Metabolomic Profiling for Identification of Potential Biomarkers in the Protective Effects of Modified Sinisan against Liver Injury in Dimethylnitrosamine Treated Rats

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Received February 10, 2013; accepted August 12, 2013

Metabolomics is a new platform based on the comprehensive analysis of low molecular weight metabolites and provides a powerful approach to discover biomarkers in biological systems. Modified Sinisan (MSNS), a traditional Chinese medicine formula, displayed bright prospects in the prevention and therapy of liver injury. However, its molecular mechanism of hepatoprotective effects remains unclear. This paper was designed to explore the effects and potential mechanisms of MSNS against dimethylnitrosamine-induced liver injury. Global metabolic profiling was performed by ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-Q-TOF-MS) in conjunction with multivariate data analysis and pathway analysis. Eleven serum biomarkers were identified and pathway analysis results showed that phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, tryptophan metabolism, retinol metabolism, tyrosine metabolism were perturbed by liver injury. Importantly, MSNS has showed satisfactory pharmacological effect on liver injury through partially regulating the perturbed pathways, correlates well to the biochemical and histopathological detection results. The present study proved that the robust metabolomics approach is promising for unraveling hepatoprotective effects of MSNS and these findings provide new insights into mechanisms of the liver injury, and its pathophysiological processes.

Key words metabolomics; serum; traditional Chinese medicine; ultra-performance liquid chromatography; electrospray ionization quadrupole time-of-flight mass spectrometry; liver injury

Sinisan is a widely used traditional Chinese medicine (TCM) formula and was first described in “Treatise on Cold-induced Febrile Diseases (Shanghan Lun),” a treatise on exogenous febrile disease written by the famous Chinese physician Zhang Zhongjing (150 to 219 A.D. in the Chinese Eastern Han Dynasty). It is composed of four herbs including bupleuri radix, paeniae radix alba, aurantii fructus immaturus and glycyrrhizae radix et rhizoma. Sinisan has been popularly used in folk medicine as a hepatic protectant.1 To enhance therapeutic efficacy of liver injury, Sinisan was modified by adding astragali radix, ostrea concha and xiongdan to achieve the property of soothing liver and invigorating Qi, softening hard lumps and dispeling nodes, clearing heat and detoxicating. Modified Sinisan (MSNS) has been clinically used as a common formula and produced evident effect on liver injury,2 However, its potential remedial mechanisms are unclear.

Metabolomics, a promising ‘omics’ platform, is the comprehensive analysis of low molecular weight metabolites in a biological sample.3 In recent years, metabolomics has shown great potential in many fields such as disease diagnosis,4 cancer,5–8 natural product discovery,9 toxicology,10–12 and traditional medicine.13–17 As a significant part of systems biology, metabolomics can be expected to provide new insights into the potential mechanisms of TCM.

In this study, the mechanisms of MSNS on dimethylnitrosamine (DMN)-induced liver injury rats were studied by examining plasma extracts obtained from the rats repeatedly exposed to DMN by the LC/MS-based coupled with pattern recognition approach. At the same time, serum biochemical analysis and histopathologic examinations were performed. The goal of the study is to acquire an overall understanding on the underlying molecular mechanisms of MSNS on liver injury rats. In addition, the identification of potential biomarkers in the protective effects of MSNS against liver injury in serum of dimethylnitrosamine treated rats was investigated by metabolomic approach for the first time.

MATERIALS AND METHODS

Reagents Acetonitrile, formic acid were purchased from Merck (Darmstadt, Germany); Leucine enkephalin was purchased from Sigma-Aldrich (St. Louis, MO, U.S.A.); Dimethylnitrosamine was purchased by Tianjin Chemistry Reagent Research Institution (Tianjin, China); Deionized water was purified “in-house” using a Milli-Q ultra pure water system (Millipore, Bedford, MA, U.S.A.).

Preparation of MSNS Chinese medicines were purchased from Harbin Shiyitang Drug Store (Harbin, China) for preparation of the MSNS extract. MSNS extract is a mixture of 7 crude ingredients: *Bupleurum chinense* DC., *Citrus aurantium* L., *Paonia lactiflora* Pall., Processed Glycyrrhiza uralensis Fisch., *Astragalus membranaceus* (Fisch.) Bge. var. mongholicus (Bge.) Hsiao, *Ostrea gigas* Thunberg, xiongdan. All the crude drugs were of high quality and were authenticated by Prof. Xiaowei Du, Department of Pharmacognosy of Heilongjiang University of Chinese Medicine.

The authors declare no conflict of interest.

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MSNS was prepared at the pharmacognosy laboratory according to the method we optimized before. *Ostrea gigas* THUNBERG was immersed in 1900 mL water and refluxed for 30 min, then the other 6 drugs except xiongdan were added and refluxed for another 60 min. The solution was filtered with eight-layer gauze, and the residue was further extracted one time in the same way. The solutions of twice extractions were mixed and condensed under decompression to roughly 190 mL, and then 2 g xiongdan was added. Finally, the solution was prepared as freeze-dried powder. The dried powder was stored at 4°C before use.

**Treatment of Rats** In this study, a DMN-induced liver injury model was used because it has been well documented and demonstrated to have good repeatability and reliability.\(^{18-20}\) One experiment cycle continued for 4 weeks, and the following procedure was performed immediately prior to the experiments. Male Wistar-derived rats weighing 180–220 g were used. They were provided by The Good Laboratory Practice Centre of Heilongjiang University of Chinese Medicine (Harbin, China), and certificate number was SCXK (Beijing) 2007-0001. The animals had free access to food and water under the standard conditions of humidity (50%±10%), temperature (25±2°C) and 12 h light-dark cycle for 1 week prior to treatment. All protocols were approved by the Animal Use and Care Committee of Heilongjiang University of Chinese Medicine. In this experiment, rats were divided into a treatment group (n=8, treated with 42.45 g crude drug/kg body weight of MSNS each day for 28 d and 10 mg/kg body weight of MSNS each day for 28 d and 10 mg/kg body weight of DMN for 3 consecutive days per week for four weeks); a model group (n=8, treated with oral saline solution each day and 10 mg/kg body weight of DMN for 3 consecutive days per week for four weeks); and a control group (n=8, treated only with oral saline solution each day). After the end of the experimental period, all the animals were sacrificed for tissue sampling and blood collection. The blood samples were allowed to coagulate at 4°C for 30 min. The serum was then separated by centrifugation at 14000×g for 10 min at 4°C. The supernatants were immediately frozen and stored at −80°C until biochemical indicator detection and ultra-performance liquid chromatography/electrospray ionization quadrupole time-of-flight mass spectrometry (UPLC/ESI-Q-TOF-MS) analysis. The livers were removed immediately and used for histopathologic examinations.

**Histopathologic Examination and Biochemical Analysis** Necropsies were performed on all the animals at the end of the experiment. The liver specimens were fixed in 10% neutral buffered formalin, dehydrated, and paraffin-embedded. After being sectioned, the thin sections of the tissues were stained with hematoxylin and eosin for histological observation.

Clinical chemistry analysis of serum samples was carried out with an Automatic Analyzer using appropriate kits. The following parameters were tested: alanine aminotransferase (ALT), aspartate aminotransferase (AST), total bilirubin (TBIL), total protein (TP) and albumin (ALB).

**Serum Sample Preparation for UPLC/MS Analysis** For UPLC/MS and UPLC/MS\(^{+}\) analysis, 500 μL of serum sample was loaded into the centrifuge tube followed by precipitation with 500 μL acetonitrile. Extraction was conducted by vortexing vigorously for 1 min and centrifuged at 16000×g for 10 min, then all the supernatant was transferred to another tube and evaporated at 40°C to dryness. Finally, the residue was reconstituted in 150 μL acetonitrile–water (3:7, v/v), then filtered through 0.22 μm filter membrane and clear solution was transferred to autosampler vial.

**UPLC/MS Analysis** Chromatographic separations were performed on an ACQUITY UPLC BEH C\(_{18}\) column (1.7 μm, 2.1 mm×100 mm, Waters Corp., Milford, U.S.A.), using a ACQUITY™ UPLC system. Column was maintained at 40°C and eluted with a linear gradient of 2–100% B, where A = water with 0.1% formic acid and B = acetonitrile with 0.1% formic acid. The duration of the gradient program was 0–1.5 min, 2% B; 1.5–3 min, 2–30% B; 3–22 min, 30–80% B; 22–24 min, 80–100% B; 24–30 min wash with 100% B; 30–32, 100–2% B and a 5 min recycle time, at a flow rate of 400 μL/min. Five microLiter aliquots of sample were loaded. The column eluent was analyzed by Q-TOF mass spectrometry, using positive ion electrospray ionization (ESI). The column eluent was directed to the mass spectrometer without split.

All MS and MS\(^{+}\) mass spectra were acquired on a Micro-mass Q-TOF Micro™ Mass Spectrometer operating in the positive ion mode. The desolvation gas was set to 750 L/h at a temperature of 350°C. The cone gas was set to 90 L/h and the source temperature was set to 120°C. The capillary and cone voltages were set to 2700 V and 25 V, respectively. The Q-TOF Micro was operated with an alternating collision energy of 6eV. The data acquisition rate was set to 0.2 s, with a 0.02 s interscan delay. All analyses were acquired using the lockspray to ensure accuracy and reproducibility. Leucine enkephalin was used as the lockmass (m/z 556.2771) at a concentration of 50 fmol/L and a flow rate of 5 μL/min. The mass spectrometric full-scan data were acquired from m/z 80 to m/z 1500 Da with a lockspray frequency of 10 s, and data averaging over 10 scans.

**Statistical Analysis** The statistical analyses on UPLC/MS data were performed using Markerlynx Applications Manager Version 4.1 (Waters Corp., Manchester, U.K.). Pareto scaling was applied to the MS integration data prior to principal component analysis (PCA) and orthogonal projection to latent structures discriminate analysis (OPLS-DA). Markers of liver injury were assessed using Student’s t-test.

Clinical chemistry analysis results were expressed as the mean±standard deviation. All data were analyzed using the Statistical Package for the Social Sciences for Windows, version 11.0 (SPSS Inc., Chicago, IL, U.S.A.). The groups were compared using ANOVA and multivariate statistical analysis, and statements of statistical significance were based on p<0.05(*).

**RESULTS AND DISCUSSION**

**Serum Biochemical Measurement** Serum AST, ALT activities, ALB, TBIL and TP levels were all changed significantly from 4 weeks after the treatment of DMN (Table 1). MSNS supplementation attenuated the elevation of AST and ALT activities in the rats treated with DMN. A statistically significant restoration in ALB, TBIL and TP levels was observed in rats treated with MSNS compared with rats in model group. These results demonstrated that MSNS had a good performance in recovering the clinical biochemical in the liver injury model.

**Histopathology Examinations** Histopathological changes of rat livers were shown in Fig. 1, the liver tissue from DMN-
treated rats (Fig. 1B) showed centrilobular necrosis and had more fibrosis, necrotic hepatocytes and degenerative hepato
cytes than that of normal control rats (Fig. 1A). DMN-injured
rat livers showed histological pattern of fibrotic septa and
incorporation of degenerated hepatocytes into pseudolobules.
Central veins were surrounded by collagen fibers. MSNS
supplementation markedly alleviated the degree of liver fibro
sis and significantly reduced the collagen deposition (Fig. 1C).

Establishment of Metabolic Fingerprints Samples of
serum were analyzed by LC/MS to determine what changes in
metabolome are associated with a deleterious effect of
DMN on liver. To optimize the experimental conditions, a
preinvestigation was conducted before the full study. Finger
prints of a small batch of serum samples were acquired in
positive and negative mode, respectively. Relatively, adequate
information of metabolites could be detected in the ESI posi
tive mode. Therefore, full-scan detection was eventually set at
ESI positive mode. We investigated the response of analytes
after the addition of different concentrations (0.01, 0.1 and
1%) of formic acid into the mobile phase and found that better
separation was obtained when 0.1% formic acid was used.
Considering resolution and sensitivity meanwhile, the flow
rate was optimized at 0.4 mL/min. The flow and temperature of
desolvation gas were set at 750 L/h and 350°C, respectively
to remove redundant solvent resulting from the high flow rate
for mass spectrometer. Ten pooled QC samples were run to
“condition” and provide assurance that the system was suit
able for metabonomic analysis. During the analytical run, one
of these pooled QC samples was interspersed between every
five biological samples. After analyzing by UPLC/MS, more
than 6000 ions could be detected in serum.

OPLS-DA and PCA Processing of UPLC/MS Data In
our study, OPLS-DA was used to obtain better discrimination
between the control and model group and to identify poten
tial biomarkers. The OPLS-DA score plots (Fig. 2A) sepa
rated serum samples into two blocks, the control block and
the model block, indicating that the liver injury model was
successfully reproduced. The corresponding loading plot (Fig.
2B) indicated that differentiating metabolites were attributable
to the clustering observed in the score plot. Then S-plot (Fig.
2C) was used to exhibiting the responsibility of each ion for
these variations more intuitively. As we can observe in the S-
plot, most of the ions were clustered around the origin point,
only a few of them scattered in the margin region. Interesting-
ly, just these few ions contributed to the clustering observed
in the score plot.

PCA score plot (Fig. 3) separated serum samples into dif
ferent blocks. Samples subjected to the same treatment were
located on the same trajectory. With regard to information
analyst of PCA, the control and liver injury groups were signifi
cantly divided into two classes. MSNS group developed the
distant tendency to the diverse extent of the liver injury group,
indicating that MSNS could have some therapeutic effect on
the live injury, which was consistent with blood biochemistry
examinations. Moreover, some of the samples in the control
and MSNS groups clustered, suggesting that the MSNS and
the control groups could have similar metabolic profiles.

Identification of Liver Injury-Related Metabolites Metabolites that significantly contributed to the clustering were
identified according to a threshold of variable importance in
the projection (VIP) values, which could be generated after
OPLS-DA processing. Ions with VIP values exceeding two
were selected. Then Student’s t-test was used to select potent
ial biomarkers worthy of preferential study in the next step.
The critical p-value was set to 0.05 for significantly differen
tial variables. Following the criterion above, 11 significantly
differential endogenous metabolites were selected for further
study. The possible elemental compositions of the selected
metabolites were generated by using the MarkerLynx. The
calculated mass, mass deviation (ppm and mDa), speculated
formula, double bond equivalent and i-fit value (the isotopic
pattern) were calculated according to the selected m/z ions.
The smaller mass deviation and lower i-fit value indicate a
more accurate elemental composition. The structure informa
tion was predicted by comparing the accurate MS and MS/MS
fragments with the metabolites searching in databases (http://
The results of identification were shown in Table 2.
Biological Pathway Analysis of Identified Biomarkers

Detailed analysis of pathways and network influenced by liver injury were performed by network analysis. MetPA (Metabolomics Pathway Analysis) is a user friendly, web-based tool that dedicates to the analysis and visualization of metabolomic data within the biological context of metabolic pathways. It combines powerful pathway enrichment analysis with the analysis of pathway topological characteristics to help researchers identify the most relevant metabolic pathways involved in the conditions under study.

Biological pathway analysis revealed that metabolites that were identified together are important for the host response to liver injury and are responsible for phenylalanine, tyrosine and tryptophan biosynthesis, phenylalanine metabolism, tryptophan metabolism, retinol metabolism, tyrosine metabolism, steroid hormone biosynthesis, ubiquinone and other terpenoid-quinone biosynthesis, aminoacid-tRNA biosynthesis, fatty acid metabolism and lysine degradation (Fig. 4 and Table 3). The detailed construction of the phenylalanine, tyrosine and tryptophan biosynthesis pathway (Fig. 5) with the highest score in rats was generated using the reference map by searching KEGG. The impact (pathway impact value calculated from pathway topology analysis) threshold was set to 0.10, above this threshold, were filtered out as potential pathways. We identified 5 unique pathways (listed in Table 3) to the liver injury animals. Results suggested that these pathways showed marked perturbations over the time-course of liver injury and could contribute to the development of liver injury.

Dynamic Changes of Biomarkers

Of the 11 metabolites identified, L-phenylalanine, enol-phenylpyruvate, L-tyrosine, L-palmitoylcarnitine, testosterone, phenylpyruvic acid and carnitine were up-regulated; L-tryptophan, retinyl ester, indoleacetaldehyde and indoleacrylic acid were down-regulated after liver injury. Phenylalanine is an essential amino acid and the precursor for the amino acid tyrosine. Like tyrosine, phenylalanine is the precursor of catecholamines in the body (tyramine, dopamine, epinephrine and norepinephrine). Phenylalanine is highly concentrated in the human brain and plasma. Tyrosine is the precursor for hormones, thyroid, catecholestrogens and the major human pigment, melanin. Tyrosine is an important amino acid in many proteins, peptides and even enkephalins. It has been demonstrated by other investigators that large quantities of phenylalanine and tyrosine were detected in patients with liver injury and these amino acids were specifically released in the patients of liver injury. Significant correlations were observed between phenylalanine and
Table 2. Identification of Biomarkers in Serum of Liver Injury Rats Detected by UPLC/ESI-Q-TOF-MS

<table>
<thead>
<tr>
<th>No.</th>
<th>VIP</th>
<th>Observed [M+H]^+</th>
<th>Rt</th>
<th>Actual-M</th>
<th>Proposed compound</th>
<th>Proposed structure</th>
<th>Trend in model group^a)</th>
<th>Trend in MSNS group^b)</th>
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<tbody>
<tr>
<td>1</td>
<td>9.30</td>
<td>188.0686</td>
<td>3.20</td>
<td>187.0633</td>
<td>Indoleacrylic acid</td>
<td><img src="image" alt="Indoleacrylic acid" /></td>
<td>↓***</td>
<td>↑***</td>
</tr>
<tr>
<td>2</td>
<td>7.11</td>
<td>166.0861</td>
<td>2.89</td>
<td>165.0790</td>
<td>l-Phenylalanine</td>
<td><img src="image" alt="l-Phenylalanine" /></td>
<td>↑***</td>
<td>↓***</td>
</tr>
<tr>
<td>3</td>
<td>4.53</td>
<td>160.0728</td>
<td>2.77</td>
<td>159.0684</td>
<td>Indoleacetaldehyde</td>
<td><img src="image" alt="Indoleacetaldehyde" /></td>
<td>↓***</td>
<td>↑</td>
</tr>
<tr>
<td>4</td>
<td>4.44</td>
<td>303.2288</td>
<td>15.96</td>
<td>302.2246</td>
<td>Retinyl ester</td>
<td><img src="image" alt="Retinyl ester" /></td>
<td>↓***</td>
<td>↑*</td>
</tr>
<tr>
<td>5</td>
<td>4.42</td>
<td>165.0526</td>
<td>0.86</td>
<td>164.0473</td>
<td>Enol-phenylpyruvate</td>
<td><img src="image" alt="Enol-phenylpyruvate" /></td>
<td>↑**</td>
<td>↓</td>
</tr>
<tr>
<td>6</td>
<td>3.57</td>
<td>205.0936</td>
<td>3.20</td>
<td>204.0899</td>
<td>l-Tryptophan</td>
<td><img src="image" alt="l-Tryptophan" /></td>
<td>↓***</td>
<td>↑**</td>
</tr>
<tr>
<td>7</td>
<td>3.55</td>
<td>182.0796</td>
<td>0.86</td>
<td>181.0739</td>
<td>l-Tyrosine</td>
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<td>↓*</td>
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<tr>
<td>8</td>
<td>3.35</td>
<td>162.1089</td>
<td>0.69</td>
<td>162.1130</td>
<td>Carnitine</td>
<td><img src="image" alt="Carnitine" /></td>
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<td>↓***</td>
</tr>
<tr>
<td>9</td>
<td>3.28</td>
<td>165.0515</td>
<td>1.55</td>
<td>164.0473</td>
<td>Phenylpyruvic acid</td>
<td><img src="image" alt="Phenylpyruvic acid" /></td>
<td>↑*</td>
<td>↓*</td>
</tr>
<tr>
<td>10</td>
<td>2.44</td>
<td>289.2157</td>
<td>11.58</td>
<td>288.2089</td>
<td>Testosterone</td>
<td><img src="image" alt="Testosterone" /></td>
<td>↑***</td>
<td>↓***</td>
</tr>
<tr>
<td>11</td>
<td>2.17</td>
<td>400.3380</td>
<td>16.06</td>
<td>399.3348</td>
<td>l-Palmitoylcarnitine</td>
<td><img src="image" alt="l-Palmitoylcarnitine" /></td>
<td>↑*</td>
<td>↓</td>
</tr>
</tbody>
</table>

^a) Change trend compared with control group. ^b) Change trend compared with model group. The levels of potential biomarkers were labeled with (↓) down-regulated and (↑) up-regulated (*p<0.05; **p<0.01; ***p<0.001).
tyrosine concentrations and total bilirubin levels and albumin levels. Serum phenylalanine and tyrosine concentrations correlated well with imaging and laparotomy findings of liver injury.\textsuperscript{23} It is also well-established that L-phenylalanine is catabolized in the liver and its metabolism depends on the integrity of liver cell function, liver cell injury could potentially cause an elevated L-phenylalanine concentration in the serum.\textsuperscript{24–28} It has been demonstrated by other investigators that central plasma clearance of amino acids accurately predicts hepatocyte function in patients with liver disease and correlates with clinical outcome.\textsuperscript{29,30} In the present study, we observed that the serum levels of phenylalanine and tyrosine decreased after DMN administration, indicating that phenylalanine, tyrosine and tryptophan biosynthesis pathway was disturbed.

Tryptophan is an essential amino acid which is the precursor of serotonin. Serotonin is a brain neurotransmitter, platelet clotting factor and neurohormone found in organs throughout the body. Metabolism of tryptophan to serotonin requires nutrients such as vitamin B\textsubscript{6}, niacin and glutathione. Niacin is an important metabolite of tryptophan. Some disorders of excess tryptophan in the blood may contribute to mental retardation. Assessment of tryptophan deficiency is done through studying excretion of tryptophan metabolites in the urine or blood. Blood may be the most sensitive test because the amino acid

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Fig. 4. Summary of Pathway Analysis with MetPA

(1) Phenylalanine, tyrosine and tryptophan biosynthesis; (2) Phenylalanine metabolism; (3) Tryptophan metabolism; (4) Retinol metabolism; (5) Tyrosine metabolism; (6) Steroid hormone biosynthesis; (7) Ubiquinone and other terpenoid-quinone biosynthesis; (8) Aminoacyl-tRNA biosynthesis; (9) Fatty acid metabolism; (10) Lysine degradation.

Fig. 5. Construction of the Phenylalanine, Tyrosine and Tryptophan Biosynthesis Pathways in Rats
The map was generated using the reference map by KEGG (http://www.genome.jp/kegg/). The gray boxes: enzymatic activities with putative cases of analogy in rats.
tryptophan is transported in a unique way. Increased urination of tryptophan fragments correlates with increased tryptophan degradation, which occurs with oral contraception, depression, mental retardation, hypertension and anxiety states. The amount of tryptophan in the serum was increased significantly after administration of DMN, which might be induced by disorders of tryptophan metabolism.

Retinyl ester is a substrate for lecithin retinol acyltransferase and retinyl pigment epithelium-specific 65 kDa protein. Retinyl ester is one of the major retinoid species that are present in the body. Retinoids (vitamin A and its metabolites) are required for life and act essentially in maintaining normal cell proliferation and differentiation, vision system, normal male and female reproduction, and a healthy immune. Approximately 80% of the body vitamin A is stored in hepatic stellate cells (HSC) with in the lipid droplets as retinyl esters. After liver injury caused by exposure to hepatotoxins, the HSC becomes “activated.” A characteristic feature of HSC activation is the loss of the lipid droplets and the retinoid stores they contain. Leo and Lieber found that there is approximately 80% of the body vitamin A is stored in hepatic stellate cells (HSC) with in the lipid droplets as retinyl esters.

Taken together, our work highlights the importance of metabolomics to elucidate metabolic characters of liver injury and therapeutic effects of MSNS at the global metabolic levels. We identified 11 potential biomarkers and predicted the drug efficacy is highlighted once more.

**Acknowledgments** This work was supported by the Grants from Natural Science Foundation of Heilongjiang Province (D201037), Heilongjiang Provincial Administration of Chinese Medicine Project (ZHY10-Z47), Heilongjiang Postdoctoral Fund (LBH-Z09010), Heilongjiang University of Chinese Medicine Research Fund (200909).

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