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Original Article

Investigation of *Coreopsis tinctoria* Nutt extracts in spontaneously hypertensive rats by an NMR-based metabolomic approach

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Abstract: Purpose: *Coreopsis tinctoria* Nutt (Compositae) is used in Uyghur folk medicine to treat hypertension. In the present study, we investigate the anti-hypertensive properties and underlying mechanism of ethanol extract of *C. (Coreopsis) tinctoria* (CT) using a NMR-based (Nuclear Magnetic Resonance-based) metabolomics strategy. Methods: The changes of blood pressure left ventricular index mass index (LVWI) and heart index of rats in each experimental group compared with the normal group were detected, and the metabolite changes of plasma and urine of rats in each group were analyzed by ^{1}H-NMR technology. Results: We identified different metabolites from rat plasma and urine by employing NMR in combination with multivariate analysis. The CT treatment group significantly decreased threonine, alanine, pyruvate, lactate, and tyrosine in the serum as well as scyllo-inositol, choline in the urine; other identified metabolites including isoleucine, creatinine, creatine, β-glucose, acetone, malic acid, taurine, and allantoin in the urine were increased after CT treatment. The multiple impacted metabolic pathways including pyruvate metabolism and modulation of taurine signaling were shown to reveal the anti-hypertensive effect of CT. Conclusion: These findings suggest that the effect of CT may be linked to the regulation of amino acid metabolism and taurine metabolism.

Keywords: *Coreopsis tinctoria* Nutt, SHRs, metabonomics, NMR

Introduction

Hypertension, a common cardiovascular disease with multiple causes and pathogenic factors [1, 2], and it is characterized by elevated systemic arterial pressure. It is conservatively estimated that hypertension accounts for 13.5% of all deaths associated with vascular disease, of which more than 95% are due to hypertension. Long-term high blood pressure can easily result in atherosclerosis, stroke, and damage to other important organs, which is accompanied by varying degrees of metabolic disorder [3]. The efficacy and safety of traditional Chinese medicines for the treatment of hypertension have been proved; they can not only stabilize blood pressure, protect large vessels and target organs, but also relieve the accompanying symptoms and side effects of Western medicine.

Coreopsis tinctoria Nutt (CT), known as the "Kunlun snow chrysanthemum" [4, 5], is native to China and used in Xinjiang folk medicine to treat several disorders such as diarrhea, internal pain, and bleeding. Studies have also reported the use of the whole plant in traditional Chinese medicine for the treatment of hypertension and diabetes [6, 7]. Modern research shows that *C. tinctoria* Nutt effectively lowers BP, with a mechanism that may be related to reductions in oxidation stress, malondialdehyde (MDA), and vascular angiotensin (Ang-II) and increased nitric oxide [8-11]. However, there are only a few studies focus on the effect of *C. tinctoria* Nutt on hypertension, so further exploring its mechanisms will be necessary.

Metabonomic approach using ^{1}H-NMR and chemometric techniques have been developed [12-15] to study the metabolic differences associ-

ated with gene function, pathophysiological and toxicological stimuli. This combination technique has been successfully used to study several diseases and the toxicity of various drugs and other compounds [16-18]. In our previous study, we applied ^{1}H-NMR-based metabolomic techniques to detect the metabolic differences between the SHR model and aged-matched normotensive control rats (Wistar-Kyoto rats, WKYR) [19]; the results showed that several metabolites such as isoleucine, creatine and β-glucose were largely responsible for the separation between the two groups, which implied that the profiles can be used to provide metabolic information. Thus, it was of great interest to confirm the biochemical effects and precise mechanism of the blood pressure-lowering action of *C. tinctoria* Nutt. In the present study, we analyzed metabolic profiles in the serum and urine of spontaneously hypertensive rats (SHRs) treated with *C. tinctoria* Nutt using an NMR based metabolomic approach and aimed to explore the underlying mechanism of the anti-hypertensive activity of *C. tinctoria* Nutt.

Materials and methods

Preparation of CT

Coreopsis tinctoria Nutt (CT) was purchased from Hetian City (Xinjiang, China) and authenticated by Professor Junping Hu (College of Pharmacy, Xinjiang Medical University) and a voucher specimen was eposited in the herbarium of medical plant in Xinjiang Medical University (number 2012-01). The dried flowers were ground into a powder and extracted with 55% ethanol (500 ml × 2) by refluxing at 80°C for 2 h. After filtration, the extracts were combined, concentrated, and then spray-dried to obtain the powder used for the animal experiments.

Analysis of CT

CT was analyzed by an HPLC (eWaters 2695) equipped with a diode-array detector (Waters 2489). The column used was a shim-pack vp-ODS (250 mm × 4.6 mm, 5 μm) and the temperature was set at 35°C. The gradient mobile phase consisted of (A) 0.5% formic acid and (B) acetonitrile, with a flow rate of 1.0 ml/min. The gradient elution was conducted as follows: 5% B for 0.0-5.0 min; 20% B for 5.01-40 min; 40% B for 40.01-60 min; and then back to 5% B at 60.01 min. The injection volume was 10 μl.

Animal experiments

All animals (Ethical review number: 20140304-133) were handled and treated in compliance with the medical ethical principles of the First Affiliated Hospital of Xinjiang Medical University and the Xinjiang Diabetes Institute. Twenty-six male 11-week-old SHRs and 8 male WKYRs (200-250 g) were obtained from Beijing Weitong Lihua Experimental Animal Technology (Qualified certificate number: 2012-000, scxk, Beijing). The rats were maintained under standard laboratory conditions (temperature, 21 ± 2°C; relative humidity, 45-55%; rearing environment daily light cycle, 12 h) and allowed free access to standard chow and water.

After a 1-week acclimatization period, the BP was measured every 2 consecutive days, and then the rats were randomly divided into the following four groups in accordance with the rat BP equilibrium values and treated as indicated: model (n = 6), given distilled water containing 1% (g·100 mL^{-1}) sodium carboxymethyl cellulose for 4 weeks; captopril (n = 7), captopril tablet dissolved in distilled water at a dose of 4 mg·kg^{-1} per day by gavage for 4 weeks; low and high-dose CT (1.6 and 3.2 g·kg^{-1}) per day by oral gavage for 4 weeks; and WKYRs (n = 8), which were the normal controls, were administered the same treatment as the model group. Each group was given free access to a normal diet and water.

Sample preparation

After a 4-week period of drug treatment, the rats were fasted but allowed free access to water for 12 h prior to sacrifice. All animals were placed in metabolic cages for the collection of urine samples and then were euthanized for cervical dislocation immediately. The blood samples were collected from the abdominal aortic artery into heparinized tubes and immediately centrifuged at 3000 rpm for 20 min. The plasma was transferred into clean tubes and stored at -80°C prior to analysis. The rat's hearts were removed, dissected, and weighed to determine the left ventricular index mass index (LVMI) and cardoic index.

The plasma samples were separated in tubes for NMR analysis. The urine samples were fil-

tered through a 0.22-μm nylon filter film to eliminate particulate matter and stored at -80°C until analysis. BP was measured by using a tail-cuff method (BP-98A, Beijing Ruanlong Biotechnology Co., Ltd) during the experiment.

^{1}H NMR-based metabolic profiling analysis

Frozen urine samples were thawed at 4°C and 400-μl aliquots were mixed with 200 μl phosphate buffer solution (pH 7.4) and deuterium oxide (0.1 ml) containing 0.1% sodium 3-trimethylsilyl propionate. Subsequently, 200 μl plasma was added to 400 μl phosphate buffer solution (pH 7.4) containing deuterium oxide. All samples were then centrifuged (8000 rpm for 5 min) and 550 μl of the supernatant was transferred to a 5-mm NMR tube for the analysis.

The ^{1}H-NMR measurements of the urine and serum samples were performed by using a Varian NMR 500 MHZ spectrometer equipped with a pulse sequence [relaxation delay -90°-(τ-180°-τ) n-acquire]. For ^{1}H-NMR, we used the following settings: frequency, 500.13 MHz; cumulative scans, 64; sampling data point, 32768; spectral width, 20 mg·l^{-1}; sampling data delay, 2 s; scanning time, 1.64 s; and test temperature, 25°C.

The collected NMR spectral data were phased and baseline corrected manually by using Bruker TOPSPIN 2.1 software. All the spectral peaks were aligned manually to overcome the peak shift problem. Each spectrum was then binned into segments in the range from 10.0 to 0.5 ppm by using an adaptive binning method. To eliminate the influence of the presence of water in the metabolite concentrations, each spectrum was normalized to its total integrated area. The preprocessed NMR spectral data were imported into the SIMCA-P+ software (version 11.0, Umetrics AB, Umea, Sweden) for analysis and visualization by using multivariate statistical methods including PLS-DA. The chemical shift region of 5.20-4.67 ppm (water and urea resonances) was excluded prior to pattern recognition analysis.

Statistical analysis

The peaks and representative peaks shared by the GC-MS TIC map were retrieved and each peak area was integrated. The retention time, each peak area, and internal standard peak were imported into the Excel table for use. The ratio of the peak area of the sample to the internal standard peak (relative peak area) indicates the number of metabolites. All statistical comparisons were made by One-way analysis of variance (ANOVA) using Statistical Package for the Social Sciences version 13.0. Differences were considered statistically significant for values of $P<0.05$ or <0.1. Pattern recognition multivariate analysis of normalized data was performed using the SIMCA-P11.0 software, using principal component analysis (PCA), and partial least squares-discrimination analysis (PLS-DA), and maximizing the difference between groups.

Results

Chemical composition of CT extract

As shown in **Figure 1**, the main compounds were identified as chlorogenic acid, flavanomarein, marien, 3,5-dicaffeoyl-quinic acid, and flavanokanin. Standard solutions were used to determine their relative content as 7.29 mg/g, 53.12 mg/g, 107.09 mg/g, 7.28 mg/g, and 31.87 mg/g. It can be seen that CTEE mainly contains flavanomarein and marine.

Potential anti-hypertensive effect of CT

In this study, SHRs were assigned to be treated with different dosage CT and positive drug for 4 weeks, as shown in **Figure 2**. **Figure 2A** shows that compared with the normal group, the SBP significantly increased in rats in the model group treated at 0, 2, and 4 weeks. Compared with the model group, the SBP significantly decreased in rats treated with HCT at 2 and 4 weeks ($P<0.01$). The LVWI values significantly decreased ($P<0.01$) in the captopril and the high-dose CT group (**Figure 2B**). Furthermore, we calculated the change of cardiac index in all treated groups, **Figure 2C** shows that the model group had a significant increase in cardiac index ($P<0.01$); whereas the high dosages CT group and the captopril group had a significant decrease compared with the model group (**Figure 2C**, $P<0.05$). These results indicate that the therapeutic effects of the CT extracts on the SHRs.

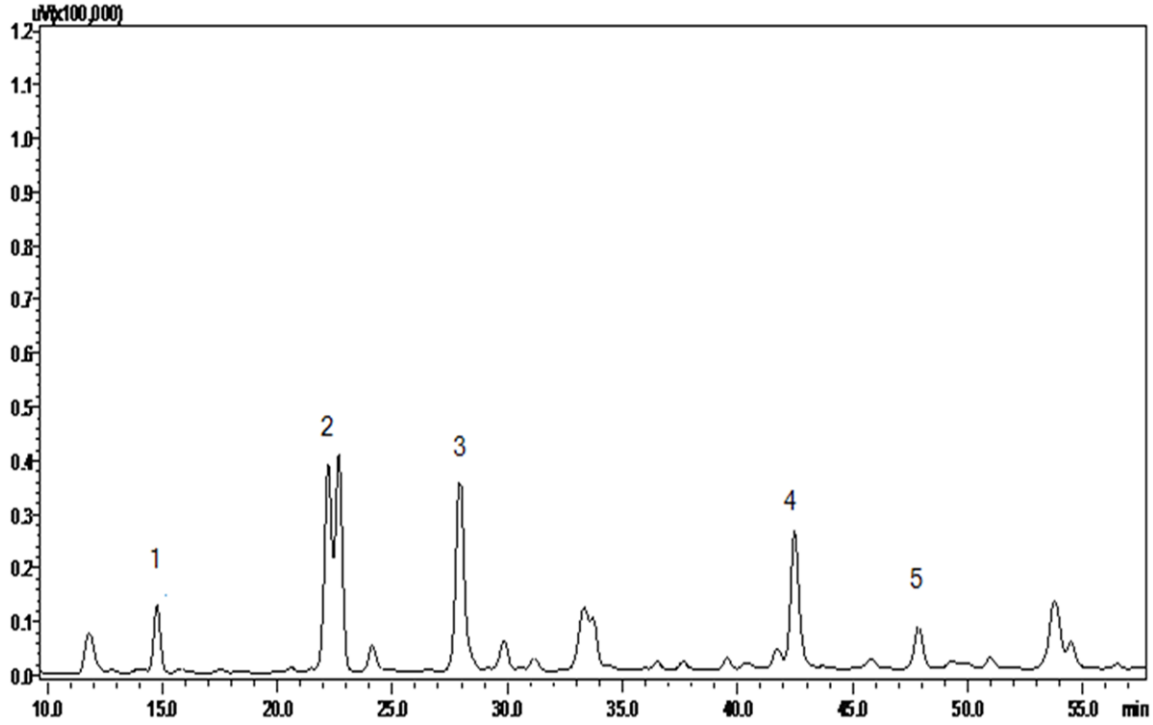


Figure 1. HPLC analysis of ethanol extract of *Coreopsis tinctoria* Nutt. The five main compounds were (1) chlorogenic acid, (2) flavanomarein, (3) flavanokanin, (4) marein, and (5) 3,5-dicaffeoyl-quinic acid.

Metabolic response to CT in the serum of SHRs

Typical ^{1}H-NMR spectra of the plasma samples from SHRs are shown in **Figure 3A**, where we identified a series of serum metabolites involving amino acid metabolism (threonine, alanine, tyrosine), energy metabolism (glucose, pyruvate, lactate). The integrated results of the ^{1}H-NMR spectra were analyzed using PLS-DA, and the obtained scatter plots are shown in **Figure 3B**. This showed obvious separation between the model, the two dose levels, captopril, and the control groups. The model group exhibited the greatest difference from the normal group, which indicated that the metabolic pattern was greatly altered. After treatment, the values in the CT-treated group were closer to those of the normal group, especially in the high-dosage group. **Figure 3C** shows the PLS-DA models were cross-validated by using a permutation analysis (200 times) and the resulting R^2 and Q^2 values, which revealed a good model performance.

Furthermore, using univariate analysis, a significant decrease in serum amino acids were observed in the CT group (**Figure 3D-H**, $P < 0.01$), the major statistically significant differences were identified as the metabolites including threonine, alanine, pyruvate, lactate, and tyrosine in the serum; all were increased in the SHR model group. After treatment, the metabolite levels were down-regulated after the treatment with different doses of CT, especially the high dose.

Metabolic response to CT in the urine of SHRS

A typical ^{1}H-NMR spectrum from the urine is illustrated in **Figure 4A**. We identified a series of metabolites from NMR-based urine metabolism, such as creatine, succinate, glucose, lactate, choline, alanine, glutathione, glutamine, histidine, isoleucine, leucine, phenylalanine, tyrosine, taurine, valine. **Figure 4B**, **4C** shows the score plot and the PLS-DA model, which revealed a good model performance. Then, we evaluated the difference of metabolite levels, among these treated groups using univariate analysis. Metabolites that were decreased after CT treatment were identified in the urine samples including Scylla-inositol and choline.

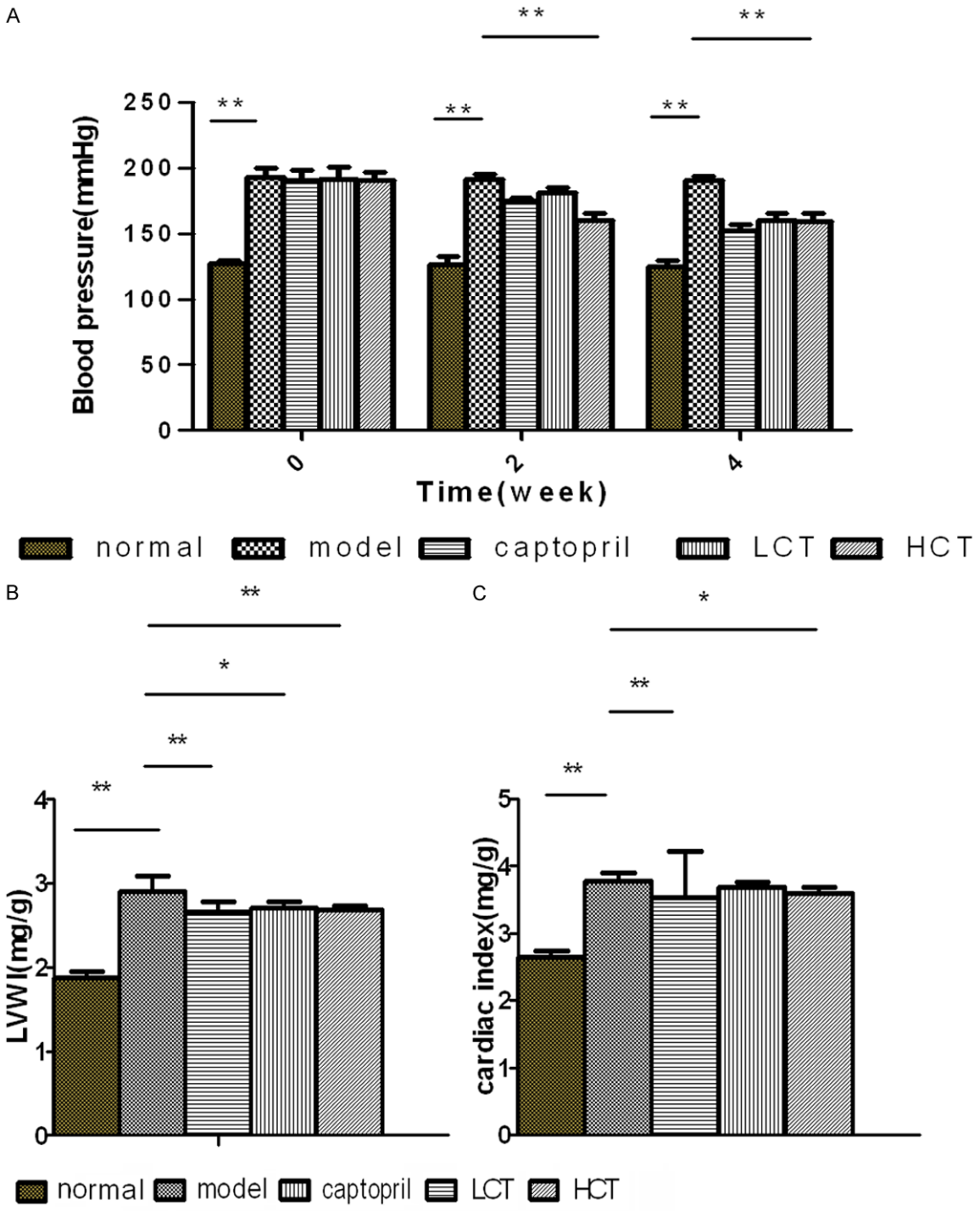
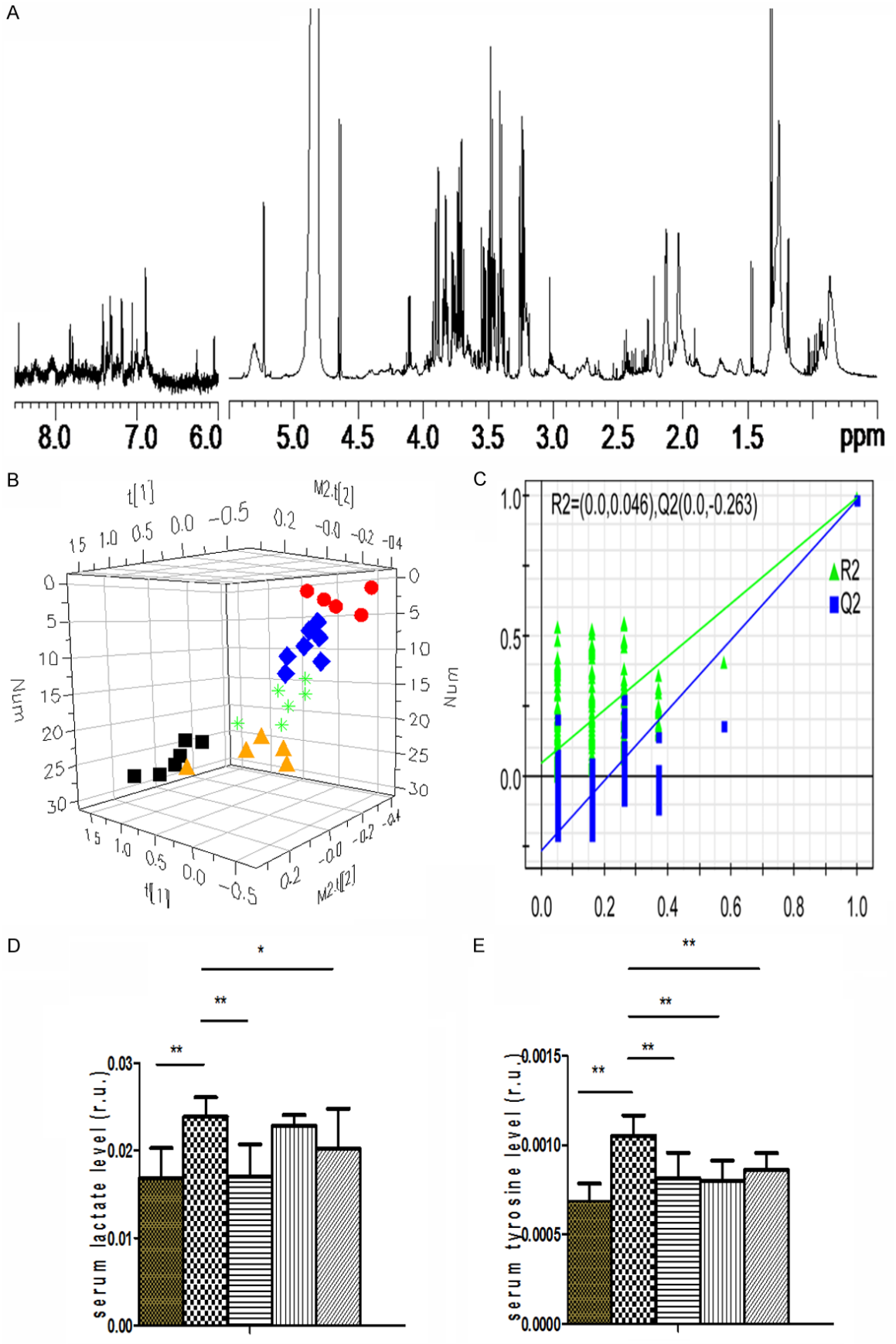


Figure 2. Change in blood pressure and LVWI of SHRs after administration of CT extract: (A) Blood pressure at 0 and 4 weeks (B) LVWI at 4 weeks (C) cardia index at 4 weeks. r.u.: relative units. Significant level: *P<0.05; **P<0.01.

In addition, the other identified metabolites, including isoleucine, creatinine, creatine, β-glucose, acetone, malic acid, taurine, and allantoin increased after CT treatment. All these metabolites may be related to the anti-hypertensive mechanism of CT. **Figure 4D-H**, (P< 0.01 or p<0.05) shows five significant changed metabolisms when treated with CT.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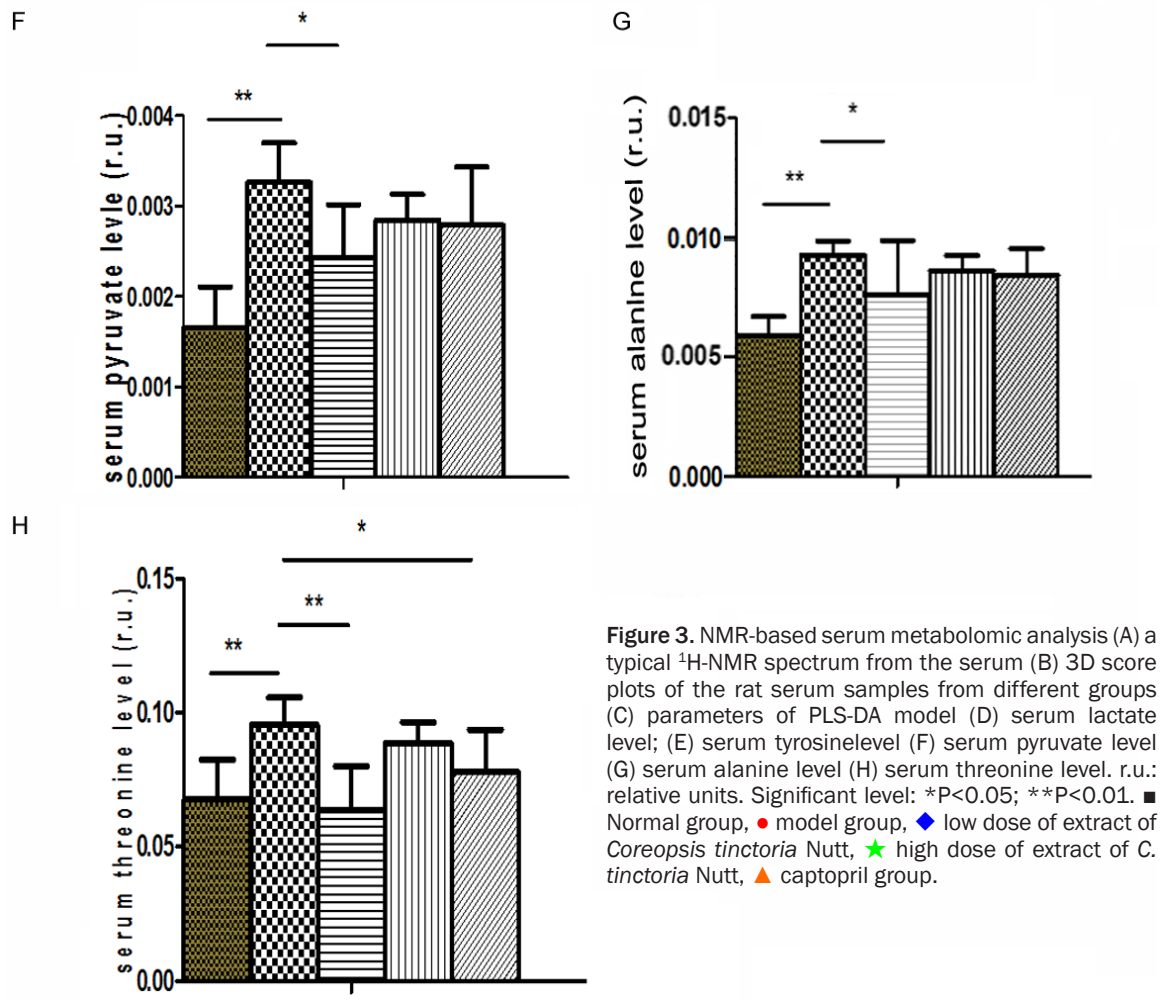


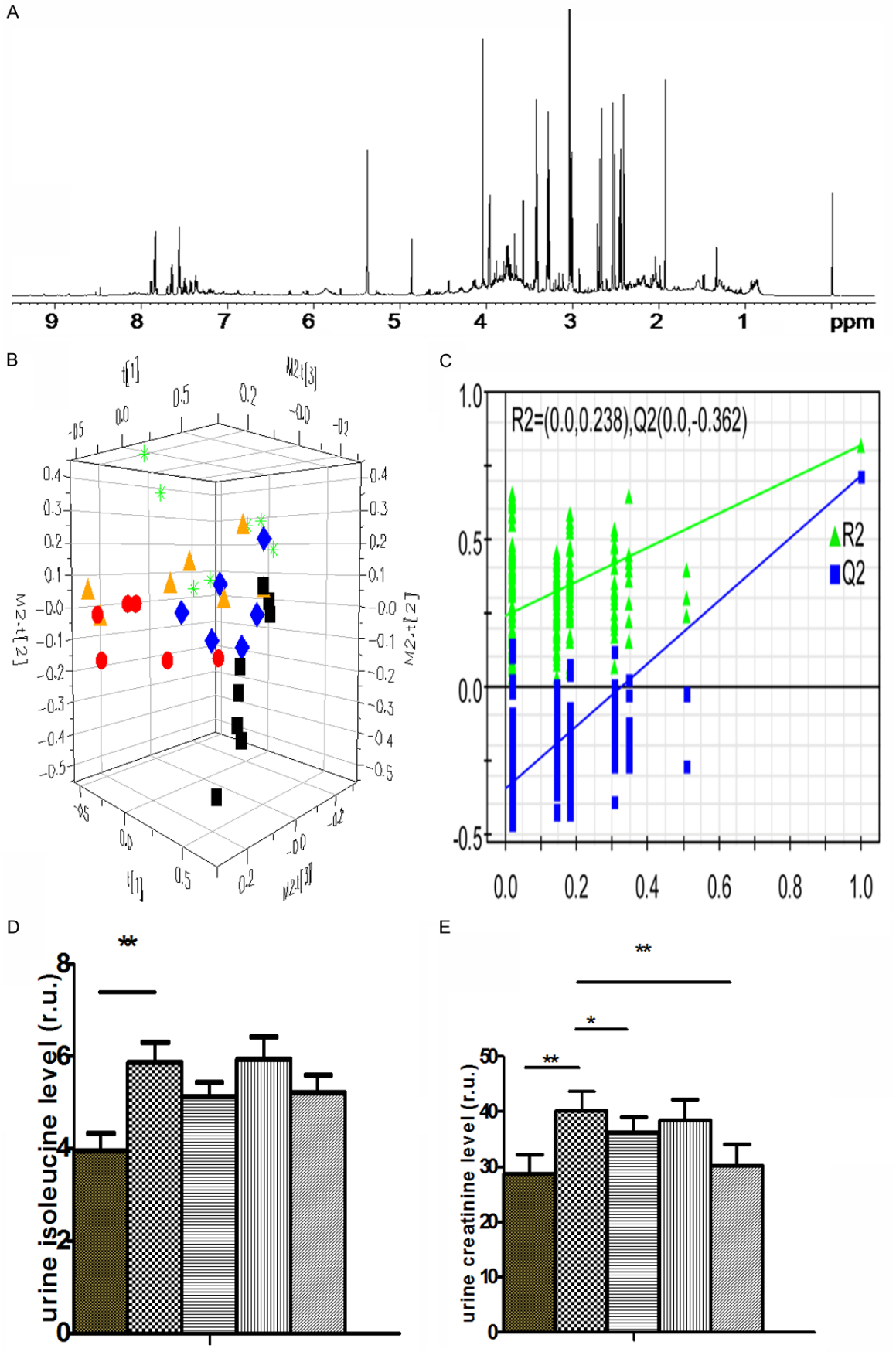
Figure 3. NMR-based serum metabolomic analysis (A) a typical ^{1}H-NMR spectrum from the serum (B) 3D score plots of the rat serum samples from different groups (C) parameters of PLS-DA model (D) serum lactate level; (E) serum tyrosinelevel (F) serum pyruvate level (G) serum alanine level (H) serum threonine level. r.u.: relative units. Significant level: *P<0.05; **P<0.01. ■ Normal group, ● model group, ◆ low dose of extract of *Coreopsis tinctoria* Nutt, ★ high dose of extract of *C. tinctoria* Nutt, ▲ captopril group.

Mechanistic considerations

MetaboAnalyst 3.0 (www.metaboanalyst.ca) was performed to discover the significantly relevant pathways affected by CT groups. **Figure 5A**, **5B** shows that the elevated pyruvate pathway and taurine and hypotaurine metabolism pathways participation was the most relevant pathways affected by CT, with the impact value >1.0. Moreover, several other important pathways, including Glycolysis or Gluconeogenesis, isoleucine biosynthesis, alanine, aspartate and glutamate metabolism were associated with response to CT administration compared with the model group. In addition, we constructed a metabolic network through the Kyoto En= cyclopedia of Genes and Genomes (KEGG, http://www.kegg.jp) pathway database. Our data suggested that the comprehensive metabolic profile changes in rats associated with CT-induced anti-hypertensive effect were mainly related to the amino acid, bile acid and TCA cycle (**Figure 5C**).

Discussion

In this study, we demonstrated the anti-hypertensive effect of CT in SHRs. After treatment with CT for 4 weeks, the BP, the LVWI and the cardiac *index* all decreased compared with the model group, which was indicative of an improvement of ventricular hypertrophy [20-24]. Moreover, we used an NMR-based metabonomics strategy to determine the differences between the serum and urine profiles of the hypertensive model rats and those administered various treated groups. The results revealed that the hypertensive model and both CT-treated groups showed a clear difference in metabolic profiles. Furthermore, we identified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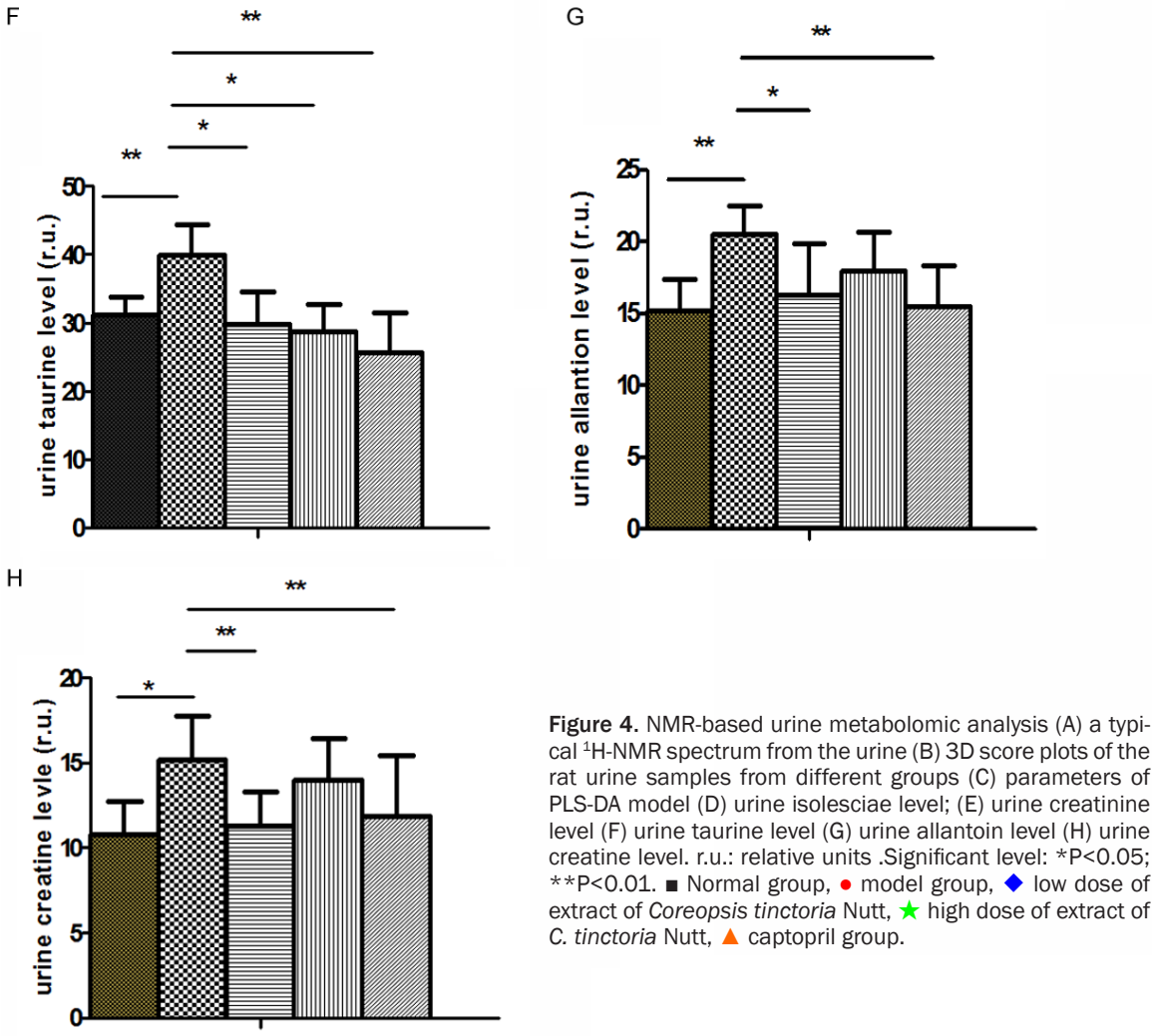

Figure 4. NMR-based urine metabolomic analysis (A) a typical ^{1}H-NMR spectrum from the urine (B) 3D score plots of the rat urine samples from different groups (C) parameters of PLS-DA model (D) urine isolesciae level; (E) urine creatinine level (F) urine taurine level (G) urine allantoin level (H) urine creatine level. r.u.: relative units .Significant level: *P<0.05; **P<0.01. ■ Normal group, ● model group, ◆ low dose of extract of *Coreopsis tinctoria* Nutt, ★ high dose of extract of *C. tinctoria* Nutt, ▲ captopril group.

potential biomarkers and the contributing pathway that can be targeted in future studies of the pathogenesis.

To accurately observe the effects of CT in the SHRs, the urine and serum samples of the rats in the model and high-dose CT groups were compared using PLS-DA to identify the metabolic differences and variables. Among all the biomarkers detected, the analysis highlighted five and ten metabolites in the serum and urine, respectively. The most important metabolic pathways that were altered for the serum and urine metabolites were the pyruvate and taurine pathways, respectively.

In our study, the regulation of pyruvate metabolism in all the treatment groups was indicated by the level of the metabolites. Pyruvate can be generated through the catabolism of various amino acids including alanine, serine, and threonine, which can enter the citric acid cycle to replenish intermediates or be converted to phosphoenolpyruvate as part of the gluconeogenic pathway [25, 26]. The disruption of pyruvate metabolism can cause severe diseases such as diabetes. In our study, a series of amino acids were highly expressed in the model group, but the levels were an accordingly relative decrease observed in the serum amino acid levels after treatment with CT. Our study is the first to report the participation of pyruvate metabolism in the pathogenesis of hypertension in SHRs and that CT treatment could reverse abnormal metabolism of pyruvate.

Among the metabolites detected in the urine, taurine was the most remarkable. Taurine is a

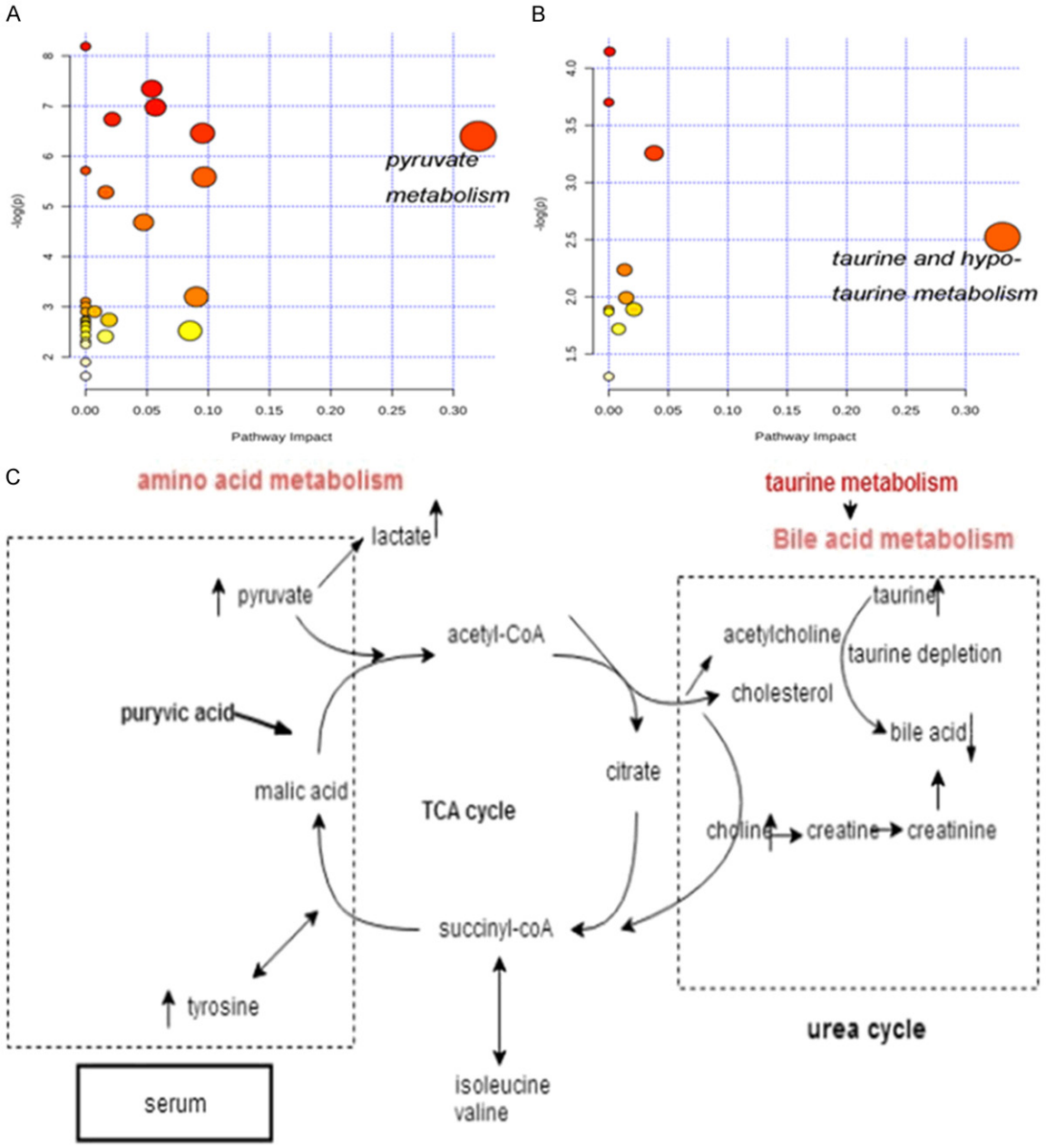

Figure 5. (A) Pathway effect of serum metabolites, Significant metabolic pathways: pyruvate metabolism (B) pathway of urine metabolites, Significant metabolic pathways: taurine and hypotaurine metabolism (C) Metabolite identified in the present study, Significant metabolites: pyruvate/puryvic acid/tyrosine/taurine etc. Significant amino acid and bile acid.

sulfur-containing amino acid, which has been termed a "functional nutrient" and may protect against diabetes mellitus and atherosclerosis [27-30]. Taurine can accelerate metabolic acidosis through the perturbation of the tricarboxylic acid cycle and modulate the intestinal microbial metabolism. In contrast, our study showed that a large amount of taurine was produced in the progression of hypertension and was decreased after treatment with CT, taken together, we suggest that the decreased level of taurine may be contributed to down-regulated of the taurine transport, some amino acid such as alaine, which can be an inhibitor of the (TAUT). The decrease in the transporter levels results in the decrease of the plasma content

of taurine with the possibility of a concomitant propensity to excrete more taurine in the urine. Although the metabolite of creatinine was also found in the urine of SHRs, its increase may be attributable to the increased creatine formation in the liver or injured skeletal muscle.

Conclusion

The present NMR-based metabonomic study revealed the metabolic changes in SHRs treated with CT. Results show that the anti-hypertensive effect of CT may be implicated in the regulation of amino acid metabolism as well as pyruvate mediated pathway and taurine pathway in the serum and urine, respectively, it showed the greatest changes during the development of hypertension. This study showed that the NMR-based Metabonomic analysis of SHRs was a useful approach to obtain new metabolic information related to hypertension and could reflect the therapeutic effect. However, further works should be considered: (1) it is of great interest to discover whether the specific main substances containing in CT play a real pharmacological role; and (2) the target gene or proteins in metabolic pathways need to be explored for better understanding of the potential mechanisms of CT to reveal the underlying mechanism.

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Disclosure of conflict of interest

None.

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