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 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.

Metabolomics 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. Metabolites are the end products of cellular adjustment processes, and their levels can be regarded as the ultimate response of biological systems. Metabolomics has been shown to have enormous potential when applied to subjects as diverse as, toxicological mechanisms, disease processes, and drug discovery. 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. 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.

Excessive alcohol consumption is the third most common cause of lifestyle-associated mortality in the world and more 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. 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. 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. As one of the main active constituents of Yinchenhao, scoparone has been proven effectively in treating liver diseases, and shows hepatoprotective and contributes directly to the therapeutic effect. 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...
understand its in vivo actions and explain a variety of events related
to efficacy and toxicity\textsuperscript{16}. Therefore, the importance of understanding
the metabolic profiles of scoparone is evident, and a correspond-
ning metabolomics study is undoubtedly required.

Common tools used for metabolomic studies include nuclear
magnetic resonance (NMR) spectroscopy and gas or high-perform-
ance 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 metabolomics because of
its ability to obtain multiparametric metabolite profiles from bio-
fluids rapidly and effectively\textsuperscript{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 metabo-
limic investigations on the liver metabolomes of rats for the endo-
genous metabolites using alcohol-fed models, with metabolite
identification via high accuracy MS\textsuperscript{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\textsuperscript{TM}
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 informa-
tion including the retention time, the exact mass and the ms/ms
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 com-
position, 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\textsuperscript{-}(1,8-
naphthylene)bis(1H-1,2,3-triazole-4,5-dicarboxylic acid) teta-
tert-butyl ester are given in Fig. 5. The metabolite, which gave an
elemental composition of C\textsubscript{34}H\textsubscript{42}N\textsubscript{6}O\textsubscript{8} and molecular mass of
[M+H]\textsuperscript{+} 663.3013, were identified. The [M+H]\textsuperscript{+} 663.3013
demonstrated even number nitrogen, the product spectra such as
m/z 607, 551, 495, and 439 contributed to [M+H]\textsuperscript{+} peak
continuous loss -C\textsubscript{4}H\textsubscript{8} when subjecting to MS/MS analysis, and
retrieving corresponding literatures. Finally, it was speculated as
1,1\textsuperscript{-}(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 1 | Chemical structures of scoparone.

Figure 2 | A typical total ion chromatograms of urine obtain from UPLC/ESI-Q-TOF/MS analysis.
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.
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 liver 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. Addition-
ally, 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 rele-
vant 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 per-
turbation is expected to be associated with characteristic changes 
in the metabolome. Metabolomics has recently demonstrated signifi-
cant 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 pheno-
type of individuals, metabolomics can help us in understanding a 
detailed analysis of complex reaction pathways and uncovering drug

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.
targets. 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. 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. Due to the increased frequency of drinking, incidence of alcoholic liver disease has increased in the world, becoming another important risk factor for morbidity and mortality in addition to viral hepatitis. However, there is no satisfactory therapy for alcoholic liver disease at present except for the combination of abstinence from alcohol and supportive care. 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. In the present study, we investigated the metabolic changes of molecular mechanism by which scoparone 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 causeds 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-
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 metabolic 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 the Milli-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

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).
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 saved for the subsequent metabolomic analysis. All the experimental procedures were approved by the Ethical Committee of Heilongjiang University of Medical Sciences 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) analysis, and the optimal 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) 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%; 2–3 min, 98–80%; 3–7 min, 80–5%; 7–9 min, 5%; 9–11 min, 5–98%; 11–15 min, 98%. 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.

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 treatment and the control 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 of known compounds, was used to match the identified metabolites. The ion spectrum of potential biomarkers was matched with the structure message of metabolites isolated 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/ChemicalBases/); MassBank, http://www.massbank.jp/; Scripps Center for Mass Spectrometry (http://massspectomics.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 means ± SD.

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**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. 2011BA03B03, 2011BA03B06, 2011BA03B08), 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).

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