MATERIALS AND METHODS

2.1 | Drugs

Sarsasapogenin, timosaponin BII, timosaponin A-III, timosaponin BIII (Yuanye Bio-Technology Ltd, Shanghai, China), dioscin (Shidande Bio-Technology Ltd, Shanghai, China) and the extracted total timosaponins of ZM and BH (Department of Pharmacognosy of the Second Military Medical University, Shanghai, China) were obtained for use in this study.

2.2 | Quality study of the total timosaponins of ZM and BH

The purities of the total timosaponins from ZM and the total saponins from BH were examined using ultraviolet spectrometry at 284 and 540 nm, respectively. Sarsasapogenin and dioscin were used as controls. The purities of the total saponins in ZM and BH were 76.5 and 61.2%, respectively. UHPLC-Q-TOF-MS was used to determine the concentrations of timosaponin BII, timosaponin AIII and timosaponin BIII (see Supporting Information). The percentage content of timosaponin BII was 37.84%. The percentage content of timosaponin AIII was 0.06%, and the percentage content of timosaponin BIII was 1.60%.

2.3 | Animals

Male Sprague–Dawley rats weighing 180–200 g (Shanghai SLAC Laboratory Animal Co Ltd) were maintained at room temperature (20–23°C), 50 ± 10% humidity and 12 h light/dark cycles with free access to water and food. They were allowed to acclimate for 1 week prior to the start of the experiment. All experiments and procedures were performed according to the Regulations of Experimental Animal Administration issued by the State Committee of Science and Technology of China. The protocol was approved by the Committee on the Ethics of Animal Experiments of the Second Military Medical University.

2.4 | Groups and drug administration

Following acclimatization, the rats were randomly divided into six groups with 12 rats per group. The groups were assigned as follows: group 1, control; group 2, CUMS model; group 3, CUMS model treated with fluoxetine (Flu; 10 mg/kg); group 4, CUMS model treated with ZM (48 mg/kg); group 5, CUMS model treated with BH (48 mg/kg); and group 6, CUMS model treated with ZB (48 mg/kg). ZB consisted of Rhizoma Anemarrhenae saponin and lily saponin and the ratio was 1:1. The administered drugs were dissolved in distilled water and given by gastric gavage once daily from day 7 to day 49. The body weight of each rat was recorded weekly.

2.5 | Chronic unpredictable mild stress procedure and sample collection

With the exception of the control group, the animals in each group were exposed to CUMS using the modified method (Willner et al., 1987). Rats were subjected to stressors once daily for six continuous weeks (day 7 to day 49). These stressors included food or water deprivation for 24 h, tail pinch for 1 min, cage tilting at 45° for 24 h, cold water swimming (at 4°C) for 5 min, exposure to 40°C for 5 min, horizontal shaking for 30 min, electric shock (1 mA for 10 s), reversal of day and night, and damp bedding (300 mL water for each cage). The rats received one of these stressors randomly each day. The identical stressor was not used for more than 2 days in a row to maintain unpredictability. Blood samples of all animals were collected on day 49 after the behavioral tests were completed. Rats were anesthetized using chloral hydrate with dose of 300 mg/kg and blood samples collected by abdominal artery sampling. After clotting at 4°C for 2 h, the blood was centrifuged at 4000 rpm for 10 min. The supernatant samples were...
transferred to Eppendorf tubes and immediately stored at –80°C until analysis.

2.6 | Behavioral tests

2.6.1 | Sucrose preference test

The sucrose preference test was conducted to evaluate the degree of anhedonia in the CUMS rats. The test was performed on day 47 in a quiet, noise-isolated environment. Before the test, two bottles of 1% sucrose solution were provided for each rat for 24 h. Then two bottles, one containing 1% sucrose solution and the other containing tap water, were offered to each rat for another 24 h. The rats were then deprived of water and food for 23 h. Following the deprivation period, one bottle of sucrose solution and one bottle of tap water were given to each rat simultaneously. The volume of sucrose solution and water consumed by the rats in 1 h was recorded. The sucrose preference percentage was calculated using the following formula:

Sucrose preference percentage (%) = sucrose solution consumption/ (sucrose solution consumption + water consumption) × 100

2.6.2 | Open-field test

Open-field tests (OFT) were performed on day 48 in a quiet room between 8:00 a.m. and 14:00 p.m. The open field system was a box (length 100 cm, width 100 cm, height 50 cm) with black peripheral walls. The floor was divided into 25 equal parts with white grid lines. Each rat was individually placed in the central square of the equipment and was allowed to move freely for 5 min. The number of horizontal activities (at least three paws crossing grid lines) and the number of standing actions (as defined as standing upright on hind legs or climbing the walls with two front legs) were recorded. Each horizontal activity or each standing action was counted as one point. The apparatus was cleaned with 75% ethanol between each test.

2.6.3 | Forced swimming test

A cylindrical glass container (height 50 cm, diameter 20 cm) filled with water (depth 30 cm, temperature 25 ± 2°C) was used for the forced swimming test (FST). Rats were individually placed into the container and forced to swim for 6 min. The total number of seconds that each animal maintained an immobile state in the water was recorded for the last 240 s. The duration of immobility was defined as the rat using only the necessary movements to maintain its head above water. This behavior occurred in the absence of active, escape-oriented behaviors, such as swimming, jumping, rearing or diving.

2.7 | Measurement of corticosterone and adrenocorticotropic hormone levels in the serum

Serum samples were collected following the behavioral tests. We measured the concentrations of corticosterone (CORT) and adrenocorticotropic hormone (ACTH) in serum using rat CORT and ACTH ELISA assay kits (Nanjing Jiancheng Bioengineering Institute) strictly following the manufacturer’s instructions. The levels of CORT and ACTH were reported in units of ng/mL and ng/L, respectively.

2.8 | Metabolic analysis of serum samples

2.8.1 | Sample preparation

Serum samples were thawed at 4°C prior to analysis. A 100 μL aliquot of the sample was drawn off, and 300 μL of methanol (containing 12.5 μg/mL 2-chloro-L-phenylalanine as an internal standard) was added to precipitate the proteins. The sample was placed on a vortex mixer for 1 min. The resulting solution was centrifuged at 13,000 rpm for 15 min at 4°C. The clear supernatant fluid (200 μL) was transferred to 2 mL glass vials with 250 μL inserts (Agilent) for UHPLC-MS data acquisition. A quality control (QC) sample was prepared by pooling aliquots (10 μL) from each serum sample after preparation.

2.8.2 | Liquid chromatography and mass spectrometry

UHPLC-Q-TOF/MS analysis was completed with an Agilent 1290 Infinity LC system (TCC, G1316C; Pump, G4220A; Sampler, G4226A) equipped with an Agilent 6538 accurate mass quadrupole time-of-flight mass spectrometer (Agilent, USA). Chromatographic separation was performed on a HSS T3 column (100 × 2.1 mm, 2.5 μm, Waters Corporation, USA). The mobile phases were composed of solution A (0.1% aqueous formic acid) and solution B (acetonitrile with 0.1% formic acid). The elution conditions applied were: 0–2 min, 5% B; 2–17 min, 5–95% B; and 17–19 min, 95% B. Post-time was 5 min. The flow rate was 0.40 mL/min and the injection volume was 3 μL. The temperature of the analytical chromatographic column was 25°C and the temperature of the autosampler was 4°C.

An electrospray ionization source was operated in both positive and negative modes. The positive mode conditions were: capillary voltage, 4 kV; drying gas flow, 11 L/min; gas pressure, 45 psig; fragmentor voltage, 120 V; and skimmer voltage, 60 V. The mass spectrum was acquired from 100 to 1100 m/z. The MS/MS analysis was completed with an Agilent 6538 accurate mass quadrupole time-of-flight mass spectrometer (Agilent, USA). The mobile phases were identical to the positive mode conditions except for capillary voltage (3.5 kV). The MS/MS method was used to validate biomarker candidates. The collision energies were set to 10, 20, 30 and 40 eV.

2.8.3 | Data analysis

Each sample was represented by a total ion chromatogram. The UHPLC-MS raw data were transformed into a common data file format (.mzdata) using MassHunter Qualitative software, version B06.00 (Agilent, USA). The interferences of isotopes were excluded and the absolute peak height was 300 counts to prevent the loss of valuable features. The program XCMS was applied for peak extraction, peak alignment and automatic integration of the extracted peaks. The 80% rule was used to filter the ions. The remainder ions were normalized to the internal standard peak area. Three-dimensional data, consisting of retention time–m/z/pair, observation name and relative ion intensity, were entered into the SIMCA-P 11.0 software package (version11.0, Umetrics, Umeå, Sweden) for principal component analysis and partial least squares discriminant analysis.

Analyses were performed using SPSS 17.0 (IBM, New York, USA). All values are expressed as the mean ± the standard deviation. Data were analyzed by one-way ANOVA followed by Tukey’s post hoc test; the least significant difference-t test was used for multiple comparison. The level of significance was p < 0.05.
3 | RESULTS

3.1 | Behavioral assessment results

3.1.1 | Bodyweight

Bodyweight was used as a visual marker for CUMS rats. Following exposure to a series of stressors, animals demonstrated significant weight loss. The bodyweights of the rats in the CUMS group decreased after the CUMS procedure (Figure 1). The mean weight of the CUMS group was lower than that of the control group on days 14, 21, 28, 35, 42 and 49 and the bodyweight of the CUMS group increased when the rats with the CUMS were administered ZB, BH, ZB and Flu.

3.1.2 | Sucrose preference test, open-field test and forced swimming test

The sucrose preference percentage was measured at the end of the experiment (Figure 2A). The sucrose consumption ratio in stressed rats decreased and was markedly lower than the control rats (p < 0.01). This phenomenon indicated that the CUMS-induced animals lacked interest. The sucrose preferences of the Flu and ZB groups were significantly higher than the CUMS-only group (p < 0.01 and p < 0.05, respectively) following the administration for 42 days. The ZM and BH groups exhibited normal sucrose preferences, but the effect was not as obvious as in the Flu and ZB groups.

The OFT indicated that CUMS group had induced depression-like behavior. Both the horizontal and vertical scores in the CUMS group were lower than those in the control group (p < 0.01, Figure 2B and 2C). Horizontal and vertical scores were improved compared with the CUMS rats when treated with Flu, ZM, BH or ZB. Treatment with Flu, BH and ZB were most effective in all the animal groups (p < 0.01, p < 0.05 and p < 0.01, respectively).

The effects of drug treatments on the FST are shown in Figure 2(D). The immobility time of the FST in the CUMS model group was longer (p < 0.01) than the control group. In the model group with drug interventions, the immobility time was significantly reduced in all treated groups, except for the ZM group.

3.2 | Serum concentrations of corticosterone and adrenocorticotropic hormone

We observed that the concentrations of CORT and ACTH were significantly elevated after the rats underwent the CUMS protocol compared with the healthy control group (Figure 3A,B). When the CUMS rats were treated with Flu, their hormonal levels of CORT and ACTH decreased compared with the CUMS rats (p < 0.05 and p < 0.001, respectively). The hormonal levels of CORT and ACTH decreased when the CUMS rats were treated with ZM, BH and ZB. The treatment with ZM was more effective in reducing CORT and ACTH levels (p < 0.01 and p < 0.05, respectively) in the CUMS rats. Decreased levels of CORT were observed when the CUMS rats were treated with BH and ZB (p < 0.05). However, the BH and ZB treatments had no statistically significant effects on the levels of ACTH.

3.3 | UHPLC-Q-TOF-MS profiling of rat serum

Representative total ion current metabolic profiles of the rat serum samples acquired in positive mode are shown in Figure 4 (Figure S3 in the Supporting Information for negative mode). A QC sample was used to validate the stability of the system during data acquisition, and an injection of QC was performed in the sequence after every eight serum samples. The relative standard deviation of the retention time and the peak area of the internal standard ions were calculated in both the positive and negative modes. The relative standard deviation of the retention time was <0.7% in both ionization modes. The peak intensity in the positive mode was 63.3%, and the peak intensity in the negative mode was 48.4%. These findings indicated that the analytical method was robust and repeatable.

3.4 | Multivariate statistical analyses

A partial least squares discriminant analysis was used to scan the tendencies of all groups (Figure S4) and also used to select potential biomarkers for depression. The scores plots are reported in Figure S5(A and B). The quality of the model was evaluated by the permutation test and was not over-fitting (Figure S5C and D). The scores plot demonstrated that the CUMS group was separated from the control group, suggesting that a metabolic perturbation had occurred in the CUMS model group. The Scatter-plots (S-plots) are shown in Fig S5E and F. The features furthest away from the origin contribute significantly to the clustering of the two groups. In this study, variable importance (VIP) values of 183 ions were >1.0 in positive mode and 140 ions had significant differences (p < 0.05). In negative mode, the VIP values of 124 ions were >1.0 and 83 ions had a significant difference (p < 0.05). Ions with VIPs >1.0 and p-values <0.05 were considered as the potential biomarkers.

3.5 | Identification of the differential metabolites and metabolic pathways

The detailed method for compound identification is discussed below. Accurate m/z values of quasi-molecular ions were entered into relevant online databases (HMDB, http://www.hmdb.ca; Metlin, https://metlin.scripps.edu/) for preliminary identification. A mass tolerance of...
±15 ppm was used. Then, MassHunter Qualitative software was used to formula match. The MS/MS fragments of the matched compounds were obtained from 10 to 40 eV and compared with those in Metlin. Potential markers were finally confirmed by comparison with the authentic compounds. Here, we took m/z 180.065 as an example (Figure S6). One-way ANOVA followed by Tukey’s post hoc test was used to examine the significant differences between the CUMS and the drug treatment groups and to evaluate the therapeutic effect of the different drugs (Table 1). To effectively visualize and characterize the differences of the groups, the heatmap and the bar charts, representing the intensity levels of 30 differential metabolic signatures in the different groups, are shown in Figs S7 and S8, respectively.

The ingenuity pathway analysis method was performed with Metaboanalyst 3.0 (http://www.metaboanalyst.ca) to evaluate the importance of the metabolic pathways. The metabolic view (Figure S9) reports all matched pathways according to p-values from pathway enrichment analysis, and the pathway impact values from pathway topology analysis.

4 | DISCUSSION

4.1 | Effect of drugs on the CUMS model

We used the CUMS model, one of the most effective models for evaluating the activity of antidepressants (Zhao et al., 2008; Zheng et al., 2010). This model simulates various environmental stresses that may cause depression. The behaviors of rats after chronic stimulation are consistent with the clinical behaviors of patients with depression. Therefore, the model is important in the evaluation of antidepressant
drugs (Xu et al., 2013). Behavioral tests, including the sucrose preference test, OFT and FST, were applied to investigate the effects of interventions. The body weights of the rats in the CUMS group were significantly reduced compared with the control group. This finding may be related to lower food intake (Su et al., 2014). In the behavioral tests, the number of activities was reduced in the OFT in the CUMS group. This result indicated that CUMS resulted in rats having less locomotor activity and a decline in curiosity in a new environment. Sucrose preference test results demonstrated that the uptake of sucrose was reduced in the CUMS rats compared with the controls. This result suggests anhedonia, a core symptom of depression. The immobility time in the FST is reflective of despair in the animals and was longer in the CUMS group (Mcarthur and Borsini, 2006). The observed body weights, locomotor activity, sucrose preference reductions and increased immobility time during the FST indicated that the rats were depressed. Rats in treatment groups, including Flu, ZM, BH and ZB, exhibited normal behaviors. ZM demonstrated an improved regulation of weight loss compared with ZB, BH and Flu. The ZB group demonstrated similar results to BH. Flu demonstrated a better effect on sucrose preference compared with ZB, BH and ZM. Flu demonstrated improved OFT (horizontal and vertical scores) and FST compared with ZB, BH, and ZM. ZB was more effective than ZM or BH in the OFT and FST. These results suggested that drug interventions could relieve the depressive symptoms induced by CUMS and the ZB combination demonstrated a better effect compared with ZM or BH individually. The effectiveness of ZB was similar to that of Flu.

4.2 | The effects of drugs on serum corticosterone and adrenocorticotropic hormone

The CUMS model results in behavioral and physiological abnormalities in animals that may relate to an imbalance of monoamine neurotransmitter in the brain, overactivity in the hypothalamic–pituitary–adrenal axis (HPA), and changes in endogenous metabolites. These abnormalities are similar to human depression. Therefore, the model is often used to study the pathogenesis of depression (Campos et al., 2013; Wang et al., 2014). The HPA axis includes the hypothalamus, the pituitary and adrenal glands, which play an important role in body homeostasis and the responses of organisms to environmental stimuli (Boyle et al., 2005; Uma et al., 2009). Stress-induced HPA axis dysfunction is one of the pathological causes of major depressive disorder (Yang et al., 2010). Studies have shown that long-term chronic stimulation may induce HPA axis hyperactivity and increased levels of ACTH and CORT may further induce the overexpression of glucocorticoid receptors (Chen et al., 2012; Pitchot et al., 2001). In our study, the elevated serum levels of ACTH and CORT in the CUMS rats were consistent with previous reports (Cai et al., 2015; Jin et al., 2015). We confirmed that the HPA axis function was over-activated in the CUMS rats. CORT and ACTH returned to normal levels in the intervention groups. Flu was the most effective at restoring CORT, followed by ZM, ZB and BH. The results for ACTH were similar. These results suggest that the antidepressant activity of ZM, BH and ZB may be related to the regulation of HPA activity. This effect was more significant for the single use of ZM.

4.3 | Biological interpretation of biomarkers

4.3.1 | Amino acid metabolism

Glutamine and glutamate metabolism is a vital metabolic pathway in CUMS-induced depression. Glutamate is an important neurotransmitter in the nervous system. It is important for learning and memory (Li et al., 2014). Glutamate and glutamine can transform into one another in astrocytes or neurons. This transformation is essential for their balance. The glutamate system also regulates HPA axis function, further regulating the stress response (Mathew et al., 2001). Glutamate-induced neurotoxicity has been demonstrated in neurological studies of depression (Palucha and Pilc, 2005). In our experiment, glutamate in CUMS rats was significantly increased. This result suggested that CUMS may affect the biosynthesis and metabolism of glutamine and glutamate.

The level of valine was reduced in the CUMS model group. Valine can be produced by pyruvate metabolism, and its reduced level in
CUMS indicated that rats applied pyruvate unreasonably (Shi et al., 2012). This response affects the transformation of succinyl coenzyme A, which plays an important role in the tricarboxylic acid cycle (TCA). Methionine and betaine decreased significantly in the model group. Betaine is a methylating agent in the methionine cycle, enabling the conversion of homocysteine into methionine (Tian et al., 2013). A decrease in betaine may result in a lower conversion rate, a decrease in methionine and an increase in homocysteine. Homocysteine levels are significantly increased in the plasma and serum of patients with depression (Dimopoulos et al., 2007). A reduction in betaine and methionine demonstrated that CUMS may result in the inhibition of the methionine cycle.

### TABLE 1  Significantly differential metabolites in the CUMS rat serum compared with the healthy control and the drug treated groups using UHPLC-MS analysis

<table>
<thead>
<tr>
<th>No.</th>
<th>m/z</th>
<th>Retention time (min)</th>
<th>Variable Importance</th>
<th>Ion</th>
<th>Formula</th>
<th>Metabolite</th>
<th>F-Value</th>
<th>Model</th>
<th>Fluoxetine</th>
<th>Zhimu</th>
<th>Baihe</th>
<th>Mu</th>
<th>He</th>
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<tbody>
<tr>
<td>1</td>
<td>205.097</td>
<td>4.93</td>
<td>5.16</td>
<td>[M + H]+</td>
<td>C₁₁H₁₂N₂O₂</td>
<td>L-Tryptophan</td>
<td>2.196</td>
<td>↑</td>
<td>↓↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>203.083</td>
<td>4.93</td>
<td>3.77</td>
<td>[M – H]⁻</td>
<td>C₁₁H₁₂N₂O₂</td>
<td>L-Tryptophan</td>
<td></td>
<td></td>
<td></td>
<td></td>
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<tr>
<td></td>
<td>162.112</td>
<td>0.73</td>
<td>2.34</td>
<td>[M + H]+</td>
<td>C₆H₁₃N₃O₃</td>
<td>L-Carnitine</td>
<td>9.812</td>
<td>↓↓</td>
<td>↓↓</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>3</td>
<td>118.086</td>
<td>0.74</td>
<td>4.91</td>
<td>[M + H]+</td>
<td>C₆H₁₃N₂O₂</td>
<td>Betaine</td>
<td>9.535</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>4</td>
<td>116.071</td>
<td>0.76</td>
<td>2.29</td>
<td>[M + H]+</td>
<td>C₆H₁₃N₂O₂</td>
<td>L-Proline</td>
<td>11.600</td>
<td>↓↓</td>
<td>↑↑</td>
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<td>5</td>
<td>150.058</td>
<td>0.90</td>
<td>1.10</td>
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<td>C₆H₁₃N₂O₅S</td>
<td>L-Methionine</td>
<td>5.883</td>
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<td>↑↑</td>
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<td>[M + H]+</td>
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<td>L-Valine</td>
<td>5.724</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<td>218.138</td>
<td>2.43</td>
<td>1.17</td>
<td>[M + H]+</td>
<td>C₂₀H₁₈N₄O₄</td>
<td>Propionylcarnitine</td>
<td>5.510</td>
<td>↓</td>
<td>↑↑</td>
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<td>[M + H]+</td>
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<td>3.76</td>
<td>1.28</td>
<td>[M + H]+</td>
<td>C₁₀H₁₉N₂O</td>
<td>Serotonin</td>
<td>4.265</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>11</td>
<td>188.070</td>
<td>4.93</td>
<td>3.52</td>
<td>[M + H]+</td>
<td>C₁₁H₁₈NO₂</td>
<td>Indoleacrylic acid</td>
<td>8.030</td>
<td>↓</td>
<td>↑↑</td>
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<tr>
<td>12</td>
<td>180.065</td>
<td>5.71</td>
<td>1.18</td>
<td>[M + H]+</td>
<td>C₄H₈N₃O₄</td>
<td>Hippuric acid</td>
<td>3.176</td>
<td>↓</td>
<td>↑↑</td>
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<td></td>
<td>178.051</td>
<td>5.71</td>
<td>1.82</td>
<td>[M – H]⁻</td>
<td>C₄H₈N₃O₃</td>
<td>Hippuric acid</td>
<td></td>
<td></td>
<td></td>
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<tr>
<td>13</td>
<td>474.257</td>
<td>6.03</td>
<td>1.42</td>
<td>[M + H]+</td>
<td>C₂₃H₄₈O₇P</td>
<td>LysoPE (0:0/18:3)</td>
<td>19.900</td>
<td>↓↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>14</td>
<td>190.085</td>
<td>8.78</td>
<td>1.86</td>
<td>[M + H]+</td>
<td>C₁₁H₁₅N₂O₂</td>
<td>3-Indolepropionic acid</td>
<td>4.918</td>
<td>↓</td>
<td>↑↑</td>
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<tr>
<td>15</td>
<td>380.256</td>
<td>11.72</td>
<td>1.42</td>
<td>[M + H]+</td>
<td>C₁₈H₃₈N₈O₈P</td>
<td>Sphinocosine-1-phosphate</td>
<td>18.952</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>16</td>
<td>508.340</td>
<td>12.86</td>
<td>1.49</td>
<td>[M + H]+</td>
<td>C₂₅H₅₀N₂O₈P</td>
<td>LysoPE(0:0/20:1)</td>
<td>4.302</td>
<td>↓</td>
<td>↑↑</td>
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<td>17</td>
<td>282.279</td>
<td>13.80</td>
<td>2.75</td>
<td>[M + H]+</td>
<td>C₁₈H₃₈NO</td>
<td>Oleamide</td>
<td>2.680</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>18</td>
<td>322.272</td>
<td>13.80</td>
<td>2.14</td>
<td>[M + Na]+</td>
<td>C₁₈H₃₈N₂O₂</td>
<td>Sphinocine</td>
<td>5.348</td>
<td>↓</td>
<td>↑↑</td>
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<tr>
<td>19</td>
<td>256.264</td>
<td>16.59</td>
<td>1.07</td>
<td>[M + H]+</td>
<td>C₁₆H₃₈NO</td>
<td>Palmiticamide</td>
<td>3.214</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<td></td>
<td>214.047</td>
<td>0.73</td>
<td>1.27</td>
<td>[M – H]⁻</td>
<td>C₁₁H₈N₄O</td>
<td>L-Glutamate</td>
<td>8.632</td>
<td>↑↓</td>
<td>↓↓</td>
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<tr>
<td>22</td>
<td>157.037</td>
<td>0.76</td>
<td>1.11</td>
<td>[M – H]⁻</td>
<td>C₁₂H₆N₃O</td>
<td>Allantoin</td>
<td>4.431</td>
<td>↓</td>
<td>↑↑</td>
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<td>23</td>
<td>145.014</td>
<td>1.13</td>
<td>1.09</td>
<td>[M – H]⁻</td>
<td>C₁₂H₆O₅</td>
<td>Oxoglutaric acid</td>
<td>3.624</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<td>24</td>
<td>191.020</td>
<td>1.28</td>
<td>2.56</td>
<td>[M – H]⁻</td>
<td>C₁₄H₆O₇</td>
<td>Citric acid</td>
<td>4.373</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td></td>
<td>281.281</td>
<td>10.69</td>
<td>9.34</td>
<td>[M – H]⁻</td>
<td>C₂₄H₄₈O₂O₂</td>
<td>Cholic acid</td>
<td>11.113</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>29</td>
<td>391.286</td>
<td>12.42</td>
<td>2.58</td>
<td>[M – H]⁻</td>
<td>C₂₄H₄₈O₄</td>
<td>Murocholic acid</td>
<td>11.113</td>
<td>↓</td>
<td>↑↑</td>
<td></td>
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<tr>
<td>30</td>
<td>303.233</td>
<td>16.95</td>
<td>2.65</td>
<td>[M – H]⁻</td>
<td>C₂ₐH₃₂O₂</td>
<td>Arachidonic acid</td>
<td>8.847</td>
<td>↑</td>
<td>↓↓</td>
<td></td>
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</tbody>
</table>

1Indicates a relative increase in signal.

*indicates a relative decrease in signal.

*p < 0.05,

**p < 0.01,

***p < 0.001 compared with the control group;

*p < 0.05,

**p < 0.01,

***p < 0.001 compared with the CUMS model group.

Metabolites validated with standards.

Metabolites confirmed by MS/MS fragments.

Metabolites putatively annotated.
Phenylalanine is a neutral amino acid. Its main metabolic process is to produce tyrosine through phenylalanine hydroxylase. Tyrosine can be further metabolized into catecholamine neurotransmitter precursors through tyrosine hydroxylase such as dopamine, norepinephrine and epinephrine, which are closely related to depression (Zhao et al., 2015). We reported that phenylalanine was significantly decreased in the model group. Decreased phenylalanine may have affected the tyrosine content and the synthesis of catecholamine neurotransmitters. Several metabolites related to tryptophan metabolism (serotonin, indoleacrylic acid and 3-indolepropionic acid) changed significantly. Serotonin is a neurotransmitter metabolized from tryptophan. Tryptophan increased in the model group while serotonin decreased. We hypothesized that this result may be attributed to the decreased hydroxylation of tryptophan, the enhanced metabolism of serotonin or the enhancement of the metabolic pathway of kynurenic acid. Abnormal regulation of the kynurenine pathway in depression has been reported in both animal and human studies (Réus et al., 2015).

### 4.3.2 Energy metabolism

TCA is correlated with CUMS-induced depression. TCA is an important metabolic pathway in vivo. We identified a variety of substances related to the metabolism of TCA. Citric acid and oxoglutaric acid were both significantly increased in the CUMS group. These substances are related to glycometabolism and energy metabolism. We also detected that carnitine, propionylcarnitine and butyryl-L-carnitine were significantly decreased in the CUMS group. L-Carnitine is an endogenous molecule that can be biosynthesized by lysine and methionine in mammals. L-Carnitine can transport long-chain fatty acids from cytosol to mitochondria for beta-oxidation (Jolanta et al., 2011). Studies have shown that uremia patients undergoing hemodialysis have lower levels of free carnitine. This finding may be associated with a high self-rating on depression scales (Fukami et al., 2014). Carnitine deficiency has been associated with depression in uremia patients on hemodialysis. Acetyl-carnitine supplementation can improve the energy metabolism of mice and increase the level of norepinephrine and serotonin in the brain (Smeland et al., 2012).

### 4.3.3 Other metabolic pathways

We reported significantly increased levels of choline and murocholic acid in the serum of the CUMS group. Bile acids promote excretion, absorption and the transport of fat and cholesterol within the intestine and liver. Cholic acid is one of the primary bile acids. It is essential for the absorption of dietary fat and lipid soluble amino acids in the intestine (Zheng et al., 2011). The up-regulation of cholic and murocholic acid in CUMS rats indicated the metabolic dysfunction of fatty nutrients in the intestines when the rats were depressed. Glycerol phospholipid maintains the integrity of nerve cell membranes and plays an important role in the storage of second messengers (Mulder et al., 2003). LysoPE were significantly lower in the model group compared with the control group. This result suggested that glycerol phospholipid metabolism is related to depression. Sphingosine and sphingosine-1-phosphate were associated with sphingolipid metabolism and they were both decreased in the CUMS group. Sphingolipid plays an important role in cell growth, differentiation, senescence and signal transduction (Birbes et al., 2002; Bourbon et al., 2002). Sphingolipid metabolism disorder may be related to the pathogenesis of depression.

### 4.4 The effects of different interventions

A total of 30 differential metabolites were considered as potential biomarkers involved in the development of depression in the CUMS rat model. Nineteen metabolites normalized after the administration of Flu, 20 metabolites normalized after the administration of ZM or BH and 22 metabolites normalized after the administration of ZB. There were 12 shared metabolites that resulted from the treatments: betaine, L-proline, indoleacrylic acid, 3-indolepropionic acid, allantoin, hydroxyphenyllactic acid, phenylacetylglucose, phenyllactic acid, indolelactic acid, cholic acid, murocholic acid and arachidonic acid. The four antidepressant drugs may target similar metabolic pathways in rats exposed to CUMS. Based on the levels of change, a number of metabolites – betaine, L-proline, L-methionine, L-valine, L-phenylalanine, serotonin, butyryl-l-carnitine, indoleacrylic acid, hippuric acid, LysoPE(0:0/18:4), 3-indolepropionic acid, indolelactic acid, LysoPE(0:0/20:1), allantoin, oxoglutaric acid, phenylacetylglucose, cholic acid, murocholic acid and propionylcarnitine – were improved more in the ZB group compared with the Flu group. These metabolites are involved in various pathways including amino acid metabolism, bile acid metabolism, lipid metabolism and sphingolipid metabolism. Treatment with ZB affected the following 11 metabolites more than ZM or BH: butyryl-l-carnitine, LysoPE(0:0/18:4), hippocric acid, sphingosine, hydroxyphenyllactic acid, murocholic acid, arachidonic acid, oleamide, 3-indolepropionic acid, sphingosine-1-phosphate and betaine. These compounds play a major role in the pathways of arachidonic acid, glyceral phospholipid and sphingolipid. In summary, the CUMS protocol resulted in metabolic dysregulation in rats and the administration of ZB restored the majority of metabolic functionalities in rats exposed to CUMS. Improved antidepressant effects were observed in some of the metabolite pathways, when BH and ZB were administered in combination. This result was consistent with the behavioral tests.

### 5 CONCLUSIONS

We applied a metabolomics approach using UHPLC-Q-TOF-MS and the CUMS model to investigate significant metabolomics changes in rat serum. Thirty-two metabolites related to depression were identified. We examined how CUMS rats responded to the treatment with antidepressant drugs comprising ZM, BH and ZB. Administration of ZM and BH to CUMS rats may restore the hyperactivity of the HPA axis or metabolomics changes. Both ZM and BH have antidepressant effects. The antidepressant effects were more potent when the two drugs were administered in combination. The administration of ZM and BH in combination displayed synergistically antidepressant effects on the CUMS rats via the regulation of multiple metabolic pathways primarily involving amino acids, the TCA cycle and phosphoglyceride metabolism. These findings illustrate the pathophysiological mechanisms underlying CUMS and suggest innovative and effective treatments for this disorder. The metabolomics approach established in this study is useful for screening the potential therapeutic efficacy of
herbs, assessing the protective benefits of herbs in combination and clarifying the mechanisms of natural drug products.

ACKNOWLEDGMENTS

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REFERENCES


SUPPORTING INFORMATION

Additional Supporting Information may be found online in the supporting information tab for this article.