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Fecal metabolomics of type 2 diabetic rats and treatment with *Gardenia jasminoides* Ellis based on mass spectrometry technique

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ABSTRACT:

Modern studies have indicated *Gardenia jasminoides* Ellis (*G. jasminoides*) showed positive effect in treating type 2 diabetes mellitus (T2DM). In this study, sixty streptozotocin induced T2DM rats were divided into four groups: type 2 diabetes control group, geniposide treated group, total iridoid glycosides treated group, and crude extraction of gardenlae fructus treated group, other ten healthy rats were the healthy control group. During 12 weeks treatment, rat’s feces samples were collected for the metabolomics study based on mass spectrometry technique. Based on the fecal metabolomics method, nineteen potential biomarkers were screened and their relative intensities in each group were compared. The results revealed *G. jasminoides* mainly regulated dysfunctions in phenylalanine metabolism, tryptophan metabolism and secondary bile acid biosynthesis pathways induced by diabetes. The current study provides a new sight for metabolomics methodology towards T2DM, and the results show that feces can preferably reflect the liver and intestines disorders.

KEYWORDS: *G. jasminoides*, Fecal metabolomics, UPLC-Q-TOF-MS, Type 2 diabetes
INTRODUCTION

Diabetes mellitus (DM) is a long-term metabolic disease resulting from absolutely or relatively absence of insulin, which its main clinical manifestation is high blood glucose, more water intake and loss of weight.\(^1\) Type 2 diabetes mellitus (T2DM) patients account for 90 percents of all DM patients.\(^2\) Insulin deficiency and insulin resistance together lead to high level of blood glucose. After a long course of disease, organs pathological changes and metabolic disorders would ensue.\(^3,4\) Particularly, researchers have found that hepatic disorder and intestinal flora change of diabetics resulted in abnormal protein, lipid and glucose metabolism.\(^5,6\) These changes may directly lead to variations in the composition of feces. There are various medicines, such as sulfonylureas, thiazolidinediones, biguanides, and α-glucosidase inhibitors etc., used for treating T2DM. However, side effects and adverse drug reactions are frequently reported.\(^7\) Compared to synthesized medicines, traditional Chinese medicine (TCM) shows more mild and persistent effects in adjusting physiological function.\(^8,9\) Thus, TCM treatment is regarded as a naturally and harmlessly therapy. Because of the above advantages, searching for TCM for treating diabetes is a feasible and valuable way.

As a common TCM, *G. jasminoides* has been extensively applied for thousands of years, such as in the therapy of hepatitis and nephritis. Modern studies have showed that *G. jasminoides* contains iridoid glycosides, diterpenes, triterpenes, flavonoids and other chemical components.\(^10,11\) Geniposide, a iridoid glycoside, is the main effective constituent in *G. jasminoides*.\(^12\) Previous studies have shown that
geniposide can significantly promote the consumption of glucose in preadipocyte cells by the activation to PPARγ receptor. Based on preventing oxidative stress-induced neuron apoptosis, activating glucagon-like peptide 1 receptor (GLP-1R) in INS-1 cells, geniposide can attenuate β-cell apoptosis, which contribute to T2DM treatment.13-15

TCM theory regards that adjusting body function is an essential factor in treating diseases. And T2DM is a long-term metabolic disease with function decline of organs. As the reasons above, metabolomics should be a favored method for researching T2DM and its treatment. Metabolomics is a science to be born and defined in 1999, as well as used to study the metabolic rule of organisms.16 The research objects of metabolomics are small-molecule metabolites (molecular weight <1000) from urine, blood, feces and other samples. By analyzing content variation of metabolites, metabolomics can reveal metabolic activities of organism when stimulated by inside and outside factors.17,18 In metabolomics analysis, nuclear magnetic resonance (NMR)19, gas chromatography-mass spectrometry (GC-MS)20 and liquid chromatography-mass spectrometry (LC-MS) are the most extensively applied techniques. Recently, ultra-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS) shows its advantage in identifying the metabolite mass accurately and rapidly. Although UPLC-Q-TOF-MS is widely applied to the analysis of the metabolites,21,22 it is not frequently used for fecal metabonomics analysis. Only H. Huang’s group adopted UPLC-Q-TOF-MS to analyze feces to reveal malabsorption in cirrhotic and hepatocellular carcinoma patients.23,24 The metabolites in feces samples were
obviously different with urine and plasma samples, which could better reflected the pathological or physiological conditions of livers and intestines. And the long course of T2DM could lead to organs pathological changes and dysfunctions such as hepatic disorder and intestinal flora change\textsuperscript{3,4}. Thus, feces metabolomics study has its unique advantages in revealing livers and intestines dysfunctions.

To date, the systematic investigation about the efficacy of \textit{G. jasminoides} in treating T2DM has not been reported. As UPLC-Q-TOF-MS used for feces metabolomics study has been reported, feces can also reflect disorders caused by T2DM, thus this study adopted UPLC-Q-TOF-MS method to detect and analyze rats’ feces samples of T2DM and treatment groups. By using chemometric methods, the evolution of T2DM and the therapeutic effect of \textit{G. jasminoides}’ ingredients will be indicated. Potential biomarkers and metabolic pathway changes between healthy rats and T2DM rats will be obtained. According to the relative intensities of potential biomarkers in each treatment group, this study will reveal how \textit{G. jasminoides} could improve symptoms and testify its effective components.

\textbf{MATERIALS AND METHODS}

\textit{Materials}

\textit{G. jasminoides} were purchased from Tongrentang drugstore (Changchun, China), and authenticated by Professor Shumin Wang (Changchun University of Traditional Chinese Medicine, China). Geniposide was purchased from Fang Cheng Biological development company (Baoji, China). Streptozotocin (STZ) was purchased from Sigma (USA). Acetonitrile and formic acid (Fisher Scientific, Loughborough, UK)
were HPLC-grade. Ultrapure water was obtained by a Milli-Q plus (Milford, MA, USA) water purification system.

**Active components extraction**

Extraction of total iridoid glycosides from *G. jasminoides* depended on laboratory experience. Powder of *G. jasminoides* was extracted twice by reflux extraction with 8 portions of 95% ethanol over 1.5 hours. After that, extracts were collected and then separated and purified by AB-8 macroporous resin. Extracts was added into column with 2 BV/h (BV: bed volume) and 0.25 g crude drug per milliliter, and then washed by water at the speed of 3 BV/h until eluent was smell-less. After washing, 95% ethanol (5 BV) was used to elute for 2.5 h. The eluent was collected, concentrated and then freeze-dried to store at -20 °C for use.

Crude extraction of *G. jasminoides* was obtained by the following method. Powder of *G. jasminoides* was immersed in 60% ethanol (8 times of their total weight), then heated to refluxing for 1.5 h, and continuously extracted another 1.5 h. The eluent was collected, concentrated, then freeze-dried and stored at -20 °C for use.

**Type 2 diabetes model construction**

Fat emulsion preparation: 40 g lard oil, 5 g cholesterol, 1 g methylthiouracil and 40 mL Tween-80 were added into beaker and mixed sufficiently. The mixture was called oil phase. To make up water phase, 1 g sodium glutamate, 5 g sucrose and 5 g glucose were dissolved in 60 mL distilled water and 60 mL 1,2- propylene glycol, and then mixed the oil phrase and water phrase so that the fat emulsion was prepared and stored at 4 °C for use.
STZ preparation: Powder of STZ was dissolved into 0.1% citric acid buffer (pH 4.5) and STZ injection was confected.

Male Wistar rats, weights 200-240 g, obtained from Experimental Animal Center of Jilin University (China) were administered with fat emulsion intragastrically at a dose of 1mL per 100 g. After gavage of 20 days, the rats were injected intraperitoneally 35 mg STZ per kilogram. A week later, the blood glucose of rats was detected, and then 90 rats whose blood glucoses high than 16.7 mmol/L were regarded as T2DM rats which were ready for treatment according to the different groups.

**Grouping and treatment**

60 T2DM rats were randomly divided into 4 groups, namely type 2 diabetes control group (DM), geniposide treated group (GP), total iridoid glycosides treated group (IG), and crude extraction of *G. jasminoides* treated group (CE). Other ten healthy male Wistar rats made up the healthy control group (HC). HC and DM rats were given distilled water in 0.5 mL/100 g by gavage. Rats in other groups were given medicines which were the same as its group names, such as geniposide treated group (GP), the medicine was geniposide, total iridoid glycosides treated group (IG), the medicine was total iridoid glycosides, and so on. The dosages respectively were: for CG, 0.125 g/kg; for IG, 0.150 g/kg; for CE, 3.00 g/kg. The dosages for treatment were according to the extraction ratios and the gavage was one time a day and performed for 12 weeks. By calculating extraction ratios, the experiment ensured each groups were given same amount of *G. jasminoides* ingredients.
**Feces sample preparation**

During the treatment of 12 weeks, feces samples were separately collected and stored at -80 °C. And time points of collecting are respectively 0 week (before treat), 4 weeks, 8 weeks and 12 weeks. As yet, technique UPLC-QTOF-MS is not widely used to analyze feces samples because of the complicated compositions. To make feces samples effectively, the method was followed according to the report and based on the preliminary experiments. Feces samples were immersed in methyl alcohol 3 times of their total weight. After blending and centrifuging at 10,000 rpm for 10 min, the supernatant was filtered through a 0.22 µm ultra-filtration membrane.

**UPLC-MS conditions**

Metabolomics analysis was performed using a Waters Acquity UPLC system coupled with a Q-TOF SYNAPT G2 High Definition Mass Spectrometer (HDMS) (Waters, UK). Separation was carried out on a Waters ACQUITY UPLC BEH C18 Column (1.7 µm, 2.1 mm × 50 mm) kept at 40 °C and at a flow rate of 0.3 mL/ min. 0.1% aqueous formic acid (v/v) (A) and acetonitrile (B) were used as mobile phase. The gradient elution of B was performed as follows: 5%–30% B at 0-1 min, 30%–50% B at 1-3 min, 50%–53% B at 3-3.6 min, 53%–60% B at 3.6-4.2 min, 60%–100% B at 4.2-8 min, 100% B at 8-9 min, 100%–5% B at 9-10 min and then kept at 5% B for 4 min. The sample injected volume was 5 µL. During the period of the analysis, all the samples were maintained at 4 °C. The ESI source in both positive and negative ion modes was used in MS analysis. Nitrogen was used as cone and desolvation gas and the flow rates were set at 50 L/h and 700 L/h, respectively. MS data were collected in
the mass range of 100-1000 Da with a 0.2 s scan time. Sodium formate was used for mass spectrometer calibration. 2 ng/mL leucine enkephalin at a flow rate of 5 µL/min was used as the lock mass. Argon was used as the collision gas.

**Data analysis**

The UPLC Q-TOF MS raw data were first processed by MassLynxV 4.1 and MarkerLynx Application Manager. After that, the peaks were detected and aligned thus the complicated data were reduced and normalized to the summed total ion intensity of each chromatogram. Afterwards, the data matrix was established based on aligning the peaks with the exact mass/retention time and normalized peak areas. Then the data matrix was imported into EZinfo 2.0 (a multivariate statistical analysis software) for principal component analysis (PCA) and orthogonal projection to latent structures squares-discriminant analysis (OPLS-DA). PASW Statistics 18.0 software was applied for independent sample t-test between groups, and p<0.01 was considered to be significant. To identify potential markers and the metabolic pathway, available biochemical databases were used, such as HMDB (http://www.hmdb.ca/), METLIN (http://metlin.scripps.edu/), Massbank (http://www.massbank.jp) and KEGG (http://www.kegg.com/). To illustrate the correlation between these biomarkers, a network diagraph was generated based on searching results from the KEGG database and literature information.

**RESULTS AND DISCUSSION**

*primal physical condition analysis*
Fasting blood-glucose can reflect the primal physical condition of T2DM rats. Thus, this experiment measured this index every four weeks one time to observe the physical condition changes and influence of treatment. The results of fasting blood-glucose are presented in Table 1. HC indicated the normal condition: the fasting blood-glucose remained around 5 mmol/L. Counter to HC, DM rats had their fasting blood-glucose to rose and then stabilized at about 24 mmol/L. To observe the effect of treatment in the same course, the data from each treatment group and type 2 diabetes control group was compared at the same time points. Independent sample t-test between DM and other groups was applied, and p<0.01 was considered to be significant. In Table 1, the fasting blood-glucose in 4 weeks and 8 weeks of each treatment group showed no significant difference with type 2 diabetes control group. However, after 12 weeks treatment, all treatment groups showed a certain symptom in fasting blood-glucose control. (P<0.01 compared to DM).

Another essential physiological indexes is body weight. As shown in Table 2, the average weight in HC increased from 332 to 437 grams gradually. In contrast with HC, DM had their average weight to decrease to only 192 grams after 12 weeks. In 12 week, body weights of GP and IG showed significant differences compared to HC (P<0.01), which gave an advice that geniposide and total iridoid glycosides postponed the loss of body weight. And these results suggested that G. jasminoides has good tendency in physical condition regulation.

**Fecal metabonomic fingerprint analysis**
This investigation adopted UPLC-Q-TOF-MS to detect feces samples from each group. Based on peak intensity chromatograms of week 12 samples in positive and negative ion modes (see Fig. 1), differences could be observed between HC and DM. For example, as shown in negative ion mode chromatogram (see Fig. 1 (B)), the peaks from 6 minutes to 8 minutes had a high intensity in HC but very low intensity in DM. However, other groups could not be clearly distinguished to compare with DM. This means that the observation might be not sufficient, thus the further survey for the therapeutic effect of *fructus gardeniae* is necessary.

**Principle component analysis (PCA)**

**PCA for disease progress:** Principle component analysis (PCA) by using the scatter diagram can be applied as a pattern recognition method. In this research, PCA was used to classify different sample groups based on the differences of metabolite species and content. In this section, only DM feces samples in various periods were detected and analyzed for demonstrating the disease progress of T2DM rats. And the results in positive and negative ion modes are described in Fig. 2. Each point represents a feces sample, and the locations of these points reflect the compositions in that feces sample. The points in different administration periods, 0, 4, 8 and 12 weeks, are marked using different colors. From this figure we can see that the metabolic changes of DM rats were not considerable in initial stage. However, the longer the administration time, such as from week 4 to week 8, the distances among the metabolite regions were larger, which indicates that the metabolic profile between the two time periods had a significant change. From week 8 to week 12, the metabolite
points were more scatter than that in other periods, which means that an important metabolic change occurred in this stage. These results reveal that with the deterioration of the disease as the time was longed, metabolic changes of rats were more and more noticeable \textit{in vivo}. One intriguing observation from \textbf{Fig. 2} is that the regions in negative ion mode were more tightness than that in positive ion mode. It might be explained that metabolism of acidic compounds put up more uniformity in feces samples.

\textbf{PCA for holistic efficacy of }\textit{G. jasminoides}: Similarly, feces after 12 weeks treatments from five groups were analyzed by the method of PCA. PCA score plots in positive and negative ion modes are showed in \textbf{Fig. 3}. From this figure we can see that after 12 weeks administration, all the points in the treated groups were separated from type 2 diabetes control group (DM), which means that \textit{G. jasminoides} showed the trends of therapeutic effect. Respectively, geniposide (GP) showed much better effects than crude extraction (CE), which should be the main active ingredients in \textit{G. jasminoides} for treatment of diabetes. As total iridoid glycosides (IG) showed considerable effect, therefore, other iridoid glycosides might have a certain effects.

\textbf{Identification of potential biomarkers}

An OPLS-DA model was established to maximize covariance between the data. By means of OPLS-DA model the potential biomarkers and metabonomic pathways of type 2 diabetic rats were investigated, i.e., the potential biomarkers from control group and model group after 12 weeks could be extracted by using OPLS-DA model. Concretely, OPLS-DA score plots, S-plots and variable importance in the projection
(VIP) values were obtained. As described in Fig. 4 (A, B), the group differences between DM and HC could be distinctly identified in this OPLS-DA score plots. The OPLS-DA score plots present good fitness and high predictability of model, with high statistical values of $R^2_Y$ and $Q^2$. In this OPLS-DA model, the parameters separately were $R^2_Y = 0.992$, $Q^2 = 0.872$ in positive mode and $R^2_Y = 0.991$, $Q^2 = 0.875$ in negative mode. In S-plots, the spots located at the ends of the plot represent endogenous components which have greater VIP values (see Fig. 4 (C, D)). The VIP of OPLS-DA model was generated using centroid scaling to identify the metabolite contribution to the discrimination. The endogenous substances with VIP value above 1.0 and $p$-value below 0.05 were considered as potential biomarkers. Based on this rule, potential biomarkers in feces were screened for further study. The possible molecular formulas of these potential biomarkers were calculated by high-accuracy quasi-molecular ion within a mass error of 10 ppm, and the fractional isotope abundance detected by UPLC-Q-TOF-MS was used to filter out the interference. According to the precise molecular masses and necessary structural information provided by MS/MS fragmentations, these potential biomarkers were identified by searching available biochemical databases. The information of these potential biomarkers is summarized in Table 3. For example, a potential biomarker had its retention time at 3.7 minutes and its measured mass 391.2856. Based on these information and its MS/MS fragmentations shown in Fig. 5, this potential biomarker was considered to be 12-ketodeoxycholic acid.

**Metabolic pathway analysis**
Diabetes is a chronic disease characterized by long-term metabolic disorders. Thus, many researchers devoted to use metabolomics to study diabetes. However, most experiments focused on urine. In this study, feces samples were adopted as the object for investigating the metabolic changes resulted from long-term diabetes. As shown in Table 3, a total of 19 potential biomarkers were identified by the method presented above, which were involved in phenylalanine, tryptophan, sphingolipid, fatty acid biosynthesis and bile acids metabolic pathways.

Fig. 6 was a network diagraph constructed based on KEGG database and literature information, which described these biomarkers and their metabolism pathways. As shown in Fig. 6, phenylacetaldehyde and trans-Cinnamic acid are markers in phenylalanine metabolism pathway, which reflect phenylalanine metabolism alterations in this research. Phenylacetaldehyde is a secondary metabolite of styrene, which is easy to hydrolysis and oxidize to yield phenylacetic acid in vivo. Trans-Cinnamic acid is the trans-structure of cinnamic acid that is a production of L-phenylalanine and involved in various metabolic pathways, tryptophan metabolism and the biosynthesis of phenylpropanoid, ubiquinone and other terpenoid-quinone besides. Recently, experiments gave a consequence that trans-Cinnamic acid could increase adiponectin and the phosphorylation of AMP-activated protein kinase through G-protein-coupled receptor signaling in 3T3-L1 adipocytes. It can be seen in Fig. 6, these two markers are all downstream mediators of phenylalanine, which played a crucial role in phenylalanine metabolism. Phenylalanine, as one of aromatic amino acids (AAA), is mainly oxidized to tyrosine
Phenylalanine is able to synthesize important neurotransmitters and hormones with tyrosine, which is essential to metabolism of sugar and fat. Abnormal phenylalanine metabolism was also demonstrated in plasma and urine samples. Since literatures had reported that elevated concentrations of branched chain (BCAA) and AAA could predict the onset of type 2 diabetes. The contents of phenylacetaldehyde and trans-cinnamic acid were quite low in HC but higher in DM, indicated that T2DM obviously changed the phenylalanine metabolism pathway of rats.

Tryptamine, 3-methyldioxyindole and 4-(2-Aminophenyl)-2,4-dioxobutanoic acid are related to tryptophan metabolism. Tryptamine is a monoamine compound generally generated by the biosynthesis of the amino acid tryptophan, in turn acting as a precursor for other compounds such as hormones and neurotransmitters. 3-Methyldioxyindole is an in vivo oxidation product formed during metabolism of 3-methylindole, which is a metabolic product of tryptophan generated by bacteria in the colon. As shown in Fig. 6, 3-methyldioxyindole could be obtained from tryptamine via three biological reactions. 4-(2-Aminophenyl)-2,4-dioxobutanoic acid is a substrate related to tryptophan as well. Tryptophan is an essential amino acid and the precursor of serotonin which is a biochemical messenger and regulator. The level of tryptamine and 4-(2-Aminophenyl)-2,4-dioxobutanoic in DM was higher compared to the level of HC. And the level of 3-Methyldioxyindole was decreased by T2DM. So in this research, abnormal tryptophan metabolism of T2DM rats was confirmed.
Sphingosine, sphinganine and phytosphingosine are intermediate products in sphingolipid metabolism. In this research, the levels of these three potential biomarkers were all decreased by T2DM. Sphingosine and its derivative sphinganine are the major bases of the sphingolipids in mammals. And it contributes to formation of a potent signaling lipid, sphingosine-1-phosphate. Sphinganine has a biological function of regulating cholesterol transport. Phytosphingosine is a phospholipid, and its physiological roles are largely unknown. Nevertheless, researchers demonstrated that phytosphingosine was able to induce apoptosis. Dihydro-ceramide, a pivotal ingredient in sphingolipid metabolism, can generate all these three markers (see Fig. 6). Sphingolipid metabolism altering has been detected in diabetic retinopathy, which was considered to be related to the mechanism of cell death. Recently, investigators chose sphingolipid metabolism as therapeutic target in treating T2DM and got satisfying results.

Tetrahydrocorticosterone is relevant to steroid hormone biosynthesis. It is one of the major metabolites from corticosterone, and was discovered in blood and urine. In this research, tetrahydrocorticosterone was detected in feces samples and it had visible level in HC. However, it had a quite low level in DM.

3-Oxohexadecanoic acid, (R)-3-hydroxy-hexadecanoic acid and elaidic acid represent fatty acid biosynthesis. Fatty acid biosynthesis reflects the function of the liver, lipid regulation by organism and other somatic functions. Thus, fatty acid has an intimate relativity with T2DM. Researchers have found that the content of fatty acid was elevated in serum and kidneys of T2DM patients. And
very-low-density-lipoprotein triglycerides (VLDL-TG) fatty acid storage in skeletal muscle in men with type 2 diabetes was also proved to be increased. Similar to the results of serum, kidneys and skeletal muscle, this study detected the changes of fatty acid content level, which demonstrated that T2DM definitely changed the fatty acid biosynthesis. And this fatty acid biosynthesis change further suggested fecal metabolomics study is feasible in diabetes research. In this research, the above three potential biomarkers were all decreased in the feces samples of T2DM rats (see Fig. 7). It could be speculated that the higher fatty acid concentrations in serum, kidneys and more fatty acid storage in body together resulted in the decrease of fatty acid in feces samples. This result further confirmed the fatty acid metabolism disorder took place in T2DM patients.

L-Urobilin and L-urobilinogen are the metabolites related to porphyrin and chlorophyll metabolism. They are both the products in bilirubin degradation showing decreased contents in T2DM feces in the current study. H. Cao's research aimed at analyzing the feces from liver cirrhosis and hepatocellular carcinoma patients, in which decrease concentrations of these two substances have been reported. It is consistent with our results (see Fig. 7). Thus, these findings suggest that T2DM increased the degree of hypohepatia.

In addition, 12-ketodeoxycholic acid, deoxycholic acid, 3b-hydroxy-5-cholenoic acid, all lithocholic acid, and bisnorcholeic acid were all bile acids. This study revealed that T2DM could increase the level of deoxycholic acid and decrease the levels of other four bile acids. Bile acids facilitate to dissolve in biliary cholesterol
and intestinal lipids,\textsuperscript{37} and possess properties of physiological detergents to facilitate excretion, absorption, and transporting the fats and sterols in the intestine and liver. Hence, the changes of these bile acids might be the response to variations of fats and sterols pathways. Evidence showed that the disturbance of glucose metabolism altered bile acid metabolism in T2DM patients.\textsuperscript{37} Thus, a suggestion was given that bile acid might have a function in maintaining glucose homeostasis.\textsuperscript{38} Deoxycholic acid is a common secondary bile acid which revealed the secondary bile acid biosynthesis in this study. Researchers have indicated that deoxycholic acid contributed to disruption of the intestinal barrier function and the changes of electrophysiological parameters in murine small intestine.\textsuperscript{39} And other investigation results revealed that elevated deoxycholic acid was related to pancreatitis.\textsuperscript{40} According to these papers, the high level of deoxycholic acid might be a factor of intestine and pancreas disorders. The presentation in Fig. 7 also demonstrates that deoxycholic acid increased observably in DM rats compared with HC rats.

To sum up, in this study, 7 unique metabolic pathways were found to be altered by T2DM. Among these, bile acids, porphyrin and chlorophyll metabolism and fatty acid biosynthesis are infrequent in urine or plasma samples.

**Regulating effects of \textit{G. jasminoides}: relative intensity analysis of potential biomarkers**

Fig. 7 figures out the relative intensities of these potential biomarkers from all 5 different groups. The potential biomarkers were exhibited separated and all the data were based on feces samples after 12 weeks treatments. By analyzing the relative
intensities of these potential biomarkers in DM and HC at first, the detailed impacts of T2DM on the above metabolic pathways were revealed. Then the treatment efficacies of \textit{G. jasminoides} in regulating the above metabolic pathways were discussed depended on the comparison of control groups and treatment groups. The same analytical method as primal physical condition analysis, the data from type 2 diabetes control group was compared with other groups. By computing the \textit{p}-value between groups, the changes of potential biomarkers’s contents in treatment groups were revealed.

As shown in \textbf{Fig. 7 (A)}, in the phenylalanine metabolism pathway, phenylacetaldehyde was quite low in HC but higher in DM. Among the 3 \textit{G. jasminoides} treatment groups, the levels of phenylacetaldehyde in GP significantly decreased (P<0.01 compared to DM), suggested better effect of geniposide in regulating the content of phenylacetaldehyde. However, crude extraction of \textit{G. jasminoides} (CE) showed no positive modulating effect. Trans-cinnamic acid, another marker related to phenylalanine metabolism whose relative intensities in treatment groups showed no significant difference compared to DM.

To observe the effect in adjusting tryptophan metabolism, the relative intensities of tryptamine, 3-Methyldioxyindole and 4-(2-Aminophenyl)-2,4-dioxo-butanoic acid would be discussed. The levels of tryptamine in DM and HC were similar, but the levels were remarkable high in the treatment groups. Thus, the medicines used in this research did not control the level of tryptamine effectively. Gratifyingly, treatment groups presented obviously functions in regulating 3-Methyldioxyindole and
4-(2-Aminophenyl)-2,4-dioxo-butanoic acid. IG presented preferable efficacy than other groups, it was even able to reverse the relative intensities to normal levels (P<0.01 compared to DM, Fig. 7 (A, D)). On the contrast, the CE showed no significant effect.

Treatment groups exhibited no significant effect as for metabolites from sphingolipid metabolism. Respectively, the level of sphingosine in DM was remarkable decreased compared to HC. However, these levels were even lower in treatment groups, which demonstrated worse trends (see Fig. 7 A). Sphinganine and phytosphingosine showed better trends in content variations but these variations were not observably. Only the level of phytosphingosine from CE improved unexpectedly. This improvement turned the relative intensities to the levels higher than normal levels (P<0.01 compared to DM, Fig. 7 B).

Tetrahydrocorticosterone had visible level in HC, but it was barely detected in other groups. The result suggested *G. jasminoides* have no effect in the only detected metabolites from steroid hormone biosynthesis.

The other markers which represent fatty acid biosynthesis, bilirubin degradation and bile acids all presented the same characteristics expect deoxycholic acid. These markers, similar to tetrahydrocorticosterone, decreased markedly by T2DM but not be altered by treatment. Deoxycholic acid is the only one regulated positively in treatment groups. Based on the interpretations about deoxycholic acid, this result suggested that *G. jasminoides* was able to improve the function of intestine and pancreas.
Above analysis gave results that geniposide was the most effective in regulating phenylalanine metabolism. Geniposide and total iridoid glycosides showed similar effect in regulating tryptophan metabolism and secondary bile acid biosynthesis.

*G. jasminoides*, as a TCM used for thousand years show efficacy in treating T2DM. In this paper, a fecal metabolomics approach based on UPLC-Q-TOF-MS was developed to observe the metabolic alternations of T2DM rats and the regulatory functions of *G. jasminoides*. The results reveal that T2DM changed various metabolic pathways including phenylalanine metabolism, tryptophan metabolism, sphingolipid metabolism, steroid hormone biosynthesis, fatty acid biosynthesis, porphyrin and chlorophyll metabolism. And the contents of numerous bile acids were changed too. The efficacy of *G. jasminoides* was discussed based on the physiological indexes and fecal metabonomic analyses. Geniposide was the major active ingredient whose adjustment to T2DM rats' physical conditions was the most remarkable, regardless of hyperglycemia control or metabolism pathway regulation. Unfortunately, these metabolism pathway regulations were incomplete. From the relative intensity analysis, we can see that phenylalanine metabolism, tryptophan metabolism and secondary bile acid biosynthesis were altered evidently by treatments. This research also found that total iridoid glycosides had similar treatment effect, but a little weaker efficacy compared to geniposide. Though the total iridoid glycosides extracted in this study contained geniposide, the other compositions also existed in the total iridoid glycosides might still play some roles in the therapeutic effect of *G. jasminoides*.
toward T2DM. Hence further experiments should be conducted to investigate the other iridoid glycosides.

**Conflicts of Interest:** On behalf of all authors, the corresponding author states that there is no conflict of interest.
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FUNDING SOURCE

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FIGURE CAPTIONS

**Figure 1.** The typical fecal base peak intensity chromatograms (positive ion (A) and negative ion (B)).

**Figure 2.** PCA score plots of fecal metabolic profiles of DM rats based on the course of disease; positive ion (A) and negative ion (B) (■ for 0 week, ■ for 4 weeks, ■ for 8 weeks, ■ for 12 weeks).

**Figure 3.** PCA score plots of fecal metabolic profiles of different groups after 12 weeks treatment; positive ion (A) and negative ion (B) (■ for CE, ■ for DM, ■ for GP, ■ for HC, ■ for IG).

**Figure 4.** OPLS-DA score plots of fecal metabolic profiling of HC and DM rats (positive ion (A) and negative ion (B)) and OPLS-DA S-plots (positive ion (C) and negative ion (D)).

**Figure 5.** Two-stage tandem mass spectrogram of 12-ketodeoxycholic acid.

**Figure 6.** Correlation networks of potential biomarkers in response to T2DM. The red marked metabolites denotes the identified potential biomarkers and blue denotes metabolic pathway represent (some bile acids are not shown).

**Figure 7.** Relative intensities of potential biomarkers in different groups (**p< 0.01, compared to DM).
Table 1. Fasting Blood-glucose (means ± SD, mmol/L) of Rats in Different Groups

(HC: healthy control group, DM: type 2 diabetes control group, GP: geniposide treated group, IG: total iridoid glycosides treated group, CE: crude extraction of Geoffroea jasminoides treated group) and different weeks (**p< 0.01, compared to DM).

<table>
<thead>
<tr>
<th>Group</th>
<th>0W</th>
<th>4W</th>
<th>8W</th>
<th>12W</th>
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<tbody>
<tr>
<td>HC</td>
<td>4.82±0.20**</td>
<td>4.79±0.25**</td>
<td>4.81±0.47**</td>
<td>4.86±0.32**</td>
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<tr>
<td>DM</td>
<td>21.14±2.92</td>
<td>22.50±2.51</td>
<td>23.39±1.61</td>
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<td>IG</td>
<td>20.82±1.88</td>
<td>21.45±3.45</td>
<td>20.13±3.53</td>
<td>19.59±3.52**</td>
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<tr>
<td>CE</td>
<td>20.93±2.67</td>
<td>21.41±2.74</td>
<td>21.16±2.15</td>
<td>20.81±4.31**</td>
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</tbody>
</table>
**Table 2.** Body Weight (means ± SD, gram) of Rats in Different Groups (HC: healthy control group, DM: type 2 diabetes control group, GP: geniposide treated group, IG: total iridoid glycosides treated group, CE: crude extraction of *G. jasminoides* treated group) and different weeks (**p< 0.01, compared to DM). 

<table>
<thead>
<tr>
<th>Group</th>
<th>0W</th>
<th>4W</th>
<th>8W</th>
<th>12W</th>
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<tr>
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<td>332.4±5.2</td>
<td>370.1±4.6**</td>
<td>410.8±6.8**</td>
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<td>323.3±4.7</td>
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<tr>
<td>GP</td>
<td>322.6±7.7</td>
<td>285.3±7.0</td>
<td>242.0±13.0</td>
<td>217.1±21.2**</td>
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<tr>
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<td>324.9±3.8</td>
<td>281.1±12.1</td>
<td>242.8±10.6</td>
<td>213.8±12.3**</td>
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<td>Measured mass</td>
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Figure 1

A

CE

IG

GP

DM

HC

B

CF

IG

GP

DM

HC

ACS Paragon Plus Environment
Figure 2
Figure 3
Figure 4
Figure 5
Figure 6

Tetracyclorsoxysterone → Spinglipid metabolism

- Cholesterol
- Glycocholate → Taurocholate
  - Cholate → Multi-step 7α-dehydroxylation
  - Deoxycholic acid
  - Secondary bile acid biosynthesis

Spinganine → Spingosine → Ceramide

- Psycholine
- Tryptophan metabolism

4-(2-Aminophenyl)-1,4-dioxo-2-butanoic acid → TCA cycle → Urea cycle

- Citric acid
- L-kynurenine → Acetyl-CoA
- trans-Cinnamic acid

- Phenylalanine

3-Oxodecanecarboxylic acid → L-Urobiloxigen

- [R]-3-Hydroxyhexadecanoic acid
- Hexa-decanoyl [acp]
- 3-Oxooctadecanoic acid
- Octadecanoyl [acp]
- L-Urobin
- Elaidic acid
- Porphyrin and chlorophyll metabolites

- Fatty acid biosynthesis
Fecal metabolomics analysis