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Supplementary Information for Original Article

Urinary and plasmatic metabolomics strategy to explore the holistic mechanism of lignans in *S. chinensis* on Alzheimer's disease using UPLC-Q-TOF-MS

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1 Step-down test

The model and treated groups were intraperitoneally injected with D-galactose (60 mg/kg/day) and intragastrically with $AlCl_3$ (5 mg/kg/day) for 9 weeks, while the normal control group was treated with the same volume of sterile saline. After 1 week treatment with D-galactose and $AlCl_3$, all the animals were administered intragastrically as indicated in 2.2 for 8 weeks. After the last intragastric administration, step-down test was performed using a passive avoidance apparatus (Yiyan Software Technology Co., Ltd, Shandong, China), formed by five chambers with each chamber (120 × 120 × 180 mm). The mice were placed in the step-down device for a 3 min acclimation period. Then, the electric grid at the bottom of chamber was (36 V) within 300 s. The frequency of the electrical stimulation caused by the electric grid after jumping down from the platform (error times of learning) was recorded. After 24 h, each mouse was exposed to a 300 s learning course with energized foot shock. The step-down latency and shock number within 300 s were recorded. If the mice did not jump off the platform within 300 s, shock number was recorded as 0, and the step-down latency was recorded as 300 s.

2 Morris Water Maze (MWM) test

The AD rat model was designed as described previously [20]. In brief, aggregated Aβ25-35 was injected into bilateral hippocampus CA1 of AD rats, whereas sterile physiological saline was injected into bilateral hippocampus CA1 of the normal control rats. Then, all animals were administered intragastrically as indicated in 2.3 for 8 weeks. After the last intragastric administration, MWM test was performed as described previously [21] by using a WMT-100 MWM analysis system and a BI2000 image analysis system (Chengdu Taimeng Technology Co. Ltd., Chengdu, China). In brief, the place navigation test was conducted in four trials per day for 4 consecutive days to help the animals recall the platformlocation. For each trial, every rat was placed in water, facing the wall randomly from a quadrant to search the platform with a ceiling time of 120 s. After climbing onto the platform, the rat was allowed to rest for 20 s. The rats that did not find the platform were guided to reach the escape platform by the operator and allowed to remain for 20 s. The space exploration experiment for the first time was performed 24 h after the place navigation test was conducted. The platform was removed from the water, and each rat was placed in the water facing the wall at a zone directly opposite the platform quadrant. The escape latency, times of crossing the original platform and trajectories were recorded using a

computer system.

3 HPLC peak purity test

3.1 Analysis condition

The HPLC analysis was performed using 2690 HPLC-DAD (Waters Co, Milford, MA, USA). The samples were separated on a Dikma Diamonsil C18 column (250 mm×4.6 mm, 5 μm). The column temperature was kept at 3 °C and the injection volume was 5 μL. The mobile phase consisted of water (A) and methanol (B) at a flow rate of 0. 5 mL/min. The gradient elution in positive mode was performed as follows: 20-30 min 83 %-85 % B; 30-45 min 85 %-100 % B; 45-60 min 100 % B.

The diode-array detector was set to monitor at 254 nm and the online UV spectra were recorded in the range 190-400 nm.

3.2 Preparation of control solution

The external reference compound method of calibration was used for HPLC peak purity test of purified lignans isolated from *S. chinensis*. Reference compound solutions of lignans including schisandrin, schisandrol B, deoxyschizandrin, schisandrin B and schisantherin A were prepared in methanol at the respective concentration of 0.2 mg/mL, 0.2 mg/mL, 0.2 mg/mL, 0.3mg/mL, 0.3 mg/mL. The 1, 2, 4, 6, 8, 10, 12, 14, 16, 18, 20 and 22 μL portions of these solutions were injected into the HPLC system. The calibration curves were drawn by the UV peak area versus the reference compounds' concentration. The results were shown in Table S1.

Table S1 The regression equation, linearity range and regression coefficient of five lignan reference compounds

Compound	Regression equation	Linearity range	regression coefficient (*r*)
Schisandrin	$y=5.4441\times10^{6}x-514978$	0.2-2.8 μg	0.9999
Schisandrol B	$y=1.2549\times10^{6}x-227993$	0.2-4.2 μg	0.9999
Schisantherin A	$y=1.4716\times10^{6}x-125155$	0.2-4.4 μg	0.9995
Deoxyschizandrin	$y=5.1534\times10^{6}x-485899$	0.2-2.4 μg	0.9999
Schisandrin B	$y=3.9135\times10^{6}x-684903$	0.2-4.2 μg	0.9992

3.3 Purity analysis of lignans isolated from *S. chinensis*

The 1.59 mg dried powders were added in the 2 mL methanol. The filtrate was

passed through a 0.45 μm membrane and then 10 μL of the filtrate was injected into the HPLC system for the analyses. The HPLC-UV chromatogram of the five lignan reference compounds (a) and the purified lignans isolated from *S. chinensis* were shown in Fig. S1. According to regression equations, the contents of schisandrin, schisandrol B, deoxyschizandrin, schisandrin B and schisantherin A were calculated which accounted for 84% of the quality of dried powders.

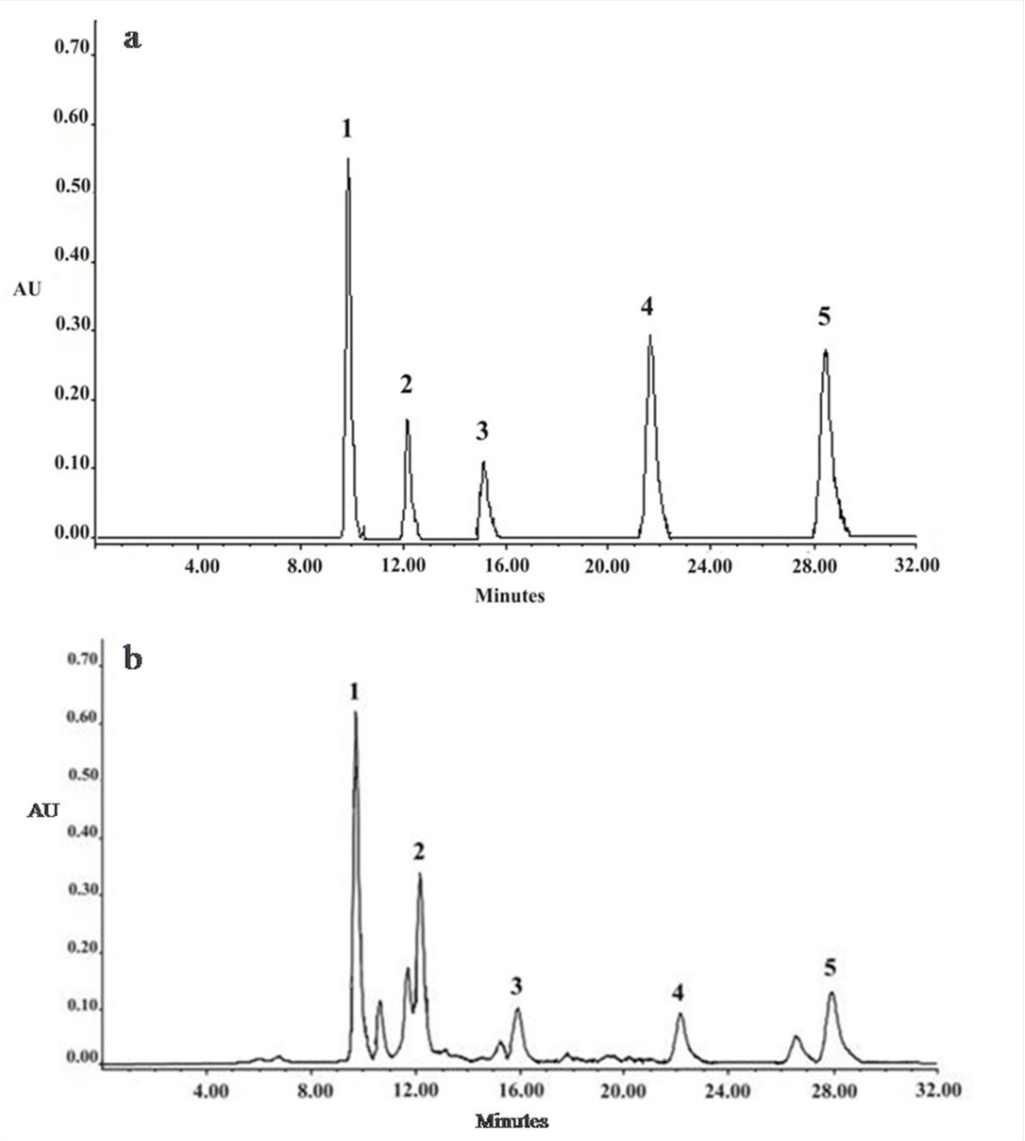

Fig.S1. HPLC-UV chromatogram of the five lignan reference compounds (a) and the purified lignans isolated from *S. chinensis*; 1. schisandrin, 2. schisandrol B, 3. schisantherin A, 4. deoxyschizandrin, 5.schisandrin B.

3.4 Identification of potential biomarkers

The ion $[M - H]^+$ was taken as an example to explain the identification procedure. The ion exhibited a retention time at 1.32 and the peak at m/z 166.0869 in positive mode of urinary sample. Via searching Human Metabolome Database, the

metabolite might be identified as phenylalanine. Then we confirmed that its retention time and accurate masses were consistent with the related reference standard. In MS/MS experiments, the fragment ions of reference standard, m/z 166.0868 were obtained respectively at m/z 149.0139, 131.004, 120.0324, 103.0058, 93.0231 and 79.0073. And the fragment ions of m/z 166.0869 in urinary sample had highly consistent fragmentation pattern with reference standard, which were shown in Fig. 5. So that fragment ion was identified to be phenylalanine.

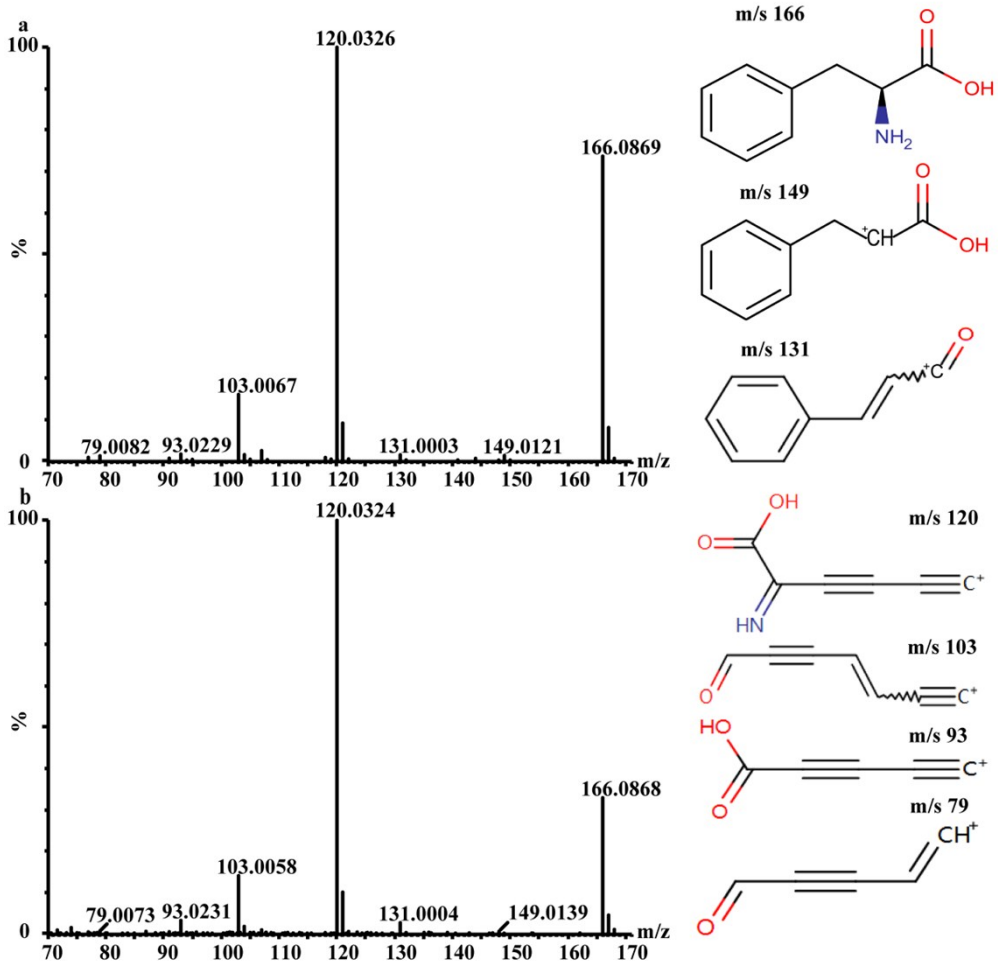

Fig.S2. MS/MS spectrum of phenylalanine and structural information of fragment ions: (a) MS/MS spectrum of standards, (b) MS/MS spectrum of sample.

Table S2 Identification of biomarkers from MS^2 data

Mode	Rt (min)	Measured mass	Compounds	MS^2
ESI^+	0.70	112.0508	Cytosine	95.0243, 85.0399, 69.0451, 68.0135, 67.0295, 52. 0186
Urine	0.91	154.0505	3-Hydroxyanthranilic acid	136.0399, 124.0398, 110.0603, 108.0448
	0.82	166.0730	7-Methylguanine	150.0416, 149.0464, 139.0621, 123.0671, 122.0355, 112.0512, 96.0561, 82.0406, 69.0453
	1.32	166.0869	Phenylalanine	149.0121, 120.0326, 131.0003, 103.0067, 93.0229, 79.0082
	1.00	184.0607	4-Pyridoxic acid	166.0501, 148.0395, 140.0709, 138.0551, 122.0602, 120.0445
	2.18	206.0453	Xanthurenic acid	188.0347, 162.0554, 160.0398,
	2.42	212.0926	3-Methoxytyrosine	195.0659, 194.0818, 177.0553, 166.0870, 149.0604, 137.0604
	1.52	220.1182	Pantothenic acid	202.1078, 184.0975, 174.1131, 103.0762, 88.0399, 73.0289, 72.0449, 71.0134, 70.0294
	1.16	257.1126	1-(beta-D-Ribofuranosyl)-1,4-dihydronicotinamide	240.0863, 239.1023, 222.0758, 212.0914, 125.0706, 108.0442
	1.78	268.1031	Adenosine	250.0937, 241.0932, 136.0620, 134.0463
ESI^+	0.49	118.0864	Betaine	73.0916, 59.0116, 58.0702
	7.04	256.2640	Palmitic amide	239.2375, 238.2534, 221.2269, 213.2581, 211.2426, 209.2268, 197.2268, 183.2113, 181.1955, 169.1955, 167.1799, 155.1799, 153.1642, 141.1642, 139.1486, 127.1486, 125.1329, 113.1329, 111.1174, 99.1174, 97.1017, 85.1017, 83.0861, 71.0861, 69.0703, 57.0703
	3.94	299.2009	Stearidonic acid	259.2059, 241.1953, 231.2110, 217.1954

Mode	Rt (min)	Measured mass	Compounds	MS^2
	3.71	300.2900	Sphingosine	283.2636, 282.2795, 265.2529, 264.2689, 195.2110, 60.0448,
	4.39	302.3063	Sphinganine	285.2796, 284.2957, 267.2689, 266.2850
	4.15	382.2679	Sphinganine 1-phosphate	365.2453, 364.2614, 347.2350, 284.2950, 267.2684, 266.28444, 264.2685
ESI^-	0.59	157.0379	Allantoin	114.0305, 71.0246, 69.0087, 97.0041, 59.0247
Urine	0.65	167.0219	Uric acid	149.0113, 124.0153, 81.0120
	0.76	175.0257	Ascorbic acid	157.0139, 145.0140, 115.0036, 112.9878, 101.0242, 61.0292, 59.0137
	2.69	178.0495	Hippuric acid	160.0396, 134.0604, 132.0447, 100.0033, 77.0390,
	0.70	243.0608	Uridine	200.0551, 152.0345, 140.0350, 110.0216, 82.0303
	0.79	254.9823	Ascorbic acid-2-sulfate	236.9712, 224.9713, 194.9605, 101.0245, 61.0297, 59.0139
ESI^-	7.53	301.2172	Eicosapentaenoic acid	283.2063, 257.2273, 255.2114, 253.1958, 241.1958, 59.0137
	7.38	277.2153	Gamma-linolenic acid (GLA)	259.2061, 233.0991, 231.2107, 59.0131
	7.57	327.2306	Docosahexaenoic acid	309.2201, 283.2418, 281.2241, 59.0133
	8.01	303.2325	Arachidonic acid (AA)	272.6103, 234.7812, 162.7103, 161.7142, 160.7189, 159.7189, 156.6159
	8.58	279.2322	Linoelaidic acid	261.2216, 235.2424, 233.2268, 59.0132
	8.08	279.2321	Linoleic acid (LA)	261.2229, 219.228, 175.2417, 157.0145, 128.0105, 132.1473, 97.1634, 59.0145

Mode	Rt (min)	Measured mass	Compounds	MS^2
	11.49	283.2621	Stearic acid	265.2513, 239.2720, 237.2564, 59.0132
	3.40	242.0790	Cytidine	152.0458, 110.0371, 109.0409, 81.0465
	3.73	255.2318	Palmitic acid	237.2206, 211.2421, 59.0133

4 Method validation

4.1 Neurotransmitter standard solutions configuration

Eight standard substances were respectively dissolved to 5.0 mmol/L stock solutions by water. Then 8 stock solutions were mixed evenly and diluted to be 1000 μmol/L mixed standard solution. The diluent was prepared by mixing 0.12 % formic acid-water and artificial cerebrospinal fluid (aCSF) at the ratio of 9:1 (v/v). Final standard solutions were obtained by dilution based on required concentrations.

4.2 Standard curve, linearity and limit of quantitation (LOQ)

N,N-Dimethyl phenylalanine was selected as internal standard substance for quantitative measurement. Equations of linear regressions for 8 neurotransmitters were calculated by concentrations of neurotransmitters (abscissa) and peak areas of quantification ion pair (ordinate). The LOQ of method was confirmed using 10 times signal to noise ratio (S/N) as standard. The results showed that excellent linearity was achieved with correlation coefficients (R^2>0.99) for all the analytes in the selected ranges (Table S3).

Table S3 Standard curve, linearity and limit of quantitation (LOQ) for 8 neurotransmitters

Analytes	Dynamic range (μmol/L)	Regression equation	Correlation coefficient (R^2)	LOQs (μmol/L)
Glu	200-1000	y = 0.125x + 0.165	0.9991	0.2
GABA	100-500	y = 0.171x + 0.136	0.9923	0.01
Asp	20-200	y = 0.123x + 0.0570	0.9999	0.01
Gly	20-200	y = 0.0242x + 0.0162	0.9999	1
5-HT	2×10^{-2}-2	y=0.1926x - 0.1489	0.9988	0.005
DA	0.2-10	y=0.871x + 0.0242	0.9997	0.1
Ach	0.05-2	y = 0.0962x + 0.0951	0.9948	0.025
NE	2×10^{-2}-1	y=0.114x + 0.0172	0.9937	0.01

4.3 Precision, recovery and matrix effect (ME)

Three concentration levels (high, medium and low) of standard solutions were added into aCSF for further measurements. Six parallel measurements of each concentration level were performed within three days. Then within-day precision, day to day precision and recovery were obtained. All concentrations of standard solutions were shown in **Table**. For ME study, high, medium and low concentration levels of standard solutions and same concentrations of matrix solution was needed. Standard solutions were prepared by 0.12 % formic acid-water while matrix solution was prepared by aCSF. ME was calculated by the following formula: ME (%) = ($S_{matrix\ solution}$ -$S_{peak\ under\ test}$)/$S_{standard\ solution}$ × 100. Precision, recovery and ME results were shown in Table S4. The intra-day precision was within 14.00 % and the inter-day precision was within 13.00 %. The recovery was 92.03-112.51 % of the nominal values. The assay values of both precision and recovery were all within an acceptable range. The ME of all analytes was 89.71-112.73 %. No significant matrix effect was observed under the current analytical conditions.

Table S4 Precision, recovery and matrix effect (ME) for 8 neurotransmitters

Analytes	Added concerntion (μmol/L)	Precision (%)		Recovery (%)	ME (%)
		Intra-day	Inter-day		
Glu	800	6.25	7.12	97.64	96.28
	400	8.31	6.84	99.02	97.50
	200	11.94	8.52	112.51	98.17
GABA	400	4.63	10.28	107.32	105.81
	200	6.91	12.41	106.16	108.61
	100	9.53	8.93	107.63	103.09
Asp	160	7.82	11.35	98.37	96.68
	80	11.72	9.32	92.59	96.17
	40	5.05	6.83	99.49	105.79
Gly	160	8.61	10.17	92.03	102.35
	80	11.56	8.61	98.43	99.71
	40	9.90	12.61	103.37	102.02

Analytes	Added concerntion (μmol/L)	Precision (%)		Recovery (%)	ME (%)
		Intra-day	Inter-day		
5-HT	2	9.72	10.37	102.26	102.63
	0.4	9.36	12.66	111.39	97.92
	0.04	9.52	11.28	107.11	99.19
DA	8	4.93	7.18	105.31	95.52
	4	3.53	2.24	106.49	93.52
	0.4	8.95	7.04	106.82	103.92
Ach	2	3.51	12.64	106.29	95.29
	1	2.09	10.93	105.82	103.72
	0.1	8.92	10.25	100.85	99.93
NE	1	6.82	7.94	109.27	112.73
	0.4	7.92	3.54	102.91	93.01
	0.04	13.31	2.07	97.21	89.71