SCIENTIFIC REPORTS

OPEN

SUBJECT AREAS:
DRUG DISCOVERY AND DEVELOPMENT
METABOLOMICS

Received
3 October 2013

Accepted
29 September 2014

Published
24 October 2014

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Urinary metabolic profiling of rat models revealed protective function of scoparone against alcohol induced hepatotoxicity

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Alcohol-induced liver disease (ALD) is a leading cause of non-accident-related deaths in the world. Identification of an early specific signature of ALD would aid in therapeutic intervention. Scoparone is an important constituent of Yinchenhao, and displayed bright prospects in hepatoprotective effect. However, its precise molecular mechanism has not been well explored. The present study was designed to assess the effects and possible mechanisms of scoparone against alcohol-induced liver injury. UPLC/ESI-Q-TOF/MS combined with pattern recognition approaches including PCA, and PLS-DA were integrated to get differentiating metabolites for the pathways and clarify mechanisms of disease, highlight insights into drug discovery. The results indicated four ions in the positive mode were characterized as potential differentiating metabolites which can be regulated by scoparone treatment, and suggested that therapeutic effect of scoparone could regulated the dysfunctions of citrate cycle, sphingolipid metabolism, taurine and hypotaurine.

Metabonomics is concerned with the study of low molecular weight (MW) compounds (typically $<$ 1000 Da) in biofluids and tissue extracts to provide systemic views of biological processes[1]. Metabolites are the end products of cellular adjustment processes, and their levels can be regarded as the ultimate response of biological systems[2]. Metabolomics has been shown to have enormous potential when applied to subjects as diverse as, toxicological mechanisms[3,4], disease processes[5], and drug discovery[6–8]. Various analytical techniques, with multivariate data analysis, such as principal components analysis (PCA), partial least squares-discriminant analysis (PLS-DA) have been applied in metabolomic-based drug metabolism studies. Today, UPLC/ESI-Q-TOF/MS has become one of the widely applied techniques in metabolomics studies owing to its high sensitivity and reproducibility[9]. As the newest of the "omics" sciences, metabolomics has brought much excitement to the field of life sciences as a potential translational tool, and offers a global analysis of low-molecular-weight metabolite in biological samples, attempts to capture global changes and physiological status in biochemical networks and pathways in order to elucidating sites of perturbations[10].

Excessive alcohol consumption is the third most common cause of lifestyle-associated mortality in the world andmore than half if these deaths were attributed to alcohol-induced liver disease (ALD), an early, and reliable tool to assess ALD risk would be helpful for intervention[11]. Only an estimated 13% of people with identified ALD have ever received specialty treatment due to the lack of effective medications that ameliorate withdrawal syndrome and cure alcohol dependence[12,13]. There is an urgent need for the development of new, more effective medications. Yinchenhao (*Artemisia annua* L.) is one of the most popular traditional Chinese medicinal plants for treatment of liver injury and has been used more than one thousand years (Chinese Pharmacopoeia Commission 2010). Interestingly, a number of studies have shown that scoparone (Fig. 1) was an important chemical substance with activities to cure hepatic injury in *Artemisia annua* L.[14]. As one of the main active constituents of Yinchenhao, scoparone has been proven effectively in treating liver diseases, and shows hepato-protectivity and contributes directly to the therapeutic effect[15]. Therefore, all these activities suggested that scoparone may be a good lead compound for further new drug studies. However, information about scoparone's metabolomics characteristic that is very important for new drug discovery, has not been found in the published literatures up to now. It is well known that the metabolomics study of a bioactive constituent can help us to

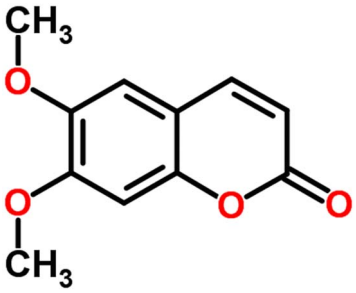

Figure 1 | Chemical structures of scoparone.

understand its *in vivo* actions and explain a variety of events related to efficacy and toxicity[16]. Therefore, the importance of understanding the metabolic profiles of scoparone is evident, and a corresponding metabolomics study is undoubtedly required.

Common tools used for metabonomic studies include nuclear magnetic resonance (NMR) spectroscopy and gas or high-performance liquid chromatography coupled to mass spectrometry. Ultra-performance liquid chromatography coupled to mass spectrometry (UPLC/MS) is the tool with the highest resolution and this sensitive technique is considered a powerful tool in metabonomics because of its ability to obtain multiparametric metabolite profiles from biofluids rapidly and effectively[17,18]. The power of mass spectrometry-based metabolomics to capture and elucidate metabolic changes during alcohol consumption has been demonstrated in this study. Here, in this paper, we describe the results of LC-MS-based metabonomic investigations on the liver metabolomes of rats for the endogenous metabolites using alcohol-fed models, with metabolite identification via high accuracy MS^n analysis.

Results

LC-MS analysis of metabolic profiling. Using the optimal reversed-phase UPLC-MS conditions described above, the representative total ion current (TIC) chromatograms of urine samples obtain from UPLC/ESI-Q-TOF/MS analysis for continuous eight days are shown in Fig. 2. All the data containing the retention time, peak intensity and exact mass were imported in the Masslynx™ software for multiple statistical analyses. Both PCA and PLS-DA often can be taken, because of their ability to cope with highly multivariate, noisy, collinear and possibly incomplete data. PCA is an unsupervised pattern recognition method initially used to discern the presence of inherent similarities in spectral profiles. Typically, the trajectory analysis of PCA score plots for the alcohol treatment in positive mode can really reflect the differences between the the 1st day and the 7th day, and showed metabolic profiles in the different days were separated clearly (Fig. 3). The PCA plot of the model group show similar behavior during the early stage of the experiment, but then gradually deviated from one another, on day 7 reached the maximum trend. The tracks of the metabolic profiles at different time points also clearly demonstrate the time dependent changes in the urine metabolites. The corresponding PLS-DA loadings plot indicated that differentiating metabolites were attributable to the clustering observed in the scores plot. The farther away from the origin, the higher the VIP value of the ions was. Four ions showed significant difference in abundance between the control and treated animals and contributed to the observed separation were selected from the loading plot of PLS-DA (Fig. 4). The UPLC-MS analysis platform provided the retention time, precise molecular mass and MS/MS data for the structural identification of biomarkers.

Identification of metabolite candidates. All collected samples were analyzed and low molecular weight metabolites were represented as the chromatographic peaks in the TIC chromatograms. The information including the retention time, the exact mass and the ms/ms

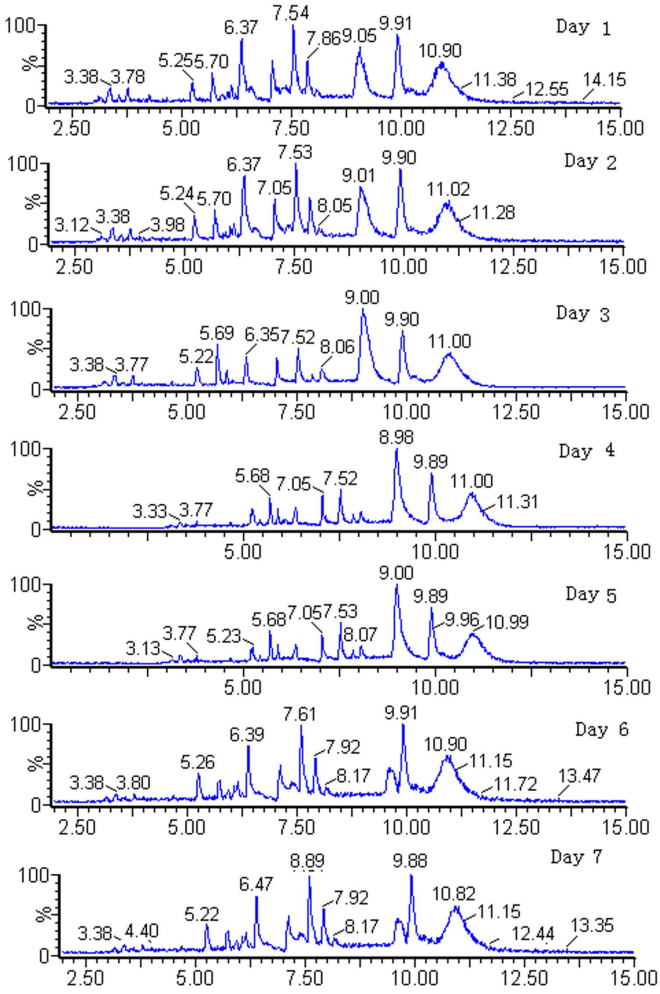

Figure 2 | A typical total ion chromatograms of urine obtain from UPLC/ESI-Q-TOF/MS analysis.

data were supplied by the robust UPLC-MS platform. The precise molecular mass was determined within a reasonable degree of measurement error using Q-TOF, and the potential element composition, degree of unsaturation and fractional isotope abundance of the compounds were also obtained. The loading plot from the PLS-DA based on UPLC/ESI-Q-TOF/MS data (10297 variables) was shown in Fig. 4. The distance of an ion from the origin represents the contribution to the clustering of different groups on the PCA. We searched for the presumed molecular formula in the ChemSpider, Human Metabolome Database, KEGG, and Small Molecule Pathway Database to confirm possible chemical compositions, and the MS/MS data were helped to identify the potential biomarkers. The collision induced dissociation experiment was implemented to get fragmentation patterns of these potential biomarkers. Furthermore, metabolite identification was conducted with high resolution MS and MS/MS fragments, as well as database analyses. For example, the postulated elemental compositions for the ions of 1,1'-(1,8-naphthylene)bis(1H-1,2,3-triazole-4,5- dicarboxylic acid) tetra-tert-butyl ester are given in Fig. 5. The metabolite, which gave an elemental composition of $C_{34}H_{42}N_6O_8$, and molecular mass of $[M+H]^+$ 663.3013, were identified. The $[M+H]^+$ 663.3013 demonstrated even number nitrogen, the product spectra such as m/z 607, 551, 495, and 439 contributed to $[M+H]^+$ peak continuous loss $-C_4H_8$ when subjecting to MS/MS analysis, and retrieving corresponding literatures. Finally, it was speculated as 1,1'-(1,8-naphthylene)bis(1H-1,2,3-triazole-4,5-dicarboxylic acid) tetra-tert-butyl ester after searching in the database. According the

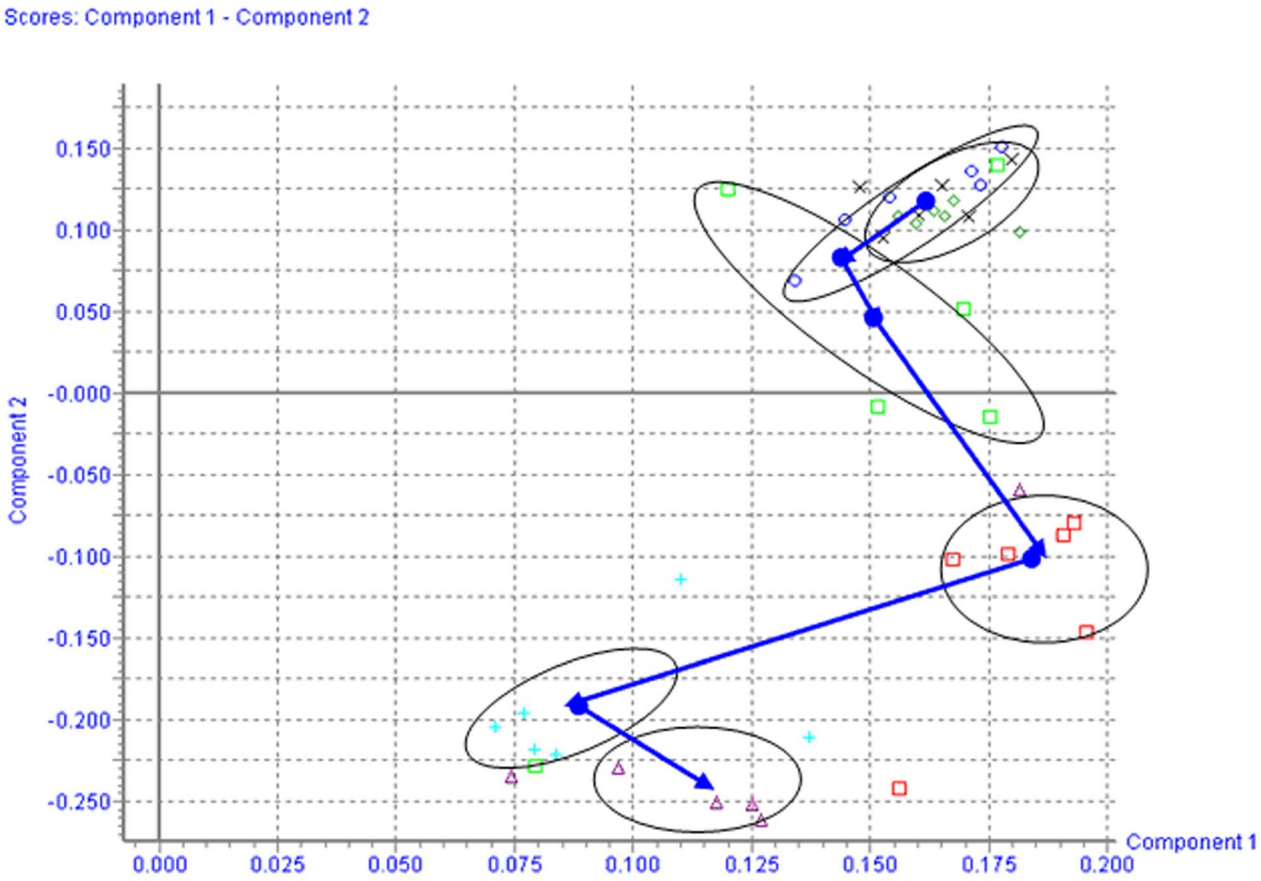

Figure 3 | **Trajectory analysis of PCA score plots for the alcohol treatment in positive mode.** (×: the 1st day; ○: the 2nd day; ◇: the 3rd day; □: the 4th day; □: the 5th day; +: the 6th day; △: the 7th day)

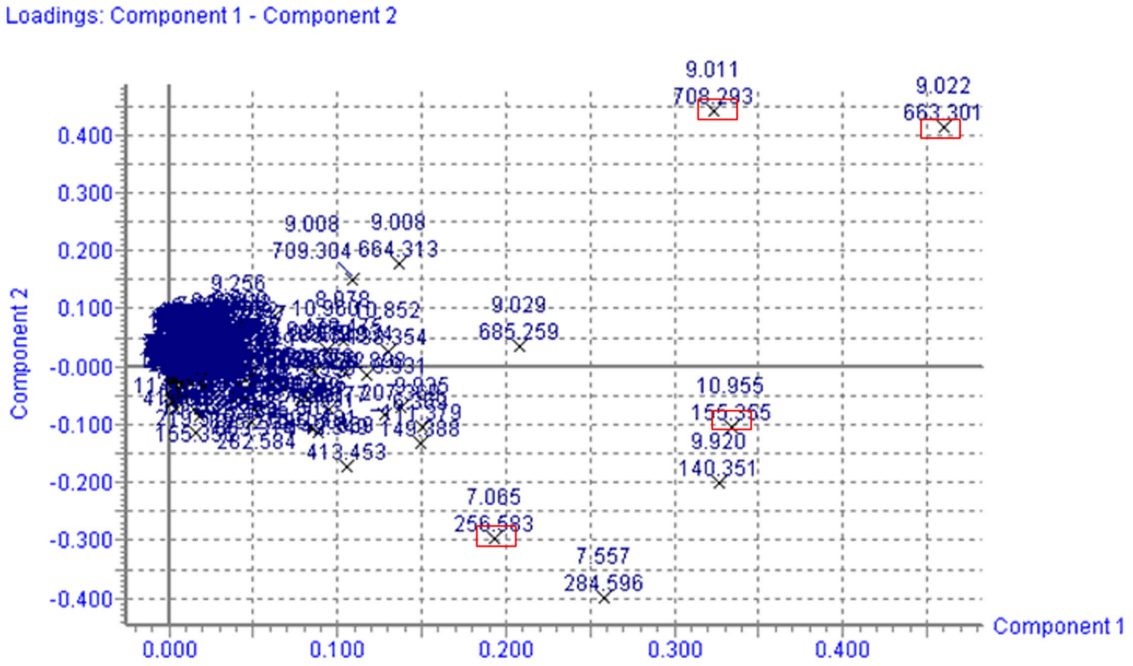

Figure 4 | **Loading plot of metabolome in rat urine from model group.** The loading plot represents the impact of the metabolites on the clustering results. PLS-DA loading plots displayed variables positively correlated with score plots. Statistically and significantly different metabolites responsible for the discrimination of the two groups were identified between the control and model group. Red data points indicate that ions most responsible for the variance in the score plot.

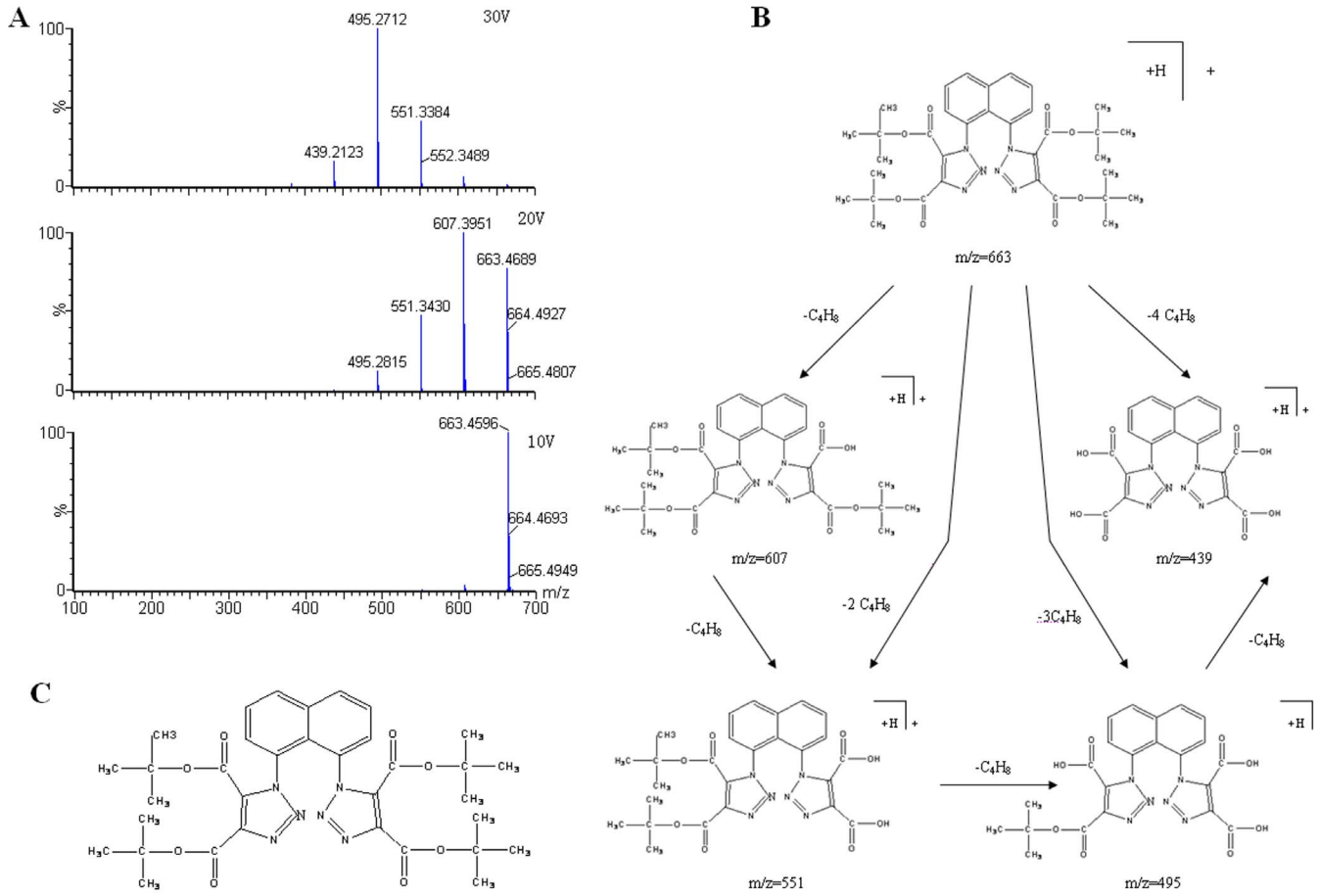

Figure 5 | Typical identification of potential biomarker 1,1'-(1,8-naphthylene)bis(1H-1,2,3-triazole-4,5-dicarboxylic acid) tetra-tert-butyl ester in urine using UPLC-ESI-QTOFMS-based metabolomics. (A). Mass spectrum of full scan and product ion scan of determined biomarkers. (B): Possible fragmentation pathway; (C): Chemical structure of glycocholate.

protocol described above, 2-pyrocatechuic acid, 3-methoxy-4-hydroxyphenylglycol sulfate, glucosylceramide (d18 : 1/18 : 0) were identified and summarized in supplementary table 1.

Changes of relative intensity of biomarkers. According to the protocol detailed above, five endogenous metabolites contributing to the separation of the model group and control group were detected in the urine samples. The ions identified by UPLC/ESI-Q-TOF/MS are summarized in supplementary table 1 with their corresponding retention time, m/z, ion mode, and related trends. Fig. 6 showed score plot of PCA for the acute livery injury after scoparone treatment in positive mode. The relative mean height intensity of different metabolites was graphed in Fig. 7. Monitoring changes of these metabolites may predict the development of liver injury. Additionally, the relative concentration of four endogenous metabolites was significantly affected by scoparone treatment. Interestingly, compared with the alterations of liver injury-related metabolites, most of them were reset to a normal state after scoparone administration.

Biomarker network and metabolic pathway reconstruction. With pattern recognition analysis of metabolites, a clear separation of model and control group was achieved, the scoparone group were located with control group. Metabolite profiling focuses on the analysis of a group of metabolites related to a metabolic pathway in biological states. To determine whether our observations of changes in the metabolites in the setting of liver injury in fact reflected coordinate changes in defined metabolic pathways, we used MetPA software to identify network pathway. This software was based on the high-quality KEGG metabolic pathways as the backend knowledgebase to help researchers identify the most relevant pathways involved in the conditions under study. Metabolic pathway analysis with MetPA revealed that potential biomarkers were identified from citrate cycle, sphingolipid metabolism, taurine and hypotaurine metabolism that changed specifically in the setting of myocardial ischemia. Of two distinct metabolites identified from these pathways, many were in various progress stages of liver injury. The detailed construction of the metabolism pathways with higher score was shown in supplementary Fig. 1. Results suggested that these target pathways showed the marked perturbations over the time-course of liver injury and could contribute to development of liver injury.

Discussion

Metabolomics is a rapidly evolving field that aims to identify and quantify the concentration changes of all the metabolites due to endogenous or exogenous perturbations. Since the production of a particular metabolite is the end result of a cascade of interactions involving numerous biological molecules, they together, i.e., the metabolome, represent the closest molecular level description of the physiological state. Thus, in principle, any physiological perturbation is expected to be associated with characteristic changes in the metabolome. Metabolomics has recently demonstrated significant potential in many fields such as toxicology, disease diagnosis, drug mechanism and development, and natural product discovery *etc*[19–21]. The metabolites that are more closely related to the phenotype of individuals, metabolomics can help us in understanding a detailed analysis of complex reaction pathways and uncovering drug

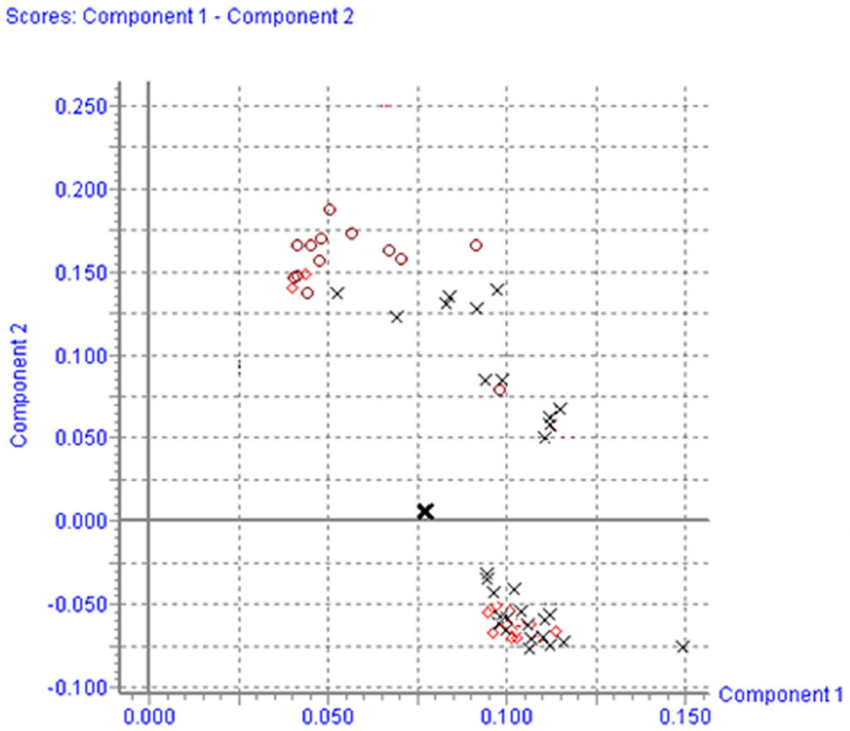

Figure 6 | PCA Score plot for the acute livery injury after scoparone treatment in positive mode. (◇: control; ×: scoparone group; ○: model group)

targets[22]. Mass-scale metabolomics suffer from some pitfalls: metabolite and metabolite expression is not significant *per se*, but only if inserted in a detailed metabolism pathways. This requires the development of a more robust and systematic tool to permit the automated construction and further analysis of molecular networks. Advent of network-based analysis methods can help in overcoming these problems but requires careful interpretation[23]. Thus metabolism pathways are emerging as an important paradigm for analysis of biological systems.

Alcohol abuse is one of the main causes of liver disease worldwide and has become a social problem[24]. Due to the increased frequency of drinking, incidence of alcoholic liver disease has increased in the world, becoming another important risk factor for morbility and mortality in addition to viral hepatitis[25]. However, there is no satisfactory therapy for alcoholic liver disease at present except for the combination of abstinence from alcohol and supportive care[26]. Despite considerable and continuous efforts, effective treatment strategies against this disease resulting in fewer side effects are still lacking. Oriental herbal medicines, widely used for treatment of various diseases, have recently attracted the interest of the modern scientific community as alternative therapies[27]. In the present study, we investigated the metabolic changes of molecular mechanism by which coparone conferred a hepatoprotective effect, using rats with alcohol-induced acute liver injury. Of note, novel metabolomic approach confirm that scoparone exhibited therapeutic efficacies on liver damage in vivo. We have also built metabolomic feature network of scoparone protects against liver injury. Interestingly, scoparone exhibited hepatoprotective role of liver injury and kept animals in the normal situation, because there were no distinct clustering differences between control and scoparone group. PCA revealed robust differences between profiles from control and alcohol-treated animals. The major metabolites seen to differ between control and alcohol-treated animals were identified using high accuracy MSn data and verified using external search engines. The main metabolite classes to show major changes in the alcoholic liver-derived samples were 1,1'-(1,8-naphthylene)bis(1H-1,2,3-triazole-4,5-dicarboxylic acid) tetra-tert-butyl ester, 2-pyrocatechuic acid, 3-methoxy-4-hydroxyphenylglycol sulfate, glucosylceramide (d18 : 1/18 : 0). In order to more clearly characterize treatment effects of scoparone, network reconstruction has led to the integration of metabolites associated with the causesd perturbation pathways including citrate cycle, sphingolipid metabolism, taurine and hypotaurine metabolism. These metabolites demonstrated that abnormal metabolism occurred in the model animals and metabolic analysis of liver injury was inferred from changes in the intermediates during substance metabolism. Urinary excretion of these metabolites was also shown to have high specificity and sensitivity as markers. It indicated that these metabolites may be the biomarkers which were related to the action mechanism of scoparone. Glucosylceramide (d18 : 1/18 : 0) is a component the cell plasma membrane which modulates cell signal transduction events. Gangliosides have been found to be highly important in immunology.

A combination of UPLC/ESI-Q-TOF/MS and chemometrics were used to identify urinary biomarkers associated with ALD. The present study was undertaken to investigate protection of scoparone against acute alcohol-induced liver injury in rat, the related mechanism of its hepatoprotective chemical compound for the first time. The overall network was significantly enriched by metabolites associated with citrate cycle, sphingolipid metabolism, taurine and hypotaurine metabolism processes. The identified metabolites were found to encompass a variety of biological processes mediated through complex networks. Application of metabolomic technologies for the study of liver injury will increase our understanding of the pathophysiological processes involved and this should help us to identify potential biomarkers to develop new therapeutic strategies. Novel metabolites and the metabolite-associated systems provide new insights into the mechanisms underlying pathogenesis. The findings demonstrate that the network-based methods are of importance for elucidating the inter-relationship between complex diseases. Generalization of the proposed method for identifying biomarkers will be the focus of future work. System analysis of metabolic networks will help us in generating more in-depth understanding of the mechanism of diseases and thus provide better guidance for drug discovery. A discussion of the newly identified biomarkers and impli-

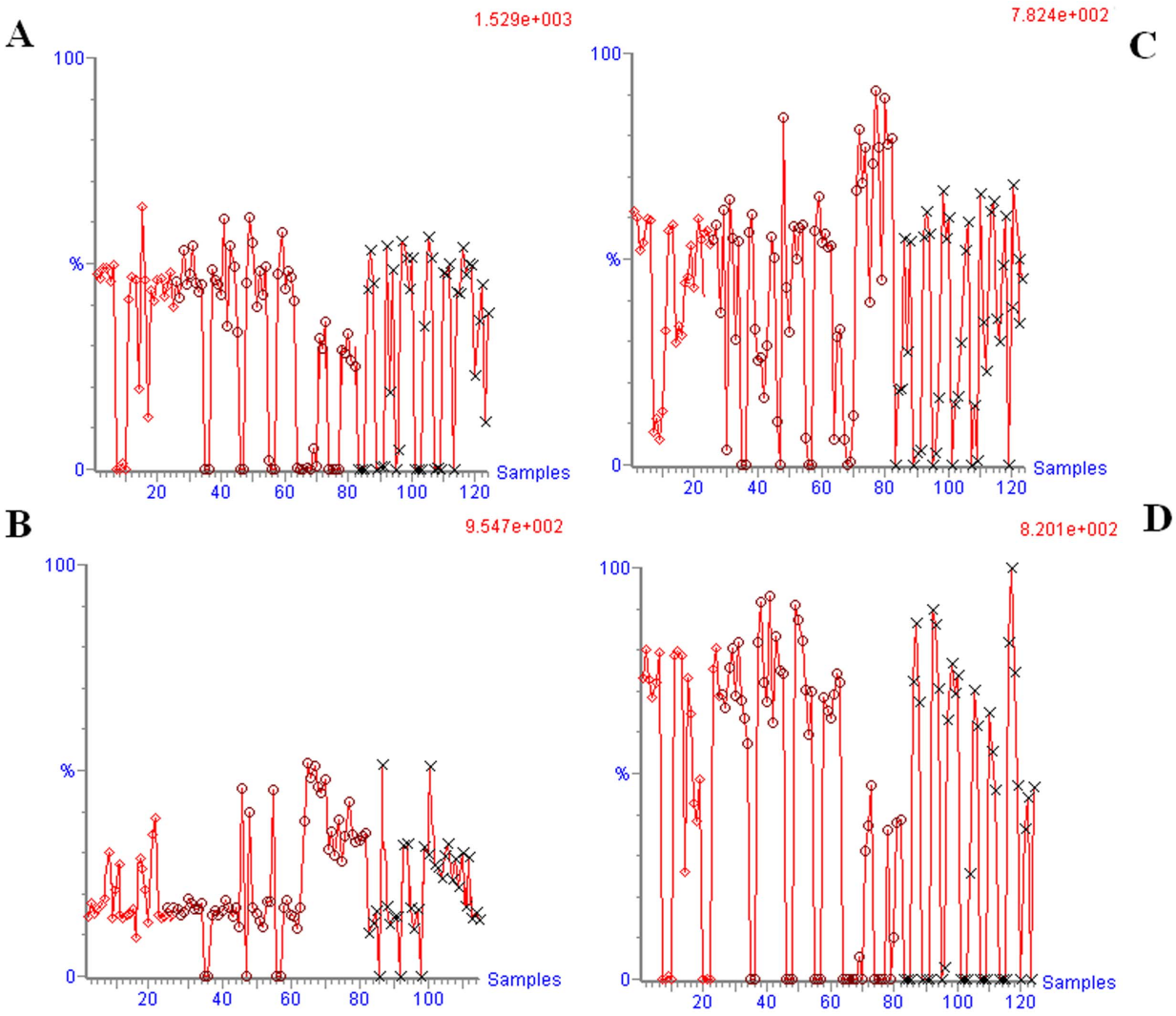

Figure 7 | The trends plot of intensity for potential urine biomarker in the urine samples. (A): 1,1'-(1,8-naphthylene)bis(1H-1,2,3-triazole-4,5-dicarboxylic acid) tetra-tert-butyl ester; (B): 3-methoxy-4- hydroxyphenylglycol sulfate; (C): 2-pyrocatechuic acid; (D): glucosylceramide (d18:1/18:0). (◇: control; ×: scoparone group; ○: model group).

cated biochemical pathways is also presented. Future metabolomic studies in human populations with ALD will be needed to validate the biomarkers found in the mouse model.

Our findings showed that the robust metabolomics techniques is promising to get biomarkers for the pathways and clarify mechanisms of disease, highlight insights into drug discovery. This paper was designed to study metabonomic characters of the hepatotoxicity induced by alcohol and the intervention effects of scoparone. It is the first demonstration of metabolomic approach to delineate metabolic changes in liver injury after dosing scoparone treatment. This study also demonstrated the ability of metabolomics approach to identify early, noninvasive biomarkers of ALD pathogenesis in rat model. Endogenous metabolites were measured and identified using a combination of high accuracy switching MS/MS data, acquired on a UPLC/ESI-Q-TOF/MS system combined with multivariate statistical analysis, and verified to internal and external databases. The results indicate 4 ions in the positive mode were characterized as potential differentiating metabolites. The identified metabolites is mechanistically related to the molecular events associated with development of ALD in alcohol-treated *rats*. They were found to encompass a variety of pathways related to citrate cycle, sphingolipid metabolism, taurine and hypotaurine metabolism. Thus, by using a metabolomic approach, this study also exemplifies that metabolomics could provide a very promising way to elucidate therapeutic mechanisms of scoparone. It provides the first metabolite network maps and may offer deeper insights into the potential pathways of ALD. Taken together, LC-MS can clearly enhance the interpretation and enrich biological discovery of urinary metabolome data of molecular mechanisms of disease.

Methods

Chemicals and reagents. Acetonitrile (HPLC grade) was purchased from Dikma Technology Inc. (Dima Company, USA). Deionized water was purified by theMilli-Q system (Millipore, Bedford, MA, USA). Formic acid (HPLC grade, FA) was purchased from honeywell Company (USA). Leucine enkephalin was purchased from Sigma-Aldrich (MO, USA). Alcohol was supplied from Chemicals Factory (Beijing, P. R. China). Olive oil (Oliver grade) was supplied by Kerry Oils & Grains Trade Co., Ltd. (Shenzhen, China). Scoparone (purify 99%) were purchased from Sichuan Provincial Institute for Food and Drug Control (Sichuan, P. R. China).

Animal handling and sample preparation. Male Wistar rats (weighting 220–260 g) were supplied by GLP Center of Heilongjiang University of Chinese Medicine (Harbin, China). The room temperature was regulated at 25 ± 1°C with 40 ± 5% humidity. A 12-h light/dark cycle was set, free access to standard diet and water. The animals were allowed to acclimatize for 7 days prior to dosing and putted in the metabolism cages during the urine collection periods specified below. After acclimatization, animals were randomly divided into 3 groups with 10 rats in each: the control, model, and scoparone groups. The rats in the control group were administrated with 0.9% saline in the whole procedure for 7 consecutive days. Rats

were orally administrated with 50% alcohol (5 ml/kg body weight) olive oil solution at 3 day (6:00 p.m.) to induce liver injury model for 5 consecutive days, and until day 8. Simultaneously, scoparone group was administrated with 50% alcohol (5 ml/kg body weight) olive oil solution and 0.7 mg/kg scoparone treatment. Urine was collected daily (at 6:00 a.m.) from metabolism cages at ambient temperature throughout the whole procedure and centrifuged at 13,000 rpm at 4°C for 5 min, and the supernatants were stored frozen at −20°C until metabolomic analysis. All the experimental procedures were approved by the Ethical Committee of Heilongjiang University of Chinese Medicine (HUCM-2012-G0178) and conducted according to the principles expressed in the Declaration of Helsinki. All efforts were made to ameliorate suffering of animals.

Metabolic profiling. *Chromatography.* UPLC/ESI-Q-TOF/MS was used for the global analysis of urine samples. Chromatographic analysis was performed in a Waters ACQUITY UPLC system controlled with Masslynx (V4.1, Waters Corporation, Milford, USA). An aliquot of 6 μL of sample solution was injected onto an ACQUITY UHPLC HSS C_{18} column (100 mm × 2.1 mm, 1.7 μm, Waters Corporation, Milford, USA) at 40°C and the flow rate was 0.4 mL/min. The optimal mobile phase consisted of a linear gradient system of (A) 0.1% formic acid in acetonitrile and (B) 0.1% formic acid in water, 0–2 min, 98% A; 2–3 min, 98–80% A; 3–7 min, 80–5% A; 7–9 min, 5% A; 9–11 min, 5–98% A; 11–15 min, 98% A. In addition, the QC sample was used to optimize the condition of UPLC-Q-TOF/MS, as it contained most information of whole urine samples. Whenever one sample injection was finished, a needle wash cycle was done to remove the remnants and prepare for the next sample. In addition, the eluent was transferred to the mass spectrometer directly, that is, without a split.

Mass spectrometry. The mass spectrometry was operated by electrospray ionization in the positive ionization mode. The eluent was introduced into the high-definition mass spectrometer (Waters Corp., Milford, USA) analysis, and the optimal conditions of analysis were as follow: the source temperature was set at 110°C, desolvation gas temperature was 300°C, cone gas flow was 100 h, desolvation gas flow was 600 L/h; the capillary voltage was 3.2 kV, the sampling cone voltage was 35 V, microchannel plate voltage was 2300 V and extraction cone voltage was 3.0 V. The data acquisition rate was set to 0.14 s/scan, with a 0.1 s inter scan delay. Data were colected in centroid mode from 100 to 900 Da. For accurate mass acquisition, a lock-mass of leucine enkephalin at a concentration of 0.2 ng/mL was used via a lock spray interface at a flowrate of 100 μl·min-1 monitoring for positive ion mode ($[M+H]^+$ = 556.2771) to ensure accuracy during the MS analysis.

Multivariate data analysis and data processing. The UPLC-MS data were processed using the MarkerLynx Application Manager (Waters Corp.). After UPLC/ESI-QTOF/MS measurement, the raw data were imported into the MassLynx software for peak detection and alignment. The intensity of each ion was normalized with respect to the total ion count to generate a data matrix that consisted of the retention time, m/z value, and the normalized peak area. The multivariate data matrix was analyzed by EZinfo software 2.0 (Waters Corp., Milford, USA). All the variables were mean-centered and Pareto-scaled prior to PCA and PLS-DA. If a separation between the control and the treatment groups was observed in the PCA scores plot, PLS-DA was performed to highlight the differences between the groups. After the analysis of all samples was finished, low-molecular weight metabolites were presented as chromatographic peaks in the base peak intensity (BPI) chromatograms.

Biomarkers identification and reconstruction of metabolic pathway. Potential markers of interest were extracted from loading plots constructed following analysis with PLS-DA, and markers were chosen based on their contribution to the variation and correlation within the data set. The MassFragment™ application manager (Waters corp., Milford, USA) was used to facilitate the MS/MS fragment ion analysis process by way of chemically intelligent peak-matching algorithms. The identities of the specific metabolites were confirmed by comparison of their mass spectra and chromatographic retention times. A full spectral library, containing MS/MS data obtained in the positive ion modes, for all metabolites reported in this work is available on request from the authors. With regard to the identification of biomarkers, the ion spectrum of potential biomarkers was matched with the structure message of metabolites acquired from available biochemical databases, such as HMDB, http://www.hmdb.ca/; KEGG, http://www.genome.jp/kegg/; METLIN, http://metlin.scripps.edu/; Chemical Entities of Biological Interest (http://www.ebi.ac.uk/Databases/); MassBank, http://www.massbank.jp/; Scripps Center for Mass Spectrometry (http://masspec.scripps.edu/index.php) and Lipidmaps (http://www.lipidmaps.org/). The reconstruction, interaction and pathway analysis of potential biomarkers was performed with MetPA software based database source to identify the metabolic pathways. The possible biological roles were evaluated by the enrichment analysis of MetaboAnalyst.

Statistical analyses. All statistical analyses were performed using the Student's t-test. Differences with a P-value of 0.05 or less were considered significant. Assays were performed in triplicate, and the results are expressed as mean ± SD.

1. Somashekar, B. S. *et al.* Metabolomic signatures in guinea pigs infected with epidemic-associated W-Beijing strains of Mycobacterium tuberculosis. *J Proteome Res* **11**, 4873–4884 (2012).
2. MacKinnon, N. *et al.* MetaboID: a graphical user interface package for assignment of 1H NMR spectra of bodyfluids and tissues. *J Magn Reson* **226**, 93–99 (2013).
3. Arnaudguilhem, C. *et al.* Selenium metabolomics in yeast using complementary reversed-phase/hydrophilic ion interaction (HILIC) liquid chromatography-electrospray hybrid quadrupole trap/Orbitrap mass spectrometry. *Anal Chim Acta.* **757**, 38–42 (2012).
4. Zhang, A. H. *et al.* Ultraperformance liquid chromatography-mass spectrometry based comprehensive metabolomics combined with pattern recognition and network analysis methods for characterization of metabolites and metabolic pathways from biological data sets. *Anal Chem* **85**, 7606–7612 (2013).
5. Tripathi, P. *et al.* HR-MAS NMR tissue metabolomic signatures cross-validated by mass spectrometry distinguish bladder cancer from benign disease. *J Proteome Res* **12**, 3519–3528 (2013).
6. Wang, X., Yang, B., Sun, H. & Zhang, A. Pattern recognition approaches and computational systems tools for ultra performance liquid chromatography-mass spectrometry-based comprehensive metabolomic profiling and pathways analysis of biological data sets. *Anal Chem.* **84**, 439–442 (2012).
7. Sun, H. *et al.* Metabolomic analysis of key regulatory metabolites in hepatitis C virus-infected tree shrews. *Mol Cell Proteomics* **12**, 710–719 (2013).
8. Zhang, A., Sun, H. & Wang, X. Power of metabolomics in diagnosis and biomarker discovery of hepatocellular carcinoma. *Hepatology.* **57**, 2072–2077 (2013).
9. Dong, H. *et al.* Ingenuity pathways analysis of urine metabolomics phenotypes toxicity of Chuanwu in Wistar rats by UPLC-Q-TOF-HDMS coupled with pattern recognition methods. *Mol Biosyst.* **8**, 1221–1125 (2012).
10. Wang, X. *et al.* Potential role of metabolomics apporoaches in the area of traditional Chinese medicine: as pillars of the bridge between Chinese and Western medicine. *J Pharm Biomed Anal.* **55**, 868–873 (2011).
11. Huang, Q. F. *et al.* Protective effect of isoorientin-2″-O-α-L-arabinopyranosyl isolated from Gypsophila elegans on alcohol induced hepatic fibrosis in rats. *Food Chem Toxicol.* **50**, 2001–2007 (2012).
12. Manna, S. K. *et al.* Identification of noninvasive biomarkers for alcohol-induced liver disease using urinary metabolomics and the Ppara-null mouse. *J Proteome Res.* **9**, 4188–4192 (2010).
13. Kwon, H. J., Kim, Y. Y. & Choung, S. Y. Amelioration effects of traditional Chinese medicine on alcohol-induced fatty liver. *World J Gastroenterol.* **11**, 5516–5569 (2005).
14. Zhang, A. *et al.* An in vivo analysis of the therapeutic and synergistic properties of Chinese medicinal formula Yin-Chen-Hao-Tang based on its active constituents. *Fitoterapia.* **82**, 1168–1176 (2011).
15. Wang, X. *et al.* Pharmacokinetics screening for multi-components absorbed in the rat plasma after oral administration traditional Chinese medicine formula Yin-Chen-Hao-Tang by ultra performance liquid chromatography-electrospray ionization/quadrupole-time-of-flight mass spectrometry combined with pattern recognition methods. *Analyst.* **136**, 5076–5083 (2011).
16. Yin, Q., Sun, H., Zhang, A. & Wang, X. Pharmacokinetics and tissue distribution study of scoparone in rats by ultraperformance liquid-chromatography with tandem high-definition mass spectrometry. *Fitoterapia.* **83**, 800–809 (2012).
17. Wang, X., Sun, H., Zhang, A., Wang, P. & Han, Y. Ultra-performance liquid chromatography coupled to mass spectrometry as a sensitive and powerful technology for metabolomic studies. *J Sep Sci.* **34**, 3459–3465 (2011).
18. Wang, X. *et al.* Urine metabolomics analysis for biomarker discovery and detection of jaundice syndrome in patients with liver disease. *Mol Cell Proteomics* **11**, 380–381 (2012).
19. Tripathi, P. *et al.* Delineating metabolic signatures of head and neck squamous cell carcinoma: phospholipase A2, a potential therapeutic target. *Int J Biochem Cell Biol* **44**, 1852–1861 (2012).
20. Somashekar, B. S. *et al.* Magic angle spinning NMR-based metabolic profiling of head and neck squamous cell carcinoma tissues. *J Proteome Res* **10**, 5232–5241 (2011).
21. Barri, T., Holmer-Jensen, J., Hermansen, K. & Dragsted, L. O. Metabolic fingerprinting of high-fat plasma samples processed by centrifugation- and filtration-based protein precipitation delineates significant differences in metabolite information coverage. *Anal Chim Acta.* **718**, 57–64 (2012).
22. Zhao, J. *et al.* Metabolomic differentiation of maca (Lepidium meyenii) accessions cultivated under different conditions using NMR and chemometric analysis. *Planta Med.* **78**, 101–109 (2012).
23. Ramautar, R., Busnel, J. M., Deelder, A. M. & Mayboroda, O. A. Enhancing the coverage of the urinary metabolome by sheathless capillary electrophoresis-mass spectrometry. *Anal Chem.* **84**, 892–899 (2012).
24. Li, S., Liu, H., Jin, Y., Lin, S., Cai, Z. & Jiang, Y. Metabolomics study of alcohol-induced liver injury and hepatocellular carcinoma xenografts in mice. *J Chromatogr B Analyt Technol Biomed Life Sci.* **879**, 2375–2379 (2011).
25. Manna, S. K. *et al.* UPLC-MS-based urine metabolomics reveals indole-3-lactic acid and phenyllactic acid as conserved biomarkers for alcohol-induced liver disease in the Ppara-null mouse model. *J Proteome Res.* **10**, 4133–4140 (2011).
26. Loftus, N. *et al.* Metabonomic investigation of liver profiles of nonpolar metabolites obtained from alcohol-dosed rats and mice using high mass accuracy MSn analysis. *J Proteome Res.* **10**, 713–719 (2011).
27. Wang, X. *et al.* Metabolic urinary profiling of alcohol hepatotoxicity and intervention effects of Yin Chen Hao Tang in rats using ultra-performance liquid

chromatography/electrospray ionization quadruple time-of-flight mass spectrometry. *J Pharm Biomed Anal.* **48**, 1168–1174 (2008).

Acknowledgments

This work was supported by grants from the Key Program of the Natural Science Foundation of the State (Grant No. 90709019, 81173500, 81373930, 81302905, 81102556, 81202639), the National Key Program on the Subject of Drug Innovation (Grant No. 2009ZX09502-005), the National Specific Program on the Subject of Public Welfare (Grant No. 200807014), National Key Technology Research and Development Program of the Ministry of Science and Technology of China (Grant No. 2011BAI03B03, 2011BAI03B06, 2011BAI03B08), and the National Program for Key Basic Research Projects in China (Grant No. 2005CB523406).

Author contributions

H.S. performed the experiments and analyzed the raw data. A.Z. wrote the manuscript, and analyzed the data. X.W. designed the experiments. All authors reviewed the manuscript.

Additional information

Supplementary information accompanies this paper at http://www.nature.com/scientificreports

Competing financial interests: The authors declare no competing financial interests.

How to cite this article: Zhang, A., Sun, H. & Wang, X. Urinary metabolic profiling of rat models revealed protective function of scoparone against alcohol induced hepatotoxicity. *Sci. Rep.* **4**, 6768; DOI:10.1038/srep06768 (2014).