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Metabolomics analysis of Semen Cuscutae protection of kidney deficient model rats using ultra high-performance liquid chromatography-quadrupole time-of-flight Mass Spectrometry

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ABSTRACT

The traditional Chinese medicine syndrome "Kidney yang deficiency" is a kind of chronic kidney disease. With the development of society, the incidence of chronic kidney disease is increasing year by year, which also brings great economic pressure to people. Semen Cuscutae is an important traditional Chinese medicine to tonify liver and kidney, mainly used to tonify deficiency of liver and kidney, spleen and kidney deficiency and diarrhea. Although there are a lot of research at the molecular and cellular level to study the Semen Cuscutae on the treatment of Kidney yang deficiency syndrome, but there's no comprehensive research complete with metabolomics method from plasma, feces and urine metabolites aspects. The purpose of this study is to find the potential differential biomarkers of the Kidney yang deficiency model and blank group rats in plasma, urine and feces, and to investigate the mechanism of Semen Cuscutae in the treatment of Kidney yang deficiency syndrome. In this study, ultra high-performance liquid chromatography-quadrupole time-of-flight Mass Spectrometry (UPLC-QTOF/MS) was used to identify potential biomarkers. Through the analysis of metabolic profiles of plasma, urine, and feces, as well as multivariate statistical analysis and pathway analysis, the therapeutic mechanism of Semen Cuscutae for Kidney yang deficiency syndrome was described. The results showed that there were 69 differential metabolites in plasma, 93 differential metabolites in feces and 62 differential metabolites in urine, and the changes of the levels of these biomarkers showed that Semen Cuscutae had a good therapeutic effect on Kidney yang deficiency syndrome. Through the analysis of the channel, the metabolite changes mainly affected the steroid hormone biosynthesis, arachidonic acid metabolism, primary bile acid biosynthesis, sheath lipid metabolism and biosynthesis of tyrosine, phenylalanine metabolism, retinol metabolism,taurine and hypotaurine metabolism, lysine degradation and vitamin B6 metabolism, tryptophan metabolism, terpenoid backbone biosynthesis and starch and sucrose metabolism. Therefore, the results suggested that Semen Cuscutae could exert a good therapeutic effect by reversing the levels of some biomarkers.

1. Introduction

Kidney yang deficiency syndrome is a common clinical TCM syndrome, which is a kind of deficiency and cold syndrome manifested by kidney yang deficiency and failure, warm dereliction of duty, and loss of power by vaporization [1,2]. Traditional Chinese medicine believes that the kidney is the main water, Kidney yang has the gasification transpiration effect on the water liquid, if the Kidney yang is insufficient, transpiration gasification is weak, the urine is clear and long and so on [3,4]. So the Kidney yang deficiency syndrome means the pathological changes of the kidney [5,6]. Hydrocortisone was used to act on the hypothalamus-pituitary-adrenal axis, thereby inhibiting the HPA axis and the pituitary gland's secretion of adrenergic hormone, which inhibited the adrenal gland's secretion of adrenaline, resulting in symptoms similar to kidney yang deficiency. In kidney yang deficiency rats, free T4 is lower, TSH is higher, and the body is mostly manifested as slow metabolism and low function. In addition, CD3 + /CD4 + and other immune factors are found to decrease in patients with kidney yang deficiency,

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indicating that the immune system of patients with kidney yang deficiency is impaired.

Semen Cuscutae was first recorded in Shennong Materia Medica Volume 2, Cuscutae has a pungent and mild medicinal property. It is mainly used to treat traumatic injuries, tonic deficiency and vitality, and can strengthen the body. Its juice can dispel freckles. It can be used for a long time to improve eyesight, light and healthy, and prolong life [7–9]. In modern medical studies, Semen Cuscutae has been found to have a wide range of biological activities, such as a positive role in male reproductive therapy [10]. For example, Semen Cuscutae was found to reduce the apoptosis of germ cells in rats with unilateral uncryptorchidism by giving Semen Cuscutae extract from rats with excised right testis [11]. Semen Cuscutae regulates H19 / IGF2 methylation by increasing DNA methyltransferase expression, thereby increasing reproductive hormone and receptor levels and reducing apoptosis, and more studies have shown that the chemical composition of Semen Cuscutae is associated with estrogen. Semen Cuscutae has antioxidant bioactivity, anti-osteoporosis, anti-inflammatory, anti-tumor and other effects [12–14].

The internal environment of diseases is a dynamic process, and traditional Chinese medicine has the characteristics of multiple components and multiple targets. Therefore, it is a great challenge to clarify the specific mechanism of traditional Chinese medicine on diseases [15]. Metabolomics obtains metabolic fingerprint characteristics and metabolic pathways that reflect the functional state of the organism through the overall and systematic analysis of endogenous small molecule metabolites, and reveals the metabolic nature of the organism's life activities and the state of the organism's microenvironment, in order to more reflect the cell's environment and other external factors [16]. Therefore, metabolomics has revealed the unique advantages of Chinese traditional medicine to treat disease metabolic pattern [17]. Although there are a lot of research at the molecular and cellular level to study the Semen Cuscutae on the treatment of Kidney yang deficiency syndrome, but there's no comprehensive research complete with metabolomics method from plasma, feces and urine metabolites aspects. In this study, LC-MS metabolomics was used to study the mechanism of Semen Cuscutae in the treatment of kidney yang deficiency syndrome. The results obtained from this research will provide a better understanding of the therapeutic effects of Semen Cuscutae.

2. Experimental

2.1. Chemical reagents

Semen Cuscutae seed and the positive drug of Jingui Kidney Qi Pill was purchased from Tianyitang TCM store (Shenyang, China). Hydrocortisone injection was purchased from Jinyao (Company Inc, Tianjin, China). Acetonitrile, methanol and formic acid of LC/MS grade were purchased from Fisher Scientific (Company Inc, USA). Distilled water prepared with Watson demineralized water was employed throughout the experiment.

2.2. Animals

Adult female Wistar rats (age: 8-weeks old, weight: 200–300 g) used for this study were obtained from the Vital River Laboratories, Beijing, China. The animal study was carried out in accordance with the Guideline for Animal Experimentation of Shenyang Medical College and the protocol was approved by the Animal Ethics Committee of the institution.

Kidney deficient model rats were induced by hydrocortisone injection method [18]. Kidney deficient group rats were intramuscularly injected with hydrocortisone injection 25 μg kg^{-1} for 15 days. Rats presenting fear of cold, loose feces, spirit wilting, reduced independent activity and weight loss were proved as the success of the kidney deficient model. During the Kidney deficient model rats process, we observed the daily habits and physical conditions of the rats by naked eyes, and found that the rats had symptoms of shrinking, clumping, hair loss, and decreased activity. The weight of normal group increased obviously, but the weight of model group increased slowly.

2.3. Analytical instruments and methods

Data was collected on UPLC-Q-TOF/MS (Waters) system. UPLC analysis was carried out in Water Acquity UPLC system. The sample (3 μL) was injected into Acquity UPLC BEH C18 column (2.1 mm × 100 mm, 1.7 μm, Waters). The column temperature was set at 40 ℃ and the flow rate was 0.4 mL/min. Feces samples positive ion mode: the gradient system consists of mobile phase A methanol and mobile phase B (0.1%) formic acid water (0–2 min, A, 5%, 2–20 min, A, 5–95%, 20–22 min, A, 95%, 22–22.10, A, 95–5%, 22.10–25 min, A, 5%). Feces samples negative ion mode: the gradient system consists of mobile phase A acetonitrile and mobile phase B water (0–2 min, A, 5%, 2–16 min, A, 5–95%. 16–18 min, A, 95%, 18–18.10 min, A, 95–5%, 18.10–21 min, A, 5%). Urine samples positive ion mode, the gradient system consists of mobile phase A methanol and mobile phase B water (0–2 min, A, 5%, 2–9 min, A, 5–55%. 9–12 min, A, 55–95%, 12–14 min, A, 95%, 14–14.10 min, A, 95–5%, 14.10–16.00 min, A, 5%). Urine samples negative ion mode, the gradient system consists of mobile phase A acetonitrile and mobile phase water (0–2 min, A, 5%, 2–9 min, A, 5–55%. 9–12 min, A, 55–95%, 12–14 min, A, 95%, 14–14.10 min, A, 95–5%, 14.10–16.00 min, A, 5%). Plasma samples positive ion mode: gradient system consists of mobile phase A (0.1%) formic acid water and mobile phase B acetonitrile (0–2 min, A, 95%, 2–8 min, A, 95–40%, 8–18 min, A, 40–5%, 18–20 min, A, 5%, 20–20.1 min, A, 5–95%, 20.1–22 min, A, 95%). Plasma samples negative ion mode: the gradient system consists of mobile phase A water and mobile phase B acetonitrile (0–2 min, A, 95%, 2–8 min, A, 95–40%, 8–18 min, A, 40–5%, 18–20 min, A, 5%, 20–20.1 min, A, 5–95%, 20.1–22 min, A, 95%). MS is performed on Waters Micro Mass Q/TOF Mass Spectrometer. The electrospray ionization sources (ESI Source) were used for mass spectrometry in positive and negative ionization modes respectively. The parameters of MS analysis are as follows: capillary voltage: plasma positive ion mode is 2.0 kV, negative ion mode is 2.0 kV; The positive ion mode of urine was 2.5 kV, the negative ion mode was 2.5 kV. The feces positive ion mode was 1.5 kV, and the negative ion mode was 2.5 kV. The desolvent gas flow was 800 (L/h), the source temperature was 120 °C, the desolvent temperature was 500 °C, and the conical air flow was 50 L/h. The collision energy is 6.0. Reference ion (positive ion mode: 556.2771; Negative ion mode 554.2615) is used to ensure the accuracy of spectral acquisition and the scanning range is *m/z* 100–1200 Da. During data collection, random lists of samples should be generated in order to avoid systematic analysis errors. Before sample analysis, 8–10 QC samples should be injected to ensure the stability of the instrument, and then one QC sample should be analyzed every 5 or 6 samples.

2.4. Preparation of Semen Cuscutae decoction

The Semen Cuscutae seeds were pulverized to fine powder. And then powders were extracted three times by refluxing in water (1:10 w/v) for 2 h. The extracted solutions were concentrated under reduced pressure to 0.5 g mL^{-1}. The decoction was stored in the refrigerator at 4 °C.

2.5. Sample collection and processing

2.5.1. Sample collection

On the 15th day of the animal experiment, urine samples were collected from the metabolic cage and centrifuged at 1300 rpm for 15 min, and then all urine samples were stored at − 80 °C.

The plasma samples were collected from the abdominal aorta and collected in a clean EP tube coated with heparin sodium. The samples were centrifuged at 4 °C and 4000 rpm for 10 min. The supernatant was collected and stored at − 80 °C before metabolomics analysis.

On the 15th day of the animal experiment, animal feces samples were collected in sterile EP tubes, and all fecal samples were stored at − 80 °C before metabolomics analysis.

2.5.2. Sample processing

The plasma samples were took out of − 80 ℃ and thawed at 4 ℃. Each 200 μL aliquot of plasma sample was mixed with 600 μL of pre-cooled methanol and vortexed for 60 s to precipitate the proteins. Then, the samples were at − 20 ℃ for 20 min and centrifuged at 12,000 rpm and 4 ℃ for 5 min. The supernatants were transferred into EP tubes and six quality control (QC) samples were generated by of all extracted samples at the same time, and evaporated to dryness at 35 °C under a slight stream of nitrogen. Then, the dried extract was reconstituted in 200 μL methanol-water (80:20, v/v), vortex-mixed for 1 min and then centrifuged at 12,000 rpm and 4 ℃ for 3 min. Followed by injection of 3 μL aliquot into UPLC-MS/MS for analysis.

The urine samples were took out of − 80 ℃ and thawed at 4 ℃. Each 200 μL aliquot of urine sample was mixed with 600 μL of pre-cooled methanol and vortexed for 60 s to precipitate the proteins. Then, the samples were at − 20 ℃ for 20 min and centrifuged at 12,000 rpm, 4 ℃ for 5 min. The supernatants were transferred into EP tubes and six quality control (QC) samples were generated by of all extracted samples at the same time. And all samples were filtered with 0.22 μm microporous membrane, then centrifuged at 12,000 rpm, 4 ℃ for 10 min and the supernatant were for analysis.

The feces samples were took out of − 80 ℃ and thawed at 4 ℃. Each 50 mg feces sample was mixed with 1000 μL of pre-cooled methanol. The mixture was homogenized at 60 Hz for 30 s and subsequently vortexed for 60 s to precipitate protein. The sample was put at − 20 °C for 20 min and centrifuged at 12,000 rpm for 10 min at 4 °C twice. The supernatants were transferred into EP tubes and six quality control (QC) samples were generated by of all extracted samples at the same time. And all samples were filtered with 0.22 μm microporous membrane, then centrifuged at 12,000 rpm, 4 ℃ for 10 min and the supernatant were for analysis.

2.6. Data analysis

Import the raw plasma sample data collected in UPLC-Q-TOF/MS into the metabolomics processing software QI2.3 (Waters) for baseline screening, peak extraction, peak matching, retention time correction and peak alignment, and then obtain the data matrix of retention time, mass-to-charge ratio (*m/z*) and peak intensity. Then the data matrix is preprocessed. First, the variables with non-zero values greater than 50% in all samples are retained, and the missing values are filled in with half of the minimum value in the original matrix. Secondly, normalize the total peaks, delete variables with relative standard deviation ≥ 30% in the quality control samples, and perform log10 conversion. Finally, after normalizing the data by using total ion intensity, a list of interesting features including retention time, *m/z* value and normalized peak intensity is used for principal component analysis (PCA) for evaluation Repeatability. Then use partial least-squares discrimination analysis (PLS-DA) and orthogonal partial least-squares discrimination analysis (OPLS-DA) to analyze the overall metabolism difference in the contour of the object m. On the basis of OPLS-DA, the metabolites selected by using variable importance (VIP) > 1 and P < 0.05 are differential metabolites, which can be used for endogenous metabolite identification and metabolic pathway enrichment analysis. All data collected were expressed as mean ± SD. Statistics significance was assessed by ANOVA test.

3. Results

3.1. UPLC-MS results

The UPLC/MS Base peak ion current chromatogram (BPI) of plasma samples, feces samples, and urine samples were shown in positive ionization modes (ESI+) and negative ionization modes (ESI-) in SFig. 1. Under the optimized gradient elution program and the metabolomics map of each sample, the BPI showed ideal separation results. In this study, a multivariate statistical analysis method was established to identify biomarkers related to the treatment of kidney yang deficiency by Semen Cuscutae.

3.2. Identification of potential differential metabolites

The raw data from UPLC/MS was analyzed by Progenesis QI software. Export RT, *m/z* and peak height intensity to EZinfo software for data analysis. Using the PLS-DA method for multivariate data analysis, there is a clear classification between the clusters of the model group and the blank group (Fig. 1) of feces samples, plasma samples and urine samples, and the blank group and the Semen Cuscutae group are relatively close, indicating biochemical disturbance has occurred. To draw OPLS-DA graphs and S-plots (SFig. 2) for each sample data to label potential biomarkers with VIP > 1. The farthest metabolite ion has a higher VIP value, which is labeled as a potential biomarker and is responsible for the difference between the blank group and the model group. Select ions with significant differences between the blank group and the model group from the S-plot, and use high-precision ion fragments and databases for metabolite identification. By consulting the online database HMDB library, the information about potential biomarkers can be obtained, and the Rt–*m/z* pairs of selected differential metabolite product ions in each group can be matched and distinguished by referring to the database. According to the above scheme, we separately labeled compounds with VIP > 1, P < 0.05 and CV ≤ 30 in feces, plasma and urine samples, and identified many potential biomarkers (Mass Error < 10 ppm) and listed them separately in STable 1. It can be seen from the table that in the plasma sample, a total of 39 differential metabolites were found in the positive ionization mode, and 30 differential metabolites were found in the negative ionization mode. In the urine sample, 24 differential metabolites were found in the positive ionization mode, and 38 different metabolites were found in negative ionization mode. In feces samples, 55 different metabolites were found in positive ionization mode, and 38 different metabolites were found in negative ionization mode.

3.3. Metabolic pathway analysis

Enter the HMDB numbers of the identified differential metabolites into the METABOANALYST online data analysis software, select Fisher's exact test for the enrichment method, and select the rat Rattusnorvegicus (rat) (KEGG) library for the access analysis. The path analysis results of plasma samples, urine samples, and feces samples were shown in Fig. 2 in the form of bubble charts. We have selected the top 15 pathways, including (1) Steroid hormone synthesis, (2) Steroid biosynthesis, (3) Arachidonic acid metabolism, (4) Linoleic acid metabolism, and primary bile acid biosynthesis, (5) Sphingolipid metabolism, (6) Phenylalanine, tyrosine and

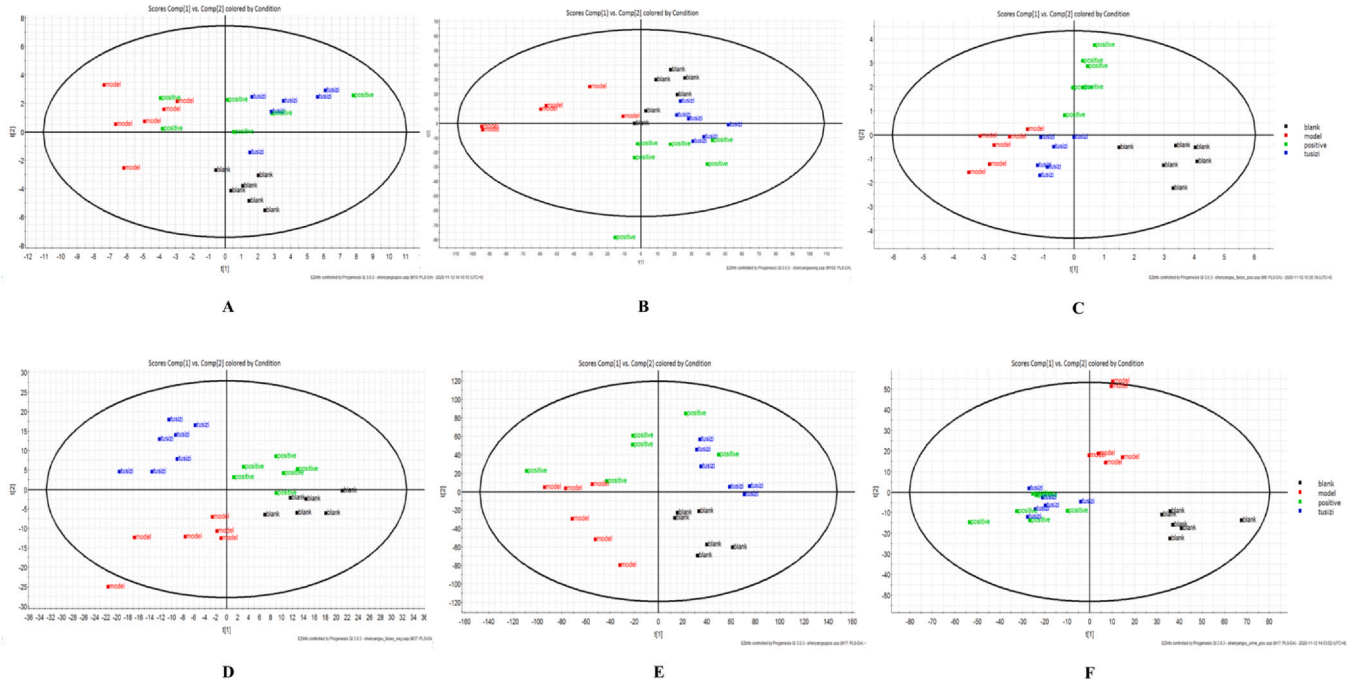

Fig. 1. PLS-DA score plots of Blank, model and positive drug , and *Cuscutae* (tusizi) groups in plasma sample, urine sample and feces sample. A. in plasma in ESI (+); B.in plasma in ESI (−); C.in feces in ESI (+), D. in feces in ESI (−); E.in urine in ESI (+), F.in urine in ESI (−). Black, red, green and blue dots were labeled as blank, model, positive drug, and *Cuscutae* (tusizi) groups, respectively. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

tryptophan biosynthesis, (7) Drug metabolism-cytochrome P450, (8) Glycerophospholipid metabolism, (9) Lysine Acid degradation, (10) Phenylalanine metabolism, (11) Tryptophan metabolism, (12) Retinol metabolism, (13) Tyrosine metabolism, (14) Taurine and taurine metabolism, (15) Starch and sucrose metabolism, etc. Perform heat map cluster analysis of the above metabolites, and select metabolites with large differences for display. The brightness change of the color represents the change of the concentration of the different metabolites. It can be seen from the heat map (Fig. 3) that compared with the blank group in plasma samples, urine samples and feces samples, there were 9 metabolites up-regulated in the feces model group, including androstandione, dehydroepiandrosterone, testosterone, corticosterone, 17alpha-hydroxyprogesterone, leukotriene A4, etc. 10 metabolites were down-regulated, including pregnenolone, cholid acid, dihydrotestosterone, Etiocholanolone, etc. 6 metabolites were up-regulated in the plasma model group, including cholid acid, testosterone, corticosterone, prostaglandin H2, 11b-hydroxyprogesterone. Down-regulation of 20 metabolites, such as arachidonic acid, prostaglandin G2, 25-hydroxycholesterol, Phenylacetylglycine, thyroxine, cholesterol, 9,10-Epoxyoctadecenoic acid, Retinyl ester, Secalciferol, etc. 6 metabolites in urine samples were up-regulated, including acetyl-CoA, LysoPE

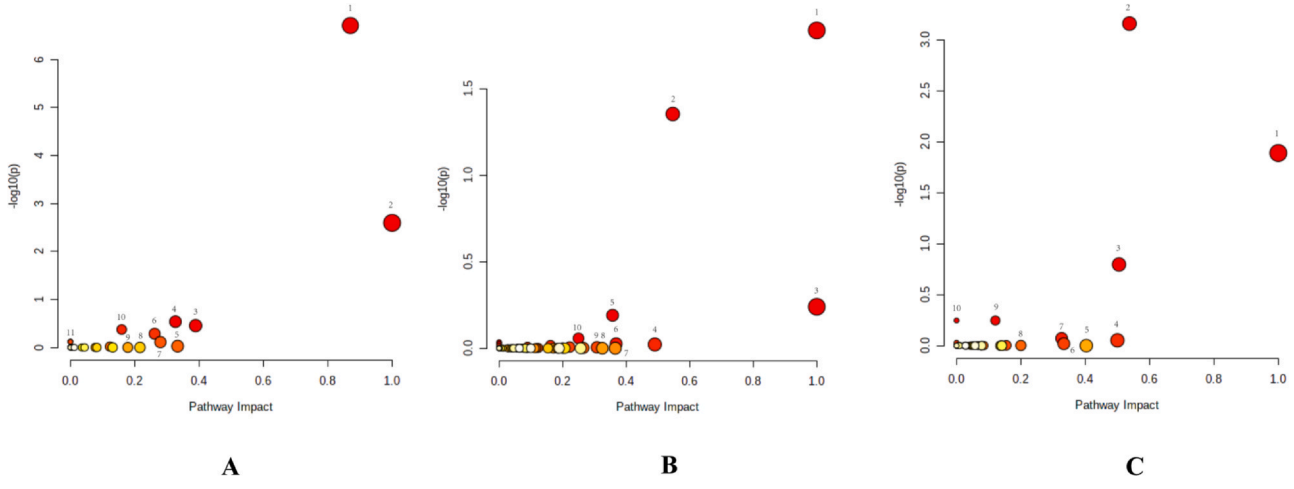

Fig. 2. Summary of pathway analysis with METABOANALYST tool. A. Plasma sample metabolic pathway: (1) Arachidonic acid metabolism (2) linoleic acid metabolism (3) Retinol metabolism (4) steroid hormone biosynthesis (5) alpha-Linolenic acid metabolism (6) Phenylalanine metabolism (7) Steroid biosynthesis (8) Glycerophospholipid metabolism (9) Pantothenate and CoA biosynthesis (10) Primary bile acid biosynthesis (11) Phenylalanine, tyrosine and tryptophan biosynthesis. B. Urine sample metabolic pathway: (1) linoleic acid metabolism (2) Arachidonic acid metabolism (3) Phenylalanine, tyrosine and tryptophan biosynthesis (4) Vitamin B6 metabolism (5) Phenylalanine metabolism (6) Terpenoid backbone biosynthesis (7) Glycerophospholipid metabolism (8) Porphyrin and chlorophyll metabolism (9) Caffeine metabolism (10) Ascorbate and aldarate metabolism C. feces sample metabolic pathway: (1) Linoleic acid metabolism (2) Arachidonic acid metabolism (3) steroid hormone biosynthesis (4) Phenylalanine, tyrosine and tryptophan biosynthesis (5) Glycerophospholipid metabolism (6) alpha-Linolenic acid metabolism (7) Sphingolipid metabolism (8) Biotin metabolism (9) One carbon pool by folate (10) Ubiquinone and other terpenoid-quinone biosynthesis.

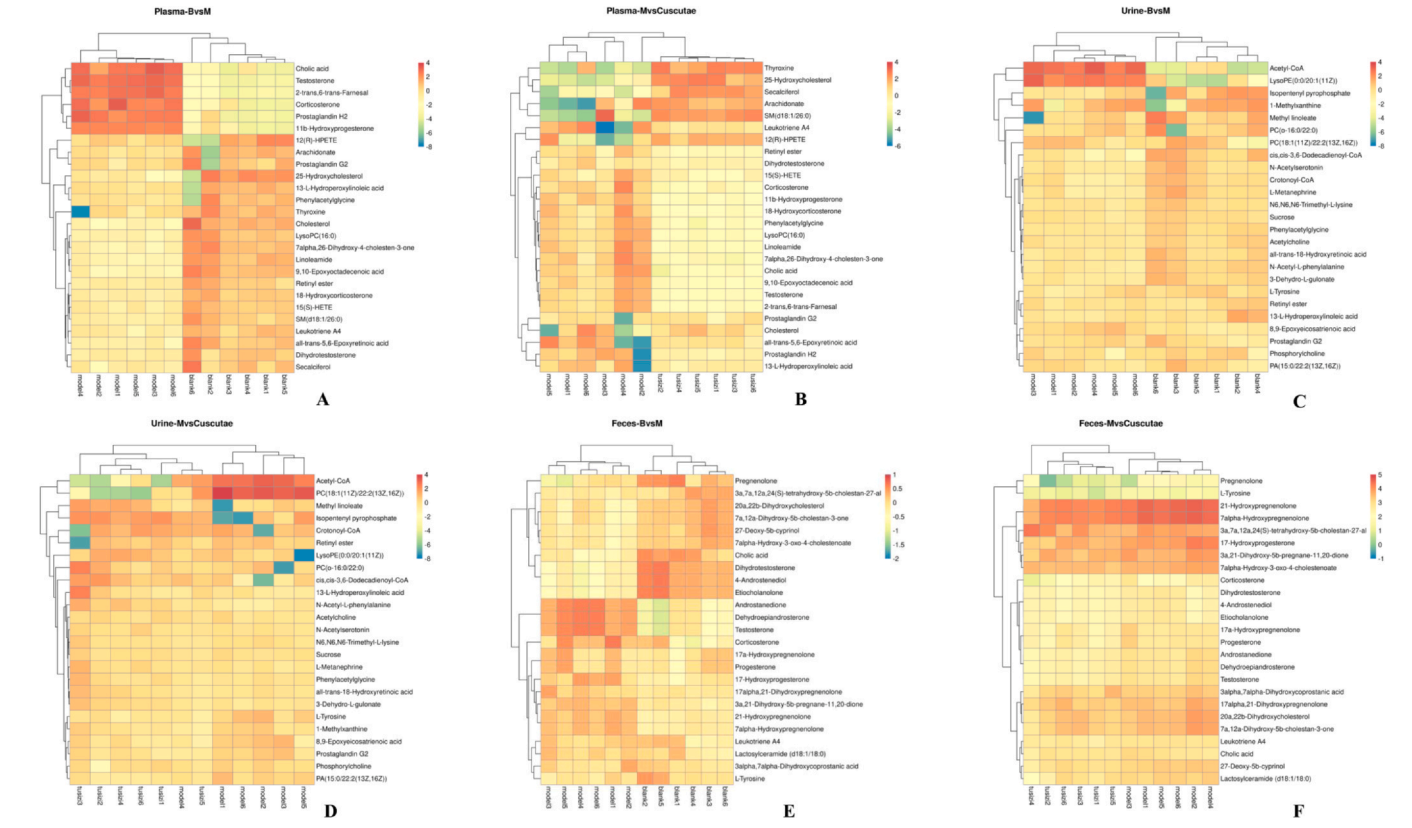

Fig. 3. Heat map cluster analysis of plasma samples, urine sample and feces sample. Heat map cluster analysis of (A). blank group and model group, (B). model group and *Cuscutae* group in plasma samples; Heat map cluster analysis of (C). blank group and model group, (D). model group and *Cuscutae* group in urine samples; Heat map cluster analysis of (E). blank group and model group, (F). model group and *Cuscutae* group in feces samples. The metabolites with large concentration differences in the model group and the blank group are selected as display, and the brightness change of the color represents the change in the concentration of the difference metabolite. (The tusizi in the picture represents *Cuscutae*). (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

(0:0/20:1(11Z)), 8,9-Epoxyeicosatrienoic acid, Prostaglandin G2, etc. 15 down-regulated metabolites, including isopentenyl pyrophosphate, methyl linoleate, N-Acetylserotonin, L-Metanephrine, N6,N6,N6-Trimethyl-L-lysine, Sucrose, Acetylcholine, N-Acetyl-L-phenylalanine, L-Tyrosine, 8,9-Epoxyeicosatrienoic acid. The above results indicated that the neuroendocrine immune system of the model group is suppressed and the Kidney yang deficient rat model was successfully established. In the Semen Cuscutae group, the levels of the above metabolites were reversed to varying degrees, indicating that the Semen Cuscutae has a better protective effect on the kidney yang deficiency rats.

4. Discussion

Deficiency of Kidney yang is a chronic kidney disease. Many Chinese medicine practitioners combine kidney deficiency syndrome with modern chronic kidney disease for complementary treatment [19]. With the development and maturity of metabolomics, more metabolomics techniques have been applied in the field of Chinese medicine. Based on metabolomics, this study analyzed the plasma, urine and feces samples on the treatment of kidney yang deficiency by Semen Cuscutae and understood the therapeutic mechanism of the kidney yang deficiency syndrome. This paper mainly elucidated the mechanism of kidney yang deficiency syndrome and the protective effect of Semen Cuscutae.

In this study, a total of 224 differential metabolites were detected. These metabolites involved about 20 metabolic pathways. Among them, arachidonic acid metabolism pathway, linoleic acid metabolism pathway, drug metabolism-cytochrome P450 pathway, phenylalanine, tyrosine and tryptophan biosynthesis pathways are commonly enriched in plasma, urine and feces. The pathways that are commonly enriched in feces and plasma include steroid hormone synthesis, primary bile acid biosynthesis pathway, and sphingolipid metabolism, feces and urine are both enriched in starch and sucrose metabolism, plasma and urine are both enriched in phenylalanine metabolism, α-linolenic acid metabolism, tryptophan metabolism, retinol metabolism, and tyrosine metabolism. The metabolism formalization shows that the metabolism of these three biological fluids complement each other.

Hydrocortisone is a glucocorticoid. Long-term use of glucocorticoid can reduce the utilization of glucose by skeletal muscle and adipose tissue and reduce the sensitivity of insulin. In this study, the shock therapy that simulates clinical hormone application was given by intragastric administration in a short time large doses of hydrocortisone in rats significantly inhibited the function of the adrenal cortex, thus showing symptoms similar to kidney yang deficiency. In the meantime, there were obvious obstacles in energy metabolism such as starch and sucrose metabolism and fatty acid metabolism in the kidney yang deficiency rats, which corresponded to the lethargy and slight weight loss in the kidney yang deficiency rats [20]. Fig. 4 shows a macro-metabolism diagram of the main metabolic pathways in this study. In patients with chronic renal failure, it was found that plasma bile acid increased due to the decreased of bile acid filtration through the kidneys, and bile acid in urine increased [21]. In this study, the level of unsaturated fatty acids such as arachidonic acid decreased, indicating that it is possible that the decreased content of arachidonic acid, linoleic acid and other unsaturated fatty acids affected the secretion of bile acids, thereby affecting renal function and energy metabolism. Bile acids are usually conjugated with glycine or taurine to promote fat absorption and cholesterol

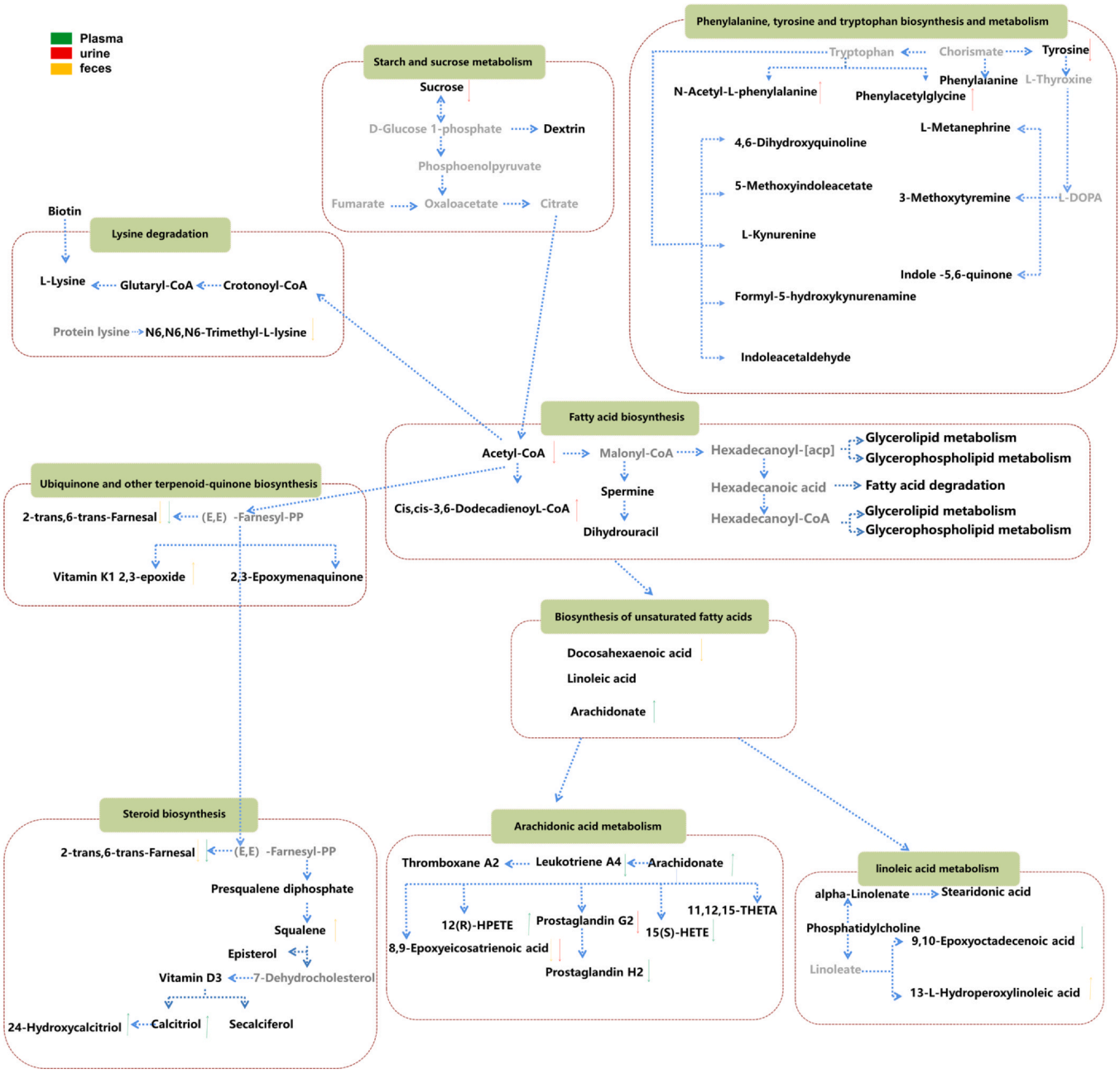

Fig. 4. Correlation metabolic networks of main potential biomarkers. Compared with the model group, the decrease in the main metabolite levels of the *Cuscutae* group is indicated by arrows, the upward arrow indicates the increase in the concentration level, the downward arrow indicates the decrease in the concentration level, the color of the arrow indicates different samples, green indicates plasma samples, and yellow indicates feces samples, red indicates urine samples, gray fonts indicate metabolites were not detected in this study. (For interpretation of the references to colour in this figure legend, the reader is referred to the web version of this article.)

excretion, the increasing of the bile acids may be the cause of adrenal insufficiency in liver disease. In the plasma model group, the level of bile acid increased, indicating that the syndrome of kidney yang deficiency rat bile acid filtration was affected. It has been reported the sperm cell apoptosis indicated that bile acid may affect male fertility. The bile acid level in the Semen Cuscutae group was lower than that in the model group, indicating that Semen Cuscutae has a certain effect on the biosynthesis of primary bile acid, which also showed that Semen Cuscutae has many protective effects on kidney yang deficiency rats.

Linoleic acid is a kind of unsaturated fatty acid. The metabolism of linoleic acid is disordered in kidney yang deficiency rats. Studies have shown that conjugated linoleic acid diet therapy can slow the progression of kidney disease. In this study, compared with the model group, the concentration of linoleamide in the Semen Cuscutae group was higher, indicating that the administration of Semen Cuscutae could enhance the activity of kidney cells, thereby improving the symptoms of kidney yang deficiency rats. 9,10-Epoxyoctadecenoic acid is a proliferator-activated receptors (PPAR) gamma2 ligand, which does not stimulate adipocyte differentiation and has anti-osteogenic properties. Flavonoids, the active ingredient of Semen Cuscutae, have effective anti-osteoporosis effects [22]. The decrease of 9,10-epoxyoctadecenoic acid indicated that the anti-osteoporosis effect of Semen Cuscutae.

Arachidonic acid and its metabolites have a wide range of functions, such as participating in inflammation, pain and fever, participating in the regulation of the immune system, reproductive system and renal function [23–25]. Microsomal prostaglandin E2

synthase 1 (mPGES-1) is an inducing enzyme that converts prostaglandin H2 (Prostaglandin H2, PGH2) into prostaglandin E2 (Prostaglandin E2, PGE2), which plays an important role in a variety of inflammatory diseases effect. In this study, the increasing of PGH2 in the plasma of model group may be caused by the suppression of mPGES-1 in kidney yang deficiency rats. The decreasing of PGH2 in the Semen Cuscutae group indicated that the inhibitory effect of mPGES-1 has been relieved.

Steroid hormones included sex hormones and adrenal cortex hormones. In the kidney yang deficiency model, the levels of sex hormones and adrenal hormones are in a suppressed state [26]. In this study, the corticosterone and testosterone increased in the model group, and the adrenal hormone, thyroid hormone, 17-hydroxycorticosteroid, 20alpha, 22beta-dihydroxycholesterol, pregnenolone and progesterone decreased, which indicated that the model is constructed successfully. Compared with the Semen Cuscutae group, the steroid hormones of the model group have different reduction, indicating that the Semen Cuscutae has a better protective effect on the kidney yang deficiency rats.

The biosynthesis of phenylalanine, tyrosine and tryptophan are mutually affected, and their biosynthesis is related to sugar metabolism [27,28]. In the kidney yang deficiency model group, the decreased of phenylacetylglycine levels in plasma and urine samples weakened the metabolic pathway of phenylalanine to tyrosine [29], resulting in lower plasma tyrosine concentrations. Decreased levels of L-Metanephrine and 3-Methoxytyramine may affect the biosynthetic pathways of dopamine and norepinephrine [30]. Compared with the model group, the levels of phenylacetylglycine and tyrosine in the Semen Cuscutae group increased, indicating that the Semen Cuscutae has a better therapeutic effect on rats with kidney yang deficiency. Tryptophan, as another essential amino acid, plays an important role in the process of protein synthesis and metabolic network regulation [31,32]. It can be seen from this study that compared with the blank group, the plasma N-Acetylserotonin level of rats in the model group was reduced. It is speculated that the decreased in N-Acetylserotonin level was the cause of lethargy in kidney yang deficiency rats. The increased of N-Acetylserotonin level in the Semen Cuscutae group indicated that the Semen Cuscutae has a good therapeutic effect.

9-cis Retinoic acid is an active retinoid that can regulate the expression of retinoid-responsive genes. Glomerular mesangial cells play a positive role in the inflammatory response to glomerular injury. Studies have found that human mesangial cells may be the target of the anti-inflammatory effect of 9-cis retinoic acid [33], and research results indicate that retinoic acid is beneficial to the treatment of nephropathy [34]. 9-cis Retinoic acid has anti-fibrosis ability by resisting TGF-β1 in glomerular mesangial cells, and the activity of 9-cis Retinoic acid is likely to depend on the mechanism of HGF/c-met receptor signal transduction is mediated [35]. Kidney yang deficiency syndrome is a chronic kidney disease, so 9-cis-retinoic acid has a certain protective effect on rats with kidney yang deficiency syndrome, and 9-cis-retinoic acid is the latest previous biomarker discovered in this study, compared with the model group, the level of 9-cis retinoic acid in the Semen Cuscutae group was higher, which indicated that the Semen Cuscutae has a good therapeutic effect.

5. Conclusion

In this study, we comprehensively studied the differential metabolites in the plasma, urine and feces of kidney yang deficiency syndrome rats through metabolomics, and discussed the therapeutic effect and mechanism of Semen Cuscutae on kidney yang deficiency syndrome rats. The results showed that there are 21 metabolic pathways mainly involved in the treatment of Semen Cuscutae on kidney yang deficiency syndrome rats, and the metabolic levels of about 70 metabolites changed significantly. The results showed that the kidney yang deficiency symptom of rats after administration of Semen Cuscutae improved to varying degrees, and the newly discovered the retinol metabolic pathway was involved in the metabolism of kidney yang deficiency rats. It is helpful to study the mechanism of Semen Cuscutae in treating kidney yang deficiency syndrome rats.

CRediT authorship contribution statement

Rong-Hua Fan: conceived and designed the experiments. **Rong-Hua Fan, Chen-Guang Liu and Tao Zhou**: performed the experiments. **Ze Zhang, Chen wang and Mei-Qi Xing**: analyzed the data. **Rong-Hua Fan**: wrote the paper. **Yu-Mo Han and Xin-Yue Wang:** reviewed the paper. All authors read and approved the final manuscript.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpba.2021.114432.

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