Electronic Supplementary Material (ESI) for RSC Advances.

The hematinic effect of *Colla corii asini* (Ejiao) using ^{1}H-NMR metabolomics coupled with correlation analysis in APH-induced anemic rats

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1. HPLC determination of amino acid in *Colla corii asini* (Ejiao)

1.1 Chemicals

Acetonitrile (chromatographic grade) and sodium acetate, hydrochloric acid, triethylamine were obained from Beijing Chemical Works (Beijing, China). Phenyl isothiocyanate (PITC), amino acid standards (L-hydroxyproline, glycine, alanine and proline) were purchased from Sangon Biotech (Shanghai, China). The water used in HPLC and sampling was prepared with a Super Purity Water System (Purite Ltd, England)

1.2 Chromatographic conditions

High performance liquid chromatograph (Agilent 1260), Ultimate C18 column (25 cm×4.6 mm, 5μm), mobile phases A consisted of acetonitrile-0.1 mol/L sodium acetate solution (v/v=7:93, pH=6.5), mobile phases B consisted of acetonitrile-water (v/v=4:1), The gradient was as follows: 0-11min, 100 %~93 % A, 0 %~7 % B; 11-13.9 min, 93 %~88 % A, 7 %~12 % B; 13.9-14 min, 88 %~85 % A, 12 %~15 % B; 14-29 min, 85 %~66 % A, 15 %~34 % B; 29-30 min, 66 %~0 % A, 34 %~100 % B. Elution was performed at a solvent flow rate of 1 ml/min. Detection was accomplished with a UV-detector and chromatograms were recorded at 254 nm. The column was maintained at 43 °C. The sample injection volume was 5 μl.

1.3 Preparation of mixed reference solution

Amino acid standards (L-hydroxyproline, glycine, alanine and proline) was weighted accurately and mixed with 0.1mol/L hydrochloric acid solution to obtain mixed reference solution (concentration: L-hydroxyproline, 80 μg/ml; glycine, 0.16 mg/ml; alanine, 70 μg/ml; proline, 0.12 mg/ml).

1.4 Preparation of sample solution

About 0.25 g of the Ejiao powder was weighed accurately and mixed with 25 ml of 0.1mol/L

hydrochloric acid solution. The mixture was extracted by ultrasonic (power: 500W, frequency: 40 kHz) 30 min. Then 2 ml of extracted solution mixed with 2 ml of hydrochloric acid was hydrolyzed for 1 hour at 150 ℃. The hydrolyzed solution was evaporated to dryness on a boiling water bath and the residue is dissolved in 25 ml of 0. 1 mol/L hydrochloric acid solution to obtain sample solution.

1.5 Derivatization of reference and sample solution

5 ml of above solution, 2.5 ml of 0.1 mol/L PITC acetonitrile solution and 2.5 ml of 0.1mol/L triethylamine acetonitrile solution was blend to a 25 mL volumetric flask. After 1 hour, Adding 50 % acetonitrile solution to 25 ml. Transfer 10 mL of this solution and 10 mL of normal hexane to separating funnel. After 10 min, the lower layer solution was filtered to obtain derivatized solution.

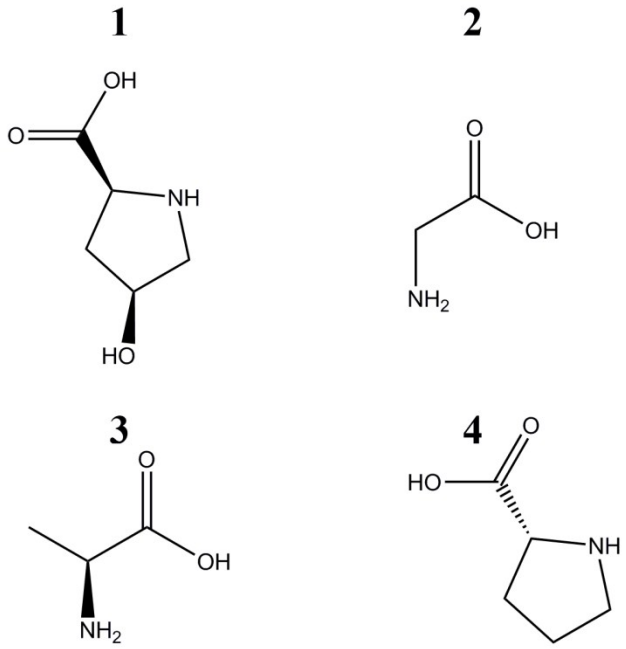

Fig. S1 Chemical structures of 4 major amino acid in Ejiao. 1, L-hydroxyproline; 2, glycine; 3, alanine; 4, proline.

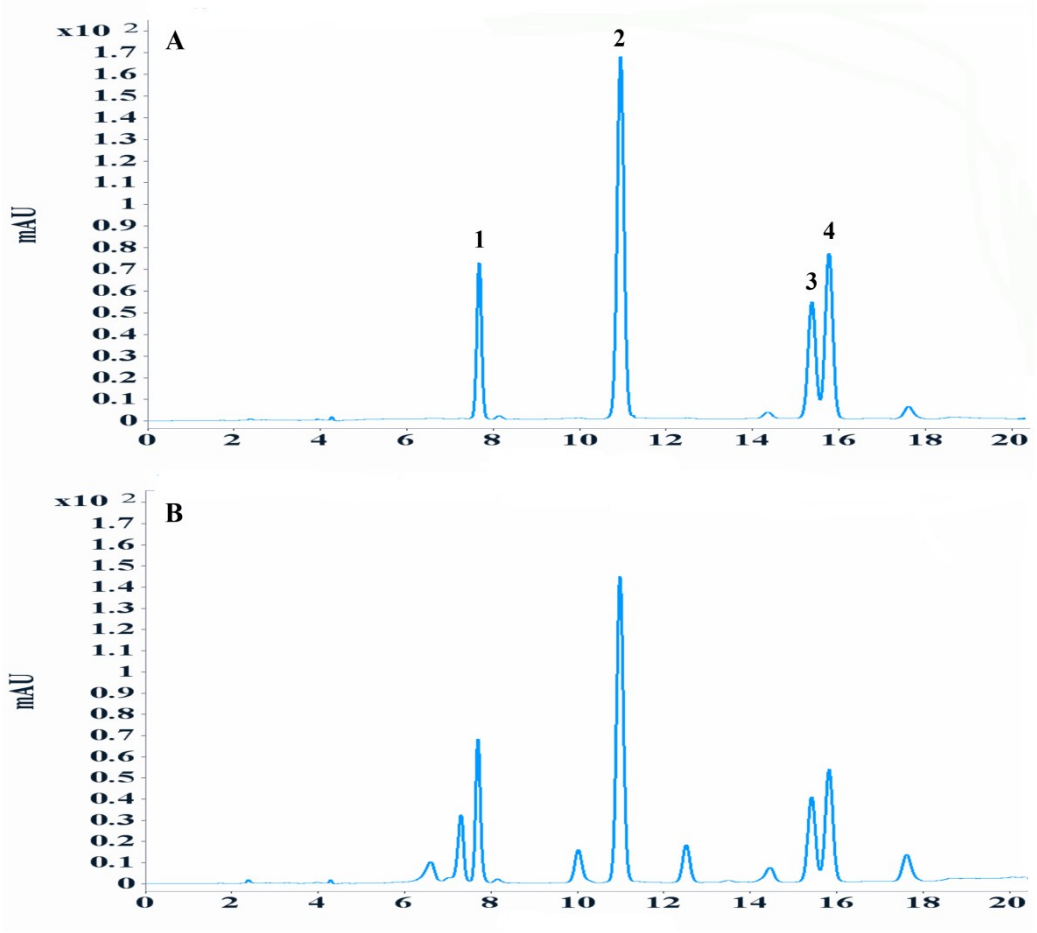

Fig. S2 HPLC chromatograms of reference solution (A), sample solution (B). 1, L-hydroxyproline; 2, glycine; 3, alanine; 4, proline.

Table S1 Determination of 4 major amino acid in 3 Ejiao extract samples.

	L-hydroxyproline	Glycine	Alanine	Proline
Sample 1	9.40 %	18.91 %	7.34 %	10.70 %
Sample 2	8.99 %	19.20 %	7.34 %	10.71 %
Sample 3	9.53 %	19.38 %	7.40 %	10.88 %

2 Body weight and viscera indexes

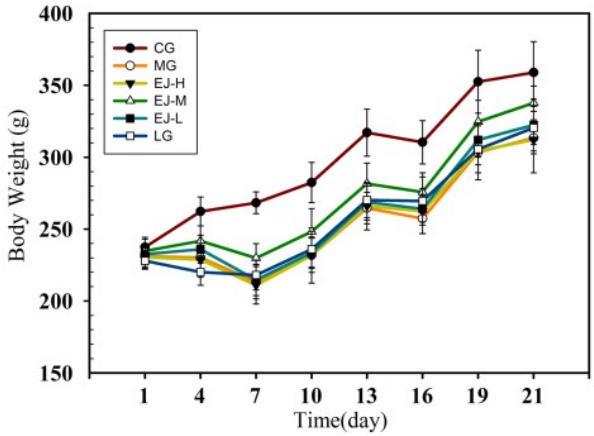

Fig. S3 Body weight of rats changed every three days by acetyl phenylhydrazine (APH) and Ejiao administration (n=10). ***p*< 0.01 compared with MG.

Table S2 Changes in the viscera index resulting from acetyl phenylhydrazine (APH) and Ejiao treatment. (n=10, the viscera index= organ weight /body weight×100 %)

	Heart (%)	Liver (%)	Spleen (%)	Lung (%)	Kidney (%)	Thymus (%)
CG	0.35±0.25	2.85±0.10	0.19±0.04	0.48±0.09	0.65±0.14	0.13±0.03
MG	0.35±0.52	2.99±0.08$^{\#}$	0.30±0.07$^{\#\#}$	0.47±0.05	0.71±0.09	0.15±0.04
EJ-H	0.37±0.25	2.95±0.05$^{\#}$	0.29±0.03$^{\#\#}$	0.46±0.06	0.70±0.04	0.15±0.05
EJ-M	0.35±0.31	2.93±0.18	0.31±0.04$^{\#\#}$	0.45±0.04	0.69±0.05	0.16±0.03
EJ-L	0.35±0.25	2.87±0.02*	0.31±0.05$^{\#\#}$	0.46±0.04	0.67±0.06	0.15±0.04
LG	0.34±0.04	2.65±0.09**$^{\#\#}$	0.28±0.03$^{\#\#}$	0.45±0.07	0.63±0.18	0.15±0.03

** Significant difference compared with MG (p< 0.01), # Significant difference compared with CG (p< 0.05), ## Significant difference compared with CG (p< 0.01)

3 2D NMR spectra and pattern recognition analysis of serum and urine samples

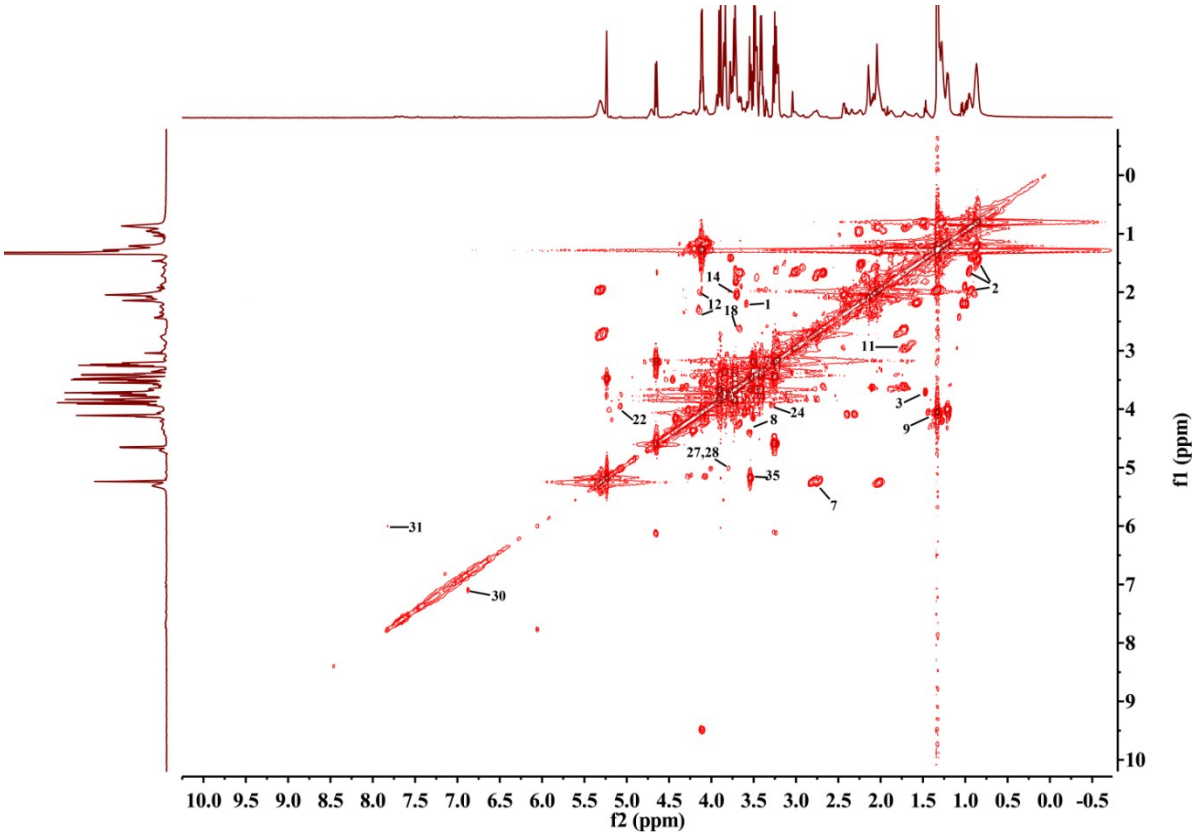

Fig. S4 Representative 600 MHz COSY 2D NMR spectra of serum sample of rat.

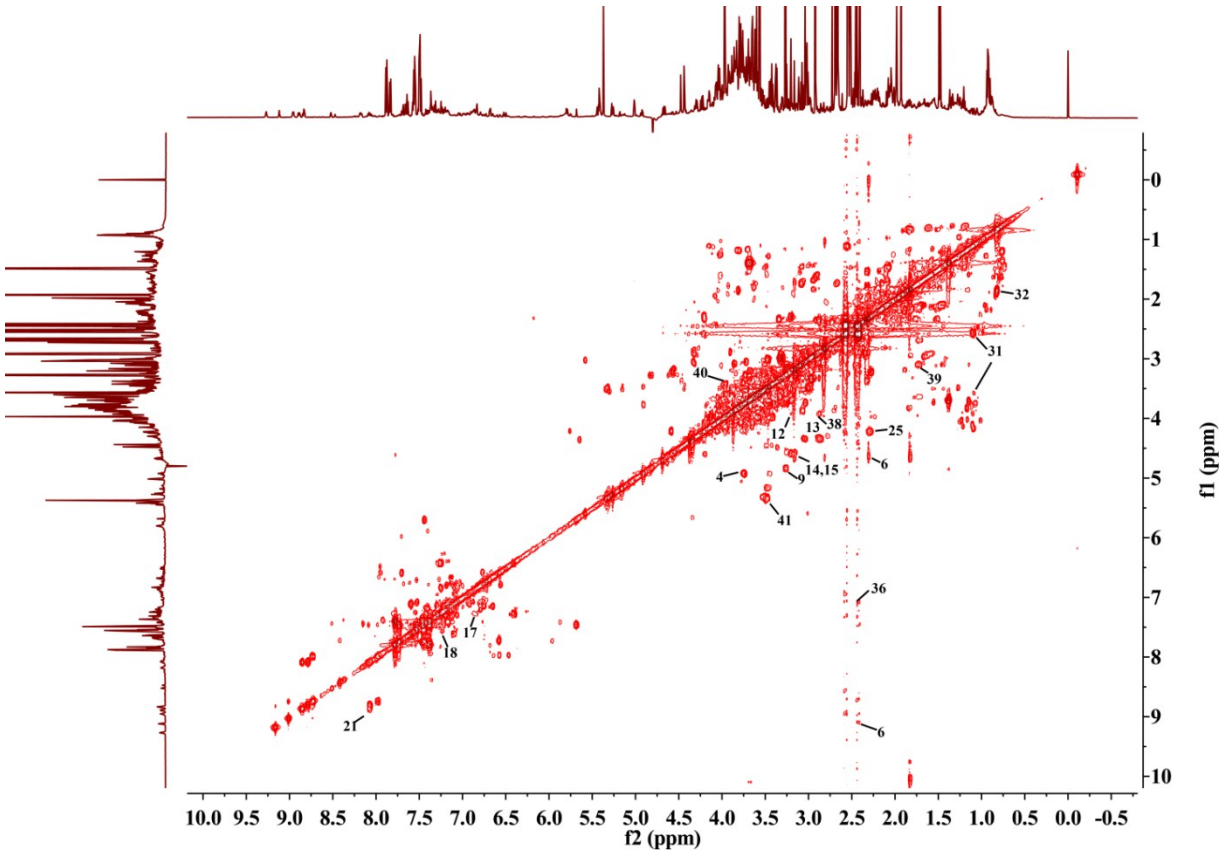

Fig. S5 Representative 600 MHz COSY 2D NMR spectra of urine sample of rat.

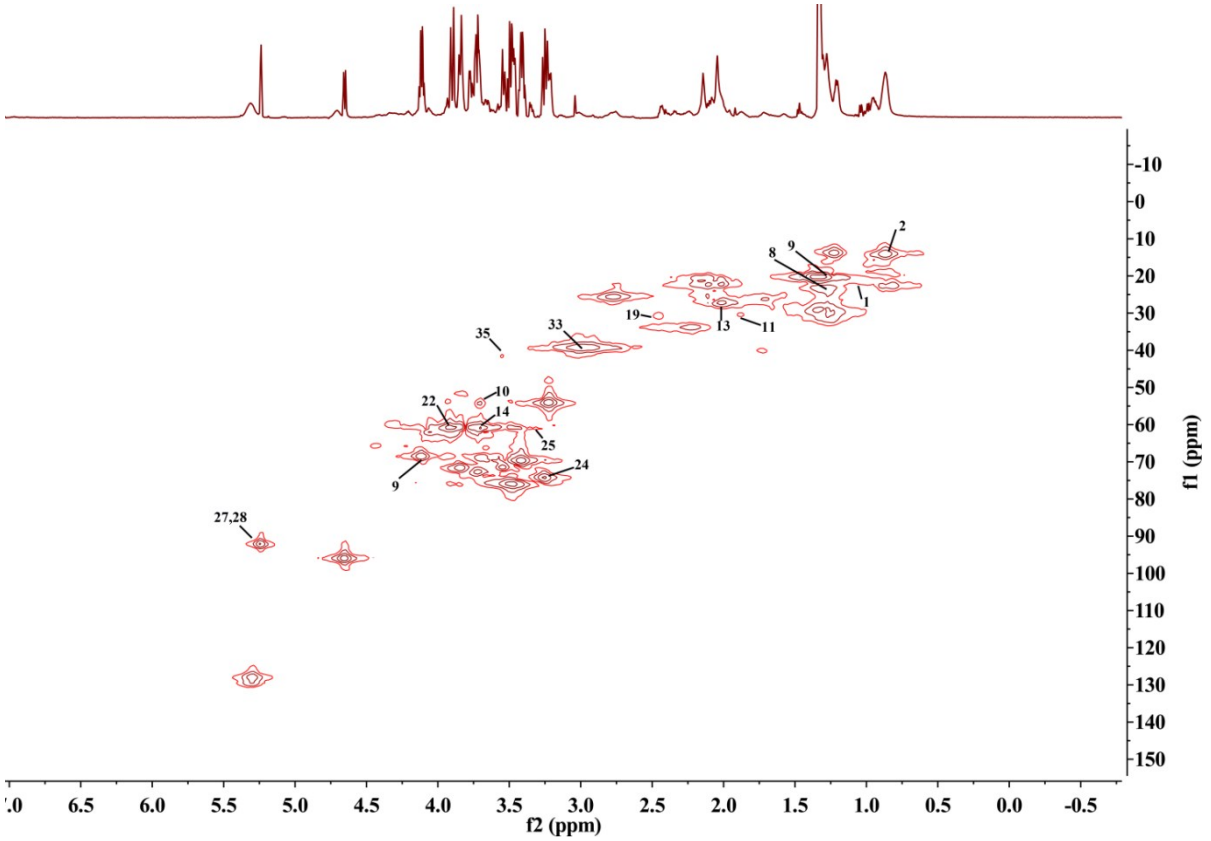

Fig. S6 Representative 600 MHz HSQC 2D NMR spectra of serum sample of rat.

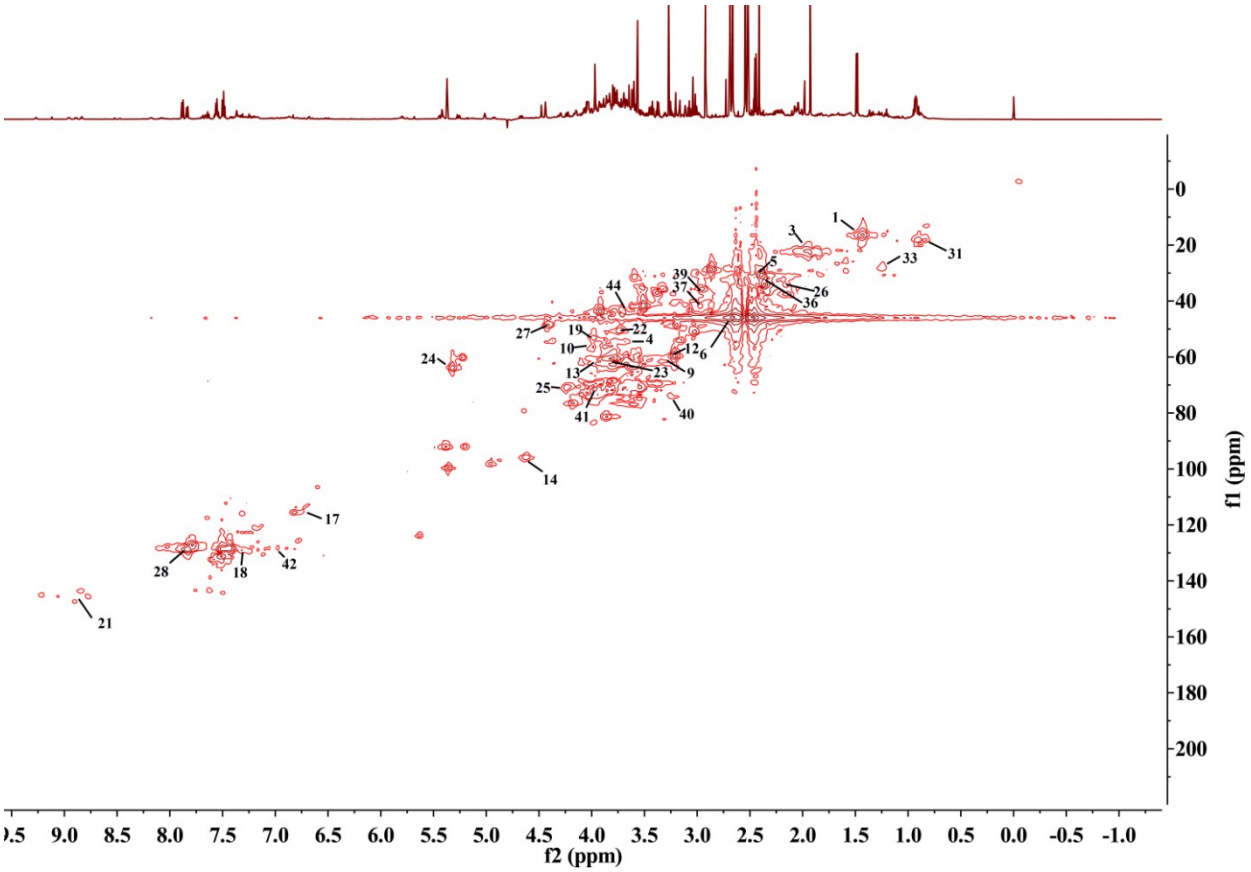

Fig. S7 Representative 600 MHz HSQC 2D NMR spectra of urine sample of rat.

Table S3 Assignments of endogenous metabolites involved in serum and urine ^{1}H-NMR spectra.

Number	Metabolites	δ ^{1}H[a]	Assignments	Samples[b]
1	Valine	0.995 0.983(d) 1.047 1.035(d) 3.606 3.593(d)	γCH3, γ′CH3, αCH	S,U
2	Isoleucine	1.003 1.015(d)	γCH3,	S,U
3	Leucine	0.94(d), 0.96(d)	δCH3, δ′ CH3	S,U
4	HDL	0.86(m)	CH3(CH2)n	S
5	LDL	0.88(m)	CH3(CH2)n	S
6	VLDL	0.89(m)	CH3CH2CH2C=	S
7	Lipid	2.77(m) 5.30(brm)	(CH2)n =C-CH2-C= -CH=CH-	S,U
8	Threonine	3.581 3.570(d)	αCH	S,U
9	Lactate	1.324 1.335(d)4.11(q)	βCH3, αCH	S,U
10	Alanine	1.472 1.484(d)	αCH	S,U
11	Lysine	1.7(m) 1.89(M)	δCH2, βCH2,	S,U
12	Proline	2.07(m)	βCH2	S
13	Acetic acid	1.918(s)	CH3	S
14	Arginine	3.25(t) 3.733(t)	δCH2, αCH	S,U
15	N-acetyl-glycoprotein	2.043(s)	CH3	S
16	O-acetyl-glycoprotein	2.142(s)	CH3	S
17	Acetate	2.278(s)	CH3	S,U
18	Glutamate	2.44(d)3.773 3.782(d)	βCH2, αCH	S,U
19	Pyruvate	2.371(s)	βCH3	S
20	Succinic acid	2.40(s)	CH2	S
21	Citrate	2.54(d), 2.70(d)	CH2(1/2)	S,U
22	Creatine	3.04(s) 3.93(s)	CH3, CH2	S,U
23	Asparate	3.89(d)	αCH	S
24	Choline	3.20(s)	N(CH3)3	S,U
25	TMAO	3.26(s)	CH3	S,U

26	Creatinine	4.06(s)	CH2	S,U
27	α-glucose	3.72(dd) 3.91(dd)	H6′H6′	S,U
28	β-glucose	5.234 5.24(d)	H1	S,U
29	Glycerophosphocholine	3.21(s)	CH3	S
30	Tyrosine	6.89(m),7.18(m)	3 or 5-CH,2 or 6-CH	S,U
31	Histidine	7.04(s),7.774(s)	2-CH,4-CH	S,U
32	Formate	8.46(s)	H−COOH	S,U
33	Dimethylglycine	2.92(s)3.714(s)	CH3 CH2	S,U
34	Acetone	2.23(s)	CH3	S
35	Glycine	3.56(s)	CH2	S
36	Phenylalanine	7.33(m) 7.43(m)	2,6-CH 2,6-CH	S,U
37	3-hydroxybutyrate	1.20(d)2.42(d)2.31(d)	γCH3 CH 2′αCH2	S,U
38	α-ketoglutarate	2.44(t)	βCH2 γCH2	U
39	Succinate	2.409(s)	CH2	U
40	DMA	2.718(s)	CH3	U
41	TMA	2.93(S)	CH3	U
42	Tauine	3.287(t) 3.44(t)	−CH2−S,−CH2−NH2	U
43	Betaine	3.229(s)3.907(s)	CH3,CH2	U
44	Fumarate	6.526(s)	CH	U
45	Hippurate	3.97(d), 8.55(s), 7.84(d), 7.56(t), 7.65(t)	CH2, NH, H2 & H6, H3 & H5, H4	U
46	NAD+	8.84(d), 9.13(s)	H3, H2	U
47	Allantoin	3.77(q) 1.49(d)	CH	U
48	Malate	4.30(dd)	CH2 CH	U
49	Propionate	2.19(q)	CH3 CH2	U
50	Trigonelline	4.44(s)	2-CH CH3	U
51	Benzoate	7.88(d)	2,6-CH 3,5-CH	U
52	Indoxyl sulfate	7.366(s)	CH3	U
53	Ethanol	1.19(t)	CH3	U
54	Pyruvic acid	2.37(s)	CH3	U
55	Asparagine	2.84(d)	βCH2	U

56	Isovalerylglycine	2.01(m)	CH	U
57	Phosphorylcholin	3.23(s)	CH3	U
58	N-acetylglutamate	1.987(s) 2.23(m) 2.02(m)1.84(m)	CH3 γCH2 βCH2′βCH2	U

[a]s, singlet; d, doublet; t, triplet; q, quartet; m, multiplet; dd, doublet of doublets.
[b]S, serum; U, urine

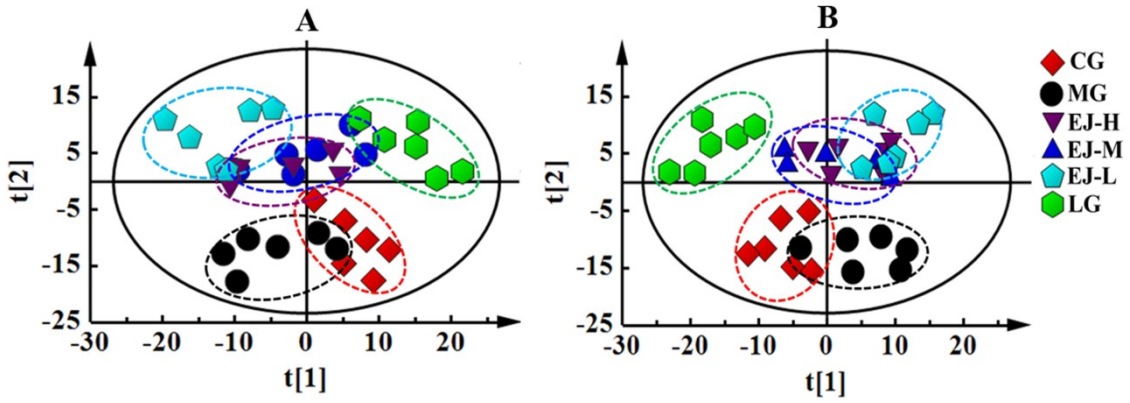

Fig.S8 Pattern recognition with Simca-P13.0. The PLS-DA score plot derived from [1]H-NMR serum spectra of all groups (A). The PLS-DA score plot derived from 1H-NMR urine spectra of all groups (B).

4 Quantitative analysis of metabolite profiles

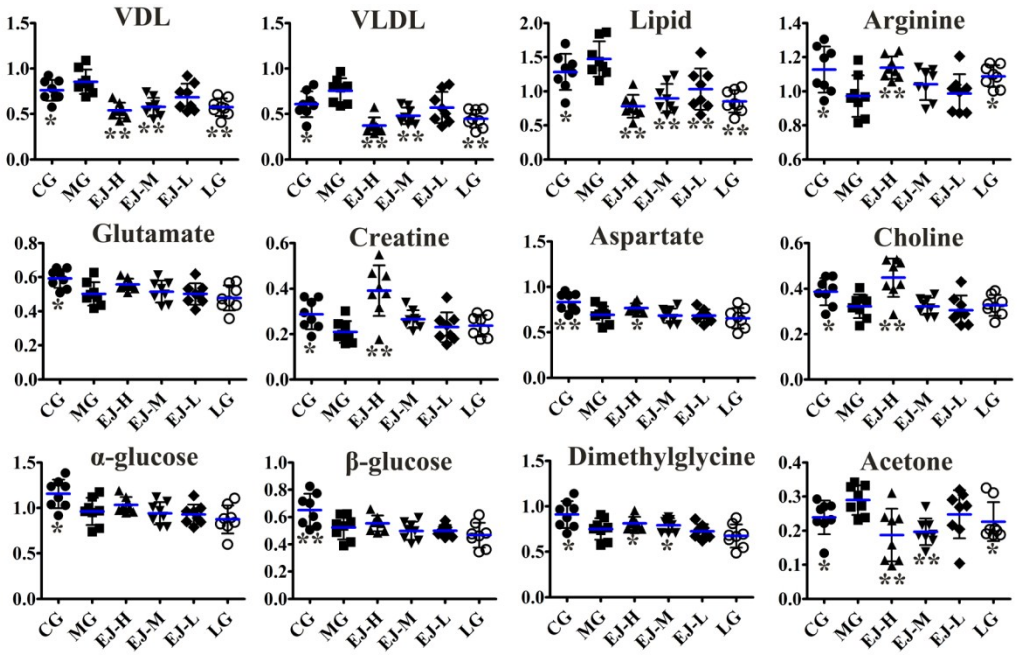

Fig. S9 Quantitative analysis of serum metabolites. Relative abundances of metabolites obtained from ^{1}H-NMR spectra of serum samples collected from all groups. *p< 0.05 and **p< 0.01 compared with MG.

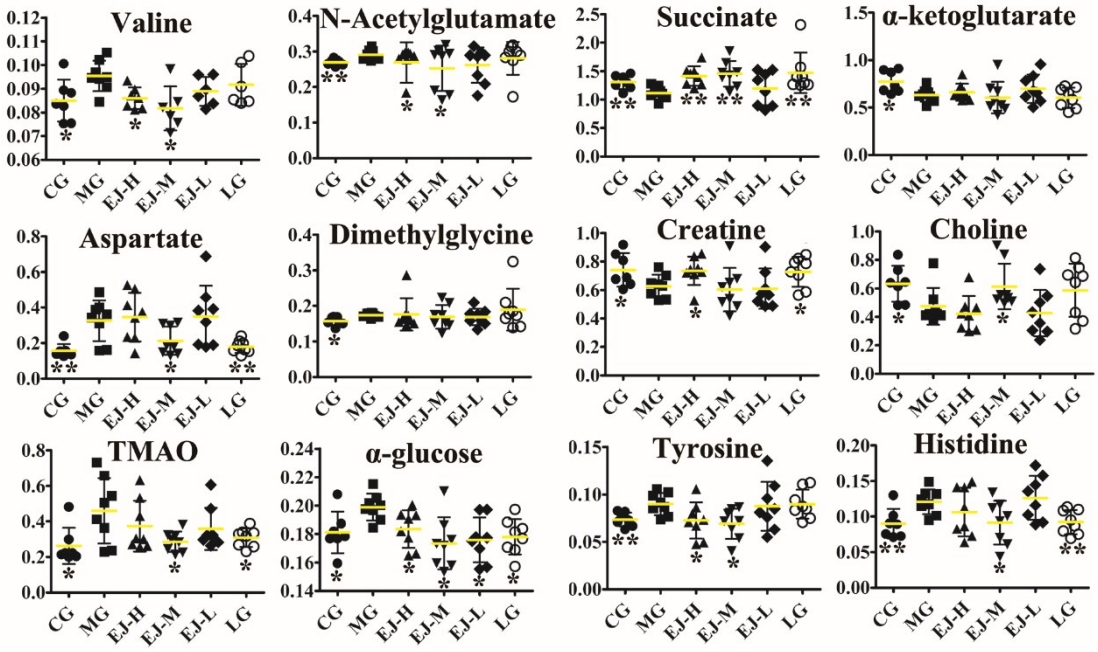

Fig. S10 Quantitative analysis of urinary metabolites. Relative abundances of metabolites obtained from ^{1}H-NMR spectra of urinary samples collected from all groups. *p< 0.05 and **p< 0.01 compared with MG.

5 The significance test plot for pairs of correlation coefficient

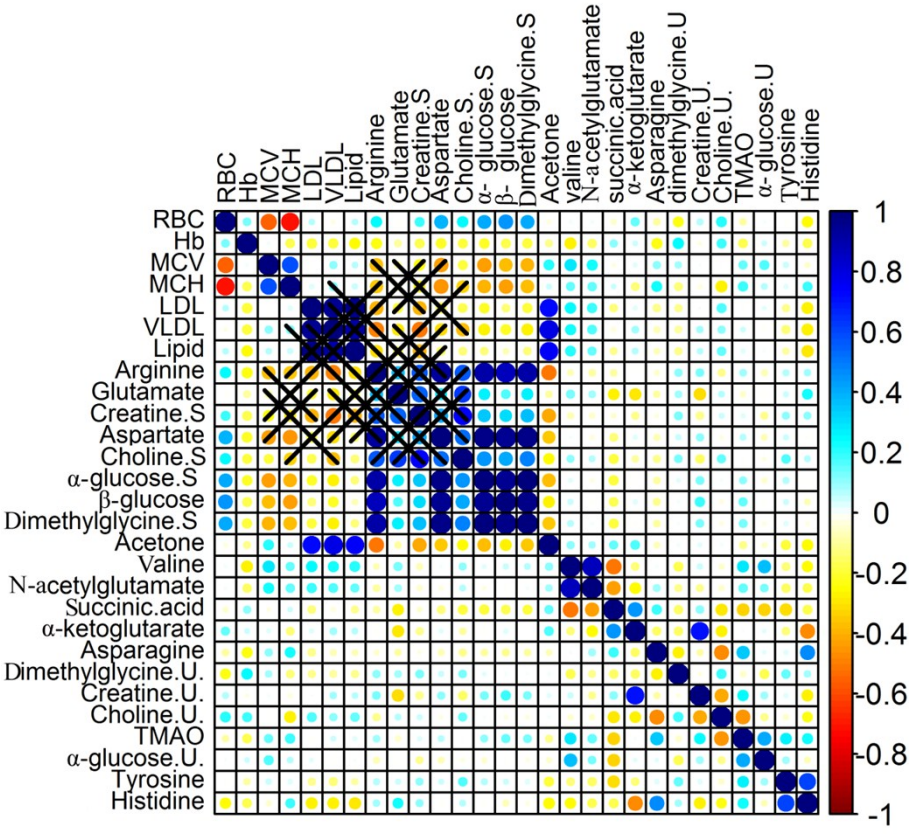

Fig. S11 The significance test plot for pairs of correlation coefficient. (×denotes no significant difference $p>0.05$)