Electronic Supplementary Material (ESI) for Analytical Methods.

Quality control for traditional Chinese medicine, Millettia Speciosa Champ, using ultra-high-performance liquid chromatography fingerprint, serum pharmacochemistry and network pharmacology

Material and method

1. Study on serum pharmacochemistry of MSC

1.1 Optimization of dosage

10 males SD rats were randomly divided into 5 groups (n=2): control group and 4 MSC administration groups with different doses. Except control group, all group rats were administrated 6.25 g/kg, 15.625 g/kg, 25.0 g/kg, 31.25 g/kg of MSC, respectively, which were equivalent twice, 5 times, 8 times, 10 times to the clinical dosage in adults. The control group was given distilled water as above method. All rats were fasting 12 h but freely drinking before experiment. After administrated 1.5 h, rats were anesthetized by intraperitoneal injection of 20% carbamate solution 0.5ml/100g. Rats blood samples were collected from the abdominal aorta and stand on ice for 30 min. Then blood samples were centrifugated at 3000 rpm for 10 min. The supernatant solution of same group rats was mixed and as pharmaceutic serum.

1.2 Optimization of blood collection time

15 males SD rats were randomly divided into 5 groups (n=3): control group and 4 MSC administrated groups. Except control group, all groups were accepted 25.0 g/kg MSC. After administrated 1 h, 3 h, 6 h, 12 h, rats were anesthetized by intraperitoneal injection of 20% carbamate solution 0.5ml/100g, respectively. Rats blood samples were collected from the abdominal aorta and stand on ice for 30 min. Then blood samples were centrifugated at 3000 rpm for 10 min. The supernatant solution of same time of blood collection was mixed and as pharmaceutic serum for subsequent analysis. The control group was given distilled water as above method and collected blood samples from the abdominal aorta as control serum.

1.3 Optimization of sample pretreatment method

2 dose of serum 500 μl were taken at 3 h time point, then 2.0 ml methanol and acetonitrile were added to precipitate protein, respectively. After that they were vortexed for 30s and centrifugated at 13000 rpm in 4 ℃ for 10 min. Finally, the supernatant solution was dry by N_2 and stored at -20 ℃ for subsequent analysis using LC-MS.

2. Quantitative analysis of lenticin

2.1 Optimization of chromatographic condition

Firstly, the lenticin standard was scan by full wavelength and its UV absorption map was recorded. Secondly, S3 test solution was used for mobile phases expedition which including methanol-water, acetonitrile-water and acetonitrile-0.1% formic acid in water. Thirdly, the flow rate with 0.2 ml/min, 0.3 ml/min and 0.4 ml/min were investigated in sequence. Finally, the column temperature for 20 ℃, 30 ℃ and 40 ℃ were also optimized.

2.2 Method validation

The specificity was confirmed by injecting 1 μl of S3 test solution and negative sample solution. For linearity evaluation, lenticin standard solutions were made up at concentrations of 10.008, 25.02, 50.04, 100.08, 150.12, 200.16 μg/ml and analysis. For precision and repeatability evaluation, S3 test solution were continuous injected 6 times to value the precision, and 6 individual S3 test solution was analyzed in parallel for repeatability determination. To confirm the stability, the S3 test solution was tested at 0,2,4,6,8,12,24 h, respectively. Recoveries was carried out by adding the know lenticin standard solution into 6 portions of S3 with known content for analysis its peak area. Recoveries were calculated as follow aquation: recovery (%) = 100*(observed content-original content) / spiked content.

Result

1. Serum pharmacochemistry analysis of MSC

1.1 Results of dosage optimization.

In our previously look for literature found that the clinical dosage of MSC is 15 to 30 g, and 30 g is the most prescribed dosage, which due to the MSC is a hard root medicine material and the prescription use larger to better play the effect [1]. So, the doses as 30 g are determined to our study, which translates to a dose of 3.125 g/kg for rats. What's more, our purposed is to find the prototypal and metabolic components that enter the bloodstream, but what components enters into the blood may be trace. To achieve the detection line of the instrument and better detect the prototype and metabolic components entering the blood, our experiment was converted 2, 5, 8 and 10 times of human clinical dose (3.125g/kg for rats) into rat oral dose to investigate the best administration dose. The serum samples of different dose groups were determinate and the identified result between medicated serum and blank serum were compared based on the Compound Discoverer 3.1 software. Then the number of ingredients which consisted in medicated serum instead of blank serum were used as a quantitative indicator. The result was showed that there were more components which absorbed into blood were identified in the dose of 25.0 g/kg of medicated serum samples (**Table S11**). Moreover, their peaks response was relatively higher which were appropriated for analyzing and identifying (**Fig S4**).

1.2 Result of blood collection time optimization

The serum samples of multiple blood collection time points were analyzed based on Compound Discoverer 3.1 software. The blank serum and medicated serum were compared and the number of ingredients which consisted in medicated serum instead of blank serum were used as a quantitative indicator. The result showed that there were more components which absorbed into blood were identified in the medicated serum after administrating for 3 h (**Table S12**). In addition, their peaks response was relatively higher which were appropriated for analyzing and identifying (**Fig S5**).

1.3 Result of pretreatment methods optimization.

The serum samples usually contained many endogenous substances which would disturb the detection of components which absorbed into blood, so it's necessary for pretreating the serum samples before detection. The result exhibited that the samples which had treated by acetonitrile were found more components that absorbed into blood (**Table S13**). Besides, it has the less impurity peaks, good peak shape and the low baseline which were good for the identification of constituents absorbed into blood (**Fig S6**).

2. Quantitative analysis of lenticin

2.1 Chromatographic condition investigation

As the **Figure S9** showed, the maximum absorption wavelength of lenticin was 280 nm through full wavelength scanning; Then the result of different mobile phase was showed at **Table S14** and **Figure S10**, the order of the peak area of each mobile phase was acetonitrile-0.1% formic acid water > acetonitrile-water > methanol-water, but the separation degree of acetonitrile-0.1% formic acid water was lower than other 2 mobile phase and take into consideration the peak of MSC from different batches was various, so acetonitrile-water was determinate as the best mobile phase; The result of flow rate and column temperatures was demonstrated in **Table S15**, **Figure S11** and **Table S16**, **Figure S12**, and the flow rate of 0.2 ml/min and the column temperature of 40 °C have excellent peak and separation degree.

Table S1 Collection information of 12 batches of *Millettia Speciosa* Champ from Guangdong province

NO.	Source	NO.	Source
S1	Yangjiang	S7	Baise
S2	Yangjiang	S8	Baise
S3	Yangjiang	S9	Baise
S4	Jiangmen	S10	Qinzhou
S5	Jiangmen	S11	Qinzhou
S6	Jiangmen	S12	Qinzhou

Table S2 The similarity of precision test

Sample	1	2	3	4	5	6	Comparison
1	1.000	0.936	0.940	0.950	0.934	0.947	0.942
2	0.936	1.000	0.999	0.997	0.992	0.985	0.999
3	0.940	0.999	1.000	0.998	0.995	0.986	0.999
4	0.950	0.997	0.998	1.000	0.991	0.988	0.999
5	0.934	0.992	0.995	0.991	1.000	0.984	0.996
6	0.947	0.985	0.986	0.988	0.984	1.000	0.988
Comparison	0.942	0.999	0.999	0.999	0.996	0.988	1.000

Table S3 The similarity of repeatability test

Sample	S1-1	S1-2	S1-3	S1-4	S1-5	S1-6	Comparison
S1-1	1.000	0.996	0.998	0.996	0.999	0.996	0.999
S1-2	0.996	1.000	0.999	0.986	0.995	0.998	0.992
S1-3	0.998	0.999	1.000	0.992	0.997	0.998	0.996
S1-4	0.996	0.986	0.992	1.000	0.996	0.989	0.999
S1-5	0.999	0.995	0.997	0.996	1.000	0.995	0.999

S1-6	0.996	0.998	0.998	0.989	0.995	1.000	0.993
Comparison	0.999	0.992	0.996	0.999	0.999	0.993	1.000

Table S4 The similarity of stability test

Sample	0 h	2 h	4 h	8 h	12 h	24 h	Comparison
0 h	1.000	0.997	0.976	0.974	0.975	0.978	0.977
2 h	0.997	1.000	0.973	0.976	0.977	0.973	0.977
4 h	0.976	0.973	1.000	0.996	0.997	0.999	0.999
8 h	0.974	0.976	0.996	1.000	1.000	0.993	0.999
12 h	0.975	0.977	0.997	1.000	1.000	0.993	0.999
24 h	0.978	0.973	0.999	0.993	0.993	1.000	0.997
Comparison	0.977	0.977	0.999	0.999	0.999	0.997	1.000

Table S5 Relative retention times of 9 common peaks in positive ion mode

NO.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	RSD%
1	0.183	0.191	0.189	0.193	0.190	0.189	0.190	0.191	0.191	0.191	0.191	0.194	1.41
2	0.426	0.440	0.436	0.439	0.438	0.437	0.438	0.440	0.441	0.441	0.441	0.441	1.00
3	0.543	0.550	0.547	0.554	0.550	0.554	0.555	0.553	0.554	0.557	0.552	0.554	0.70
4	0.586	0.586	0.585	0.589	0.588	0.586	0.588	0.588	0.587	0.590	0.590	0.587	0.28
5	0.846	0.868	0.859	0.863	0.864	0.858	0.861	0.865	0.867	0.867	0.867	0.867	0.75
6	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.00
7	1.203	1.233	1.220	1.227	1.226	1.226	1.226	1.233	1.236	1.236	1.233	1.231	0.75
8	2.474	2.538	2.512	2.534	2.528	2.519	2.526	2.539	2.545	2.554	2.545	2.545	0.85
9	2.696	2.769	2.737	2.759	2.752	2.744	2.752	2.766	2.774	2.774	2.774	2.774	0.82

Table S6 Relative retention times of 18 common peaks in negative ion mode

NO.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	RSD%
1	0.096	0.096	0.096	0.097	0.096	0.096	0.096	0.096	0.096	0.096	0.096	0.095	0.52
2	0.124	0.124	0.124	0.124	0.124	0.123	0.124	0.123	0.124	0.116	0.124	0.124	1.69
3	0.198	0.198	0.198	0.188	0.186	0.189	0.198	0.200	0.199	0.190	0.199	0.191	2.73
4	0.328	0.328	0.330	0.331	0.330	0.330	0.332	0.333	0.334	0.336	0.336	0.340	1.13
5	0.567	0.570	0.570	0.573	0.572	0.571	0.574	0.574	0.578	0.578	0.578	0.583	0.79
6	0.620	0.620	0.623	0.624	0.623	0.620	0.620	0.619	0.618	0.618	0.616	0.616	0.43
7	0.643	0.643	0.640	0.647	0.647	0.644	0.650	0.648	0.652	0.652	0.654	0.657	0.76
8	0.685	0.685	0.687	0.689	0.687	0.686	0.690	0.688	0.692	0.692	0.692	0.697	0.49
9	0.887	0.887	0.887	0.889	0.887	0.883	0.887	0.883	0.885	0.885	0.883	0.887	0.24
10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.00
11	1.218	1.218	1.218	1.222	1.220	1.214	1.220	1.216	1.220	1.220	1.220	1.225	0.24
12	1.378	1.378	1.380	1.383	1.380	1.373	1.380	1.376	1.380	1.382	1.380	1.388	0.28

13	1.453	1.453	1.453	1.458	1.455	1.448	1.455	1.451	1.455	1.455	1.455	1.462	0.23
14	1.957	1.957	1.957	1.961	1.957	1.947	1.957	1.952	1.957	1.957	1.957	1.968	0.24
15	1.979	1.979	1.982	1.986	1.979	1.972	1.979	1.974	1.982	1.982	1.982	1.991	0.25
16	2.015	2.015	2.015	2.019	2.015	2.005	2.015	2.009	2.017	2.015	2.015	2.026	0.25
17	2.242	2.239	2.239	2.244	2.239	2.228	2.239	2.233	2.239	2.239	2.239	2.248	0.23
18	2.290	2.288	2.290	2.293	2.288	2.279	2.290	2.284	2.290	2.292	2.288	2.300	0.22

Table S7 Relative peak areas of 9 characteristic peaks in positive ion mode

NO.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	RSD%
1	0.475	0.476	2.887	0.662	0.480	0.657	0.423	0.371	0.170	0.207	0.251	0.307	119.47
2	1.110	0.665	3.296	0.876	1.010	0.996	0.448	0.431	0.217	0.494	0.502	0.589	91.11
3	3.035	1.508	6.855	1.324	2.247	1.280	0.772	0.823	0.728	0.728	1.321	1.007	96.14
4	1.030	0.847	1.436	0.529	0.636	0.355	0.304	0.508	0.449	0.179	0.329	0.678	58.51
5	0.555	0.325	1.288	0.664	0.572	0.286	0.572	0.347	0.270	0.369	0.258	0.461	57.25
6	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.00
7	9.055	4.530	19.173	10.152	9.521	8.268	9.467	3.667	3.635	3.406	4.369	8.145	57.30
8	0.993	0.564	2.440	0.597	0.729	1.195	0.627	0.437	0.260	0.291	0.420	0.532	78.60
9	0.492	0.569	1.116	0.885	0.959	0.236	0.509	0.250	0.196	0.544	0.626	0.790	49.30

Table S8 Relative peak areas of 18 characteristic peaks in negative ion mode

NO.	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	RSD%
1	0.232	0.213	0.228	0.180	0.164	0.350	0.181	0.260	0.243	0.420	0.295	0.181	31.26
2	0.918	0.682	0.869	0.610	0.543	1.425	0.564	0.432	0.336	1.075	0.465	0.339	47.63
3	0.201	0.090	0.286	0.143	0.112	0.234	0.163	0.106	0.098	0.267	0.069	0.089	48.29
4	0.242	0.202	0.333	0.202	0.217	0.170	0.091	0.189	0.086	0.318	0.194	0.116	39.60
5	0.167	0.201	0.272	0.133	0.145	0.047	0.093	0.071	0.085	0.127	0.146	0.113	45.77
6	1.135	1.801	8.159	0.968	0.987	0.135	0.434	0.499	0.620	1.236	1.187	0.598	145.32
7	0.312	0.362	0.394	0.206	0.280	0.079	0.141	0.101	0.122	0.413	0.464	0.228	51.04
8	0.843	0.828	0.820	0.988	0.700	0.438	0.963	1.136	1.191	1.361	0.803	0.867	26.52
9	0.959	1.170	3.330	1.112	1.201	0.341	0.494	0.317	0.672	1.946	1.653	1.100	70.09
10	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	1.000	0.00
11	0.112	0.255	0.358	0.361	0.392	0.099	0.099	0.071	0.090	0.534	0.472	0.308	62.65
12	0.406	0.392	0.521	0.262	0.293	0.551	0.270	0.641	0.535	0.265	0.296	0.262	35.18
13	0.270	0.400	1.076	0.285	0.298	0.080	0.165	0.047	0.206	0.432	1.185	0.394	89.58
14	0.228	0.611	0.309	0.587	0.615	0.164	0.343	0.406	0.383	1.472	1.230	0.525	68.99
15	0.127	0.432	0.235	0.376	0.543	0.107	0.181	0.207	0.142	0.981	0.839	0.465	73.79
16	0.230	0.468	0.226	0.594	0.712	0.209	0.317	0.338	0.256	1.399	1.098	0.521	70.98
17	0.954	1.235	0.813	1.017	1.836	0.303	0.797	1.195	0.946	1.992	2.302	1.489	46.05

18	0.323	0.593	0.741	0.658	1.010	0.810	0.642	0.685	0.436	4.314	1.548	0.680	103.88

Table S9 Similarity evaluation results of 12 batches of MSC in positive ion mode

Sample	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	Comparison
S1	1.000	0.982	0.985	0.969	0.983	0.976	0.964	0.977	0.975	0.937	0.965	0.970	0.984
S2	0.982	1.000	0.974	0.964	0.980	0.963	0.946	0.987	0.978	0.966	0.982	0.967	0.985
S3	0.985	0.974	1.000	0.962	0.978	0.969	0.947	0.961	0.949	0.935	0.959	0.955	0.973
S4	0.969	0.964	0.962	1.000	0.992	0.986	0.992	0.974	0.977	0.961	0.969	0.996	0.991
S5	0.983	0.980	0.978	0.992	1.000	0.980	0.979	0.976	0.974	0.969	0.983	0.989	0.992
S6	0.976	0.963	0.969	0.986	0.980	1.000	0.986	0.978	0.977	0.940	0.956	0.982	0.988
S7	0.964	0.946	0.947	0.992	0.979	0.986	1.000	0.968	0.976	0.942	0.949	0.992	0.985
S8	0.977	0.987	0.961	0.974	0.976	0.978	0.968	1.000	0.994	0.963	0.971	0.976	0.991
S9	0.975	0.978	0.949	0.977	0.974	0.977	0.976	0.994	1.000	0.960	0.969	0.982	0.990
S10	0.937	0.966	0.935	0.961	0.969	0.940	0.942	0.963	0.960	1.000	0.986	0.961	0.972
S11	0.965	0.982	0.959	0.969	0.983	0.956	0.949	0.971	0.969	0.986	1.000	0.968	0.981
S12	0.970	0.967	0.955	0.996	0.989	0.982	0.992	0.976	0.982	0.961	0.968	1.000	0.991
Comparison	0.984	0.985	0.973	0.991	0.992	0.988	0.985	0.991	0.990	0.972	0.981	0.991	1.000

Table S10 Similarity evaluation results of 12 batches of MSC in negative ion mode

Sample	S1	S2	S3	S4	S5	S6	S7	S8	S9	S10	S11	S12	Comparison
S1	1.000	0.879	0.713	0.842	0.815	0.743	0.840	0.814	0.876	0.617	0.752	0.817	0.914
S2	0.879	1.000	0.758	0.919	0.846	0.602	0.806	0.727	0.802	0.697	0.810	0.851	0.938
S3	0.713	0.758	1.000	0.619	0.567	0.323	0.473	0.418	0.538	0.435	0.533	0.504	0.718
S4	0.842	0.919	0.619	1.000	0.908	0.708	0.909	0.777	0.855	0.764	0.845	0.894	0.951
S5	0.815	0.846	0.567	0.908	1.000	0.652	0.844	0.780	0.822	0.833	0.922	0.915	0.943
S6	0.743	0.602	0.323	0.708	0.652	1.000	0.821	0.705	0.700	0.603	0.557	0.611	0.709
S7	0.840	0.806	0.473	0.909	0.844	0.821	1.000	0.838	0.878	0.702	0.763	0.826	0.888
S8	0.814	0.727	0.418	0.777	0.780	0.705	0.838	1.000	0.933	0.665	0.747	0.830	0.840
S9	0.876	0.802	0.538	0.855	0.822	0.700	0.878	0.933	1.000	0.664	0.771	0.851	0.895
S10	0.617	0.697	0.435	0.764	0.833	0.603	0.702	0.665	0.664	1.000	0.831	0.776	0.825
S11	0.752	0.810	0.533	0.845	0.922	0.557	0.763	0.747	0.771	0.831	1.000	0.920	0.908
S12	0.817	0.851	0.504	0.894	0.915	0.611	0.826	0.830	0.851	0.776	0.920	1.000	0.925
Comparison	0.914	0.938	0.718	0.951	0.943	0.709	0.888	0.840	0.895	0.825	0.908	0.925	1.000

Table S11 The amounts of transitional components in the blood of different dose groups

Group	Number of components absorbed into the blood
6.25 g/kg	215
15.625 g/kg	128

25.0 g/kg	220
31.25 g/kg	210

Table S12 The amounts of transitional components in blood at different blood sampling tines

Group	Number of components absorbed into the blood
1 h	49
3 h	65
6 h	43
12 h	30

Table S13 The amounts of transitional components in blood at different blood sampling times

Group	Number of components absorbed into the blood
Methanol	65
Acetonitrile	85

Table S14 Optimization of different mobile phases

Mobile phase type	Retention time (min)	Peak area	Separation degree	Theoretical plates
Methanol-water	7.667	163162	12.280321	13186.893480
Acetonitrile-water	3.031	166783	10.453897	12378.883302
Acetonitrile-0.1%formic water	4.105	171453	5.4169755	12470.326432

Table S15 Optimization of different flow rates

Flow rate (ml/min)	Retention time (min)	Peak area	Separation degree	Theoretical plates
0.2	5.641	252563	10.993685	15421.615969
0.3	3.031	166783	10.453897	12378.883302
0.4	3.031	127874	4.376920	11066.852614

Table S16 Investigation of different column temperatures

Column temperature	Retention time (min)	Peak area	Separation degree	Theoretical plates
20℃	6.532	244150	13.147771	16046.684122

30℃	5.641	252563	10.993685	15421.615969
40℃	5.097	254039	10.916847	14911.895132

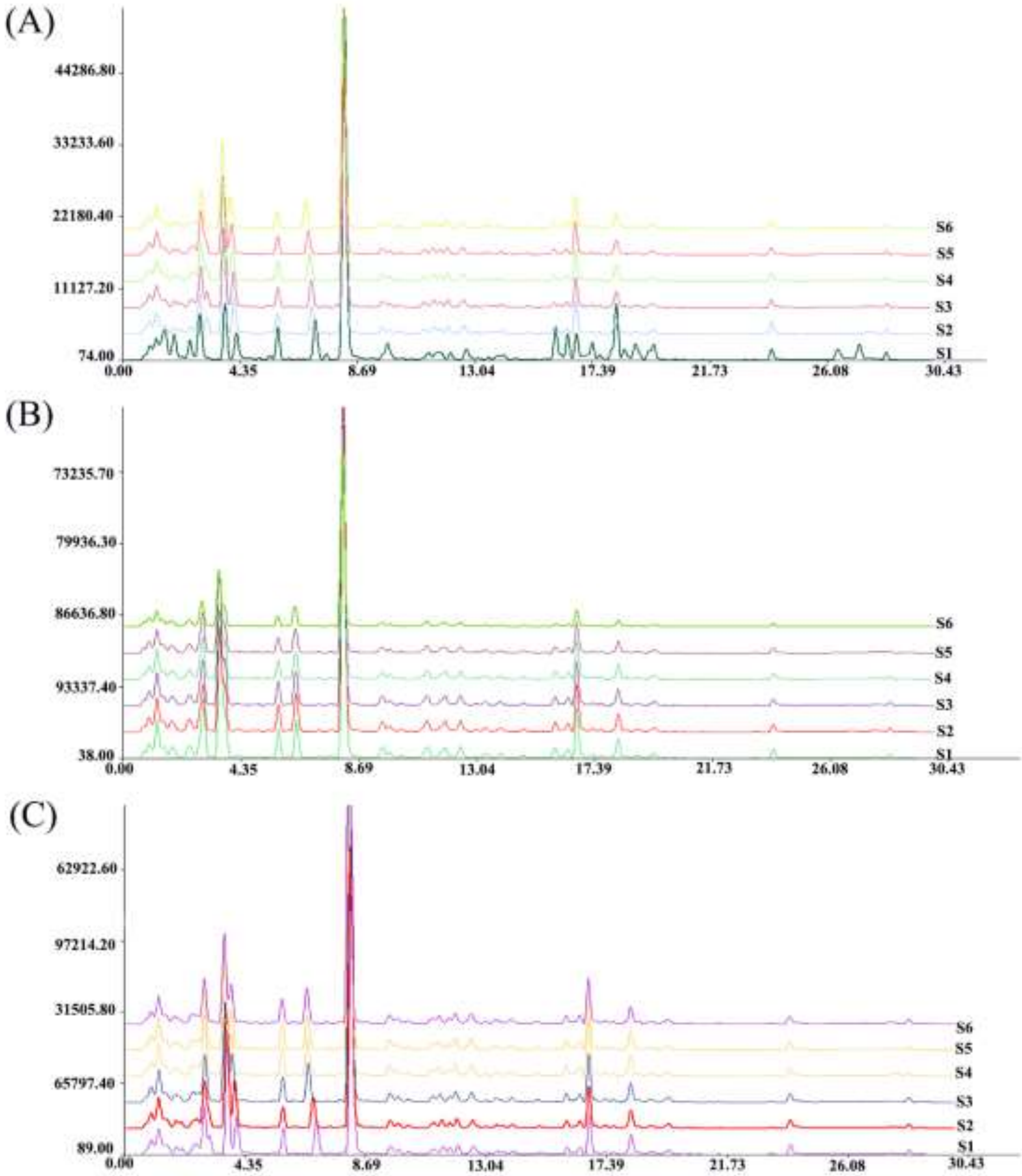


Fig S1 Chromatogram of (A) precision; (B)repeatability;(C) stability test

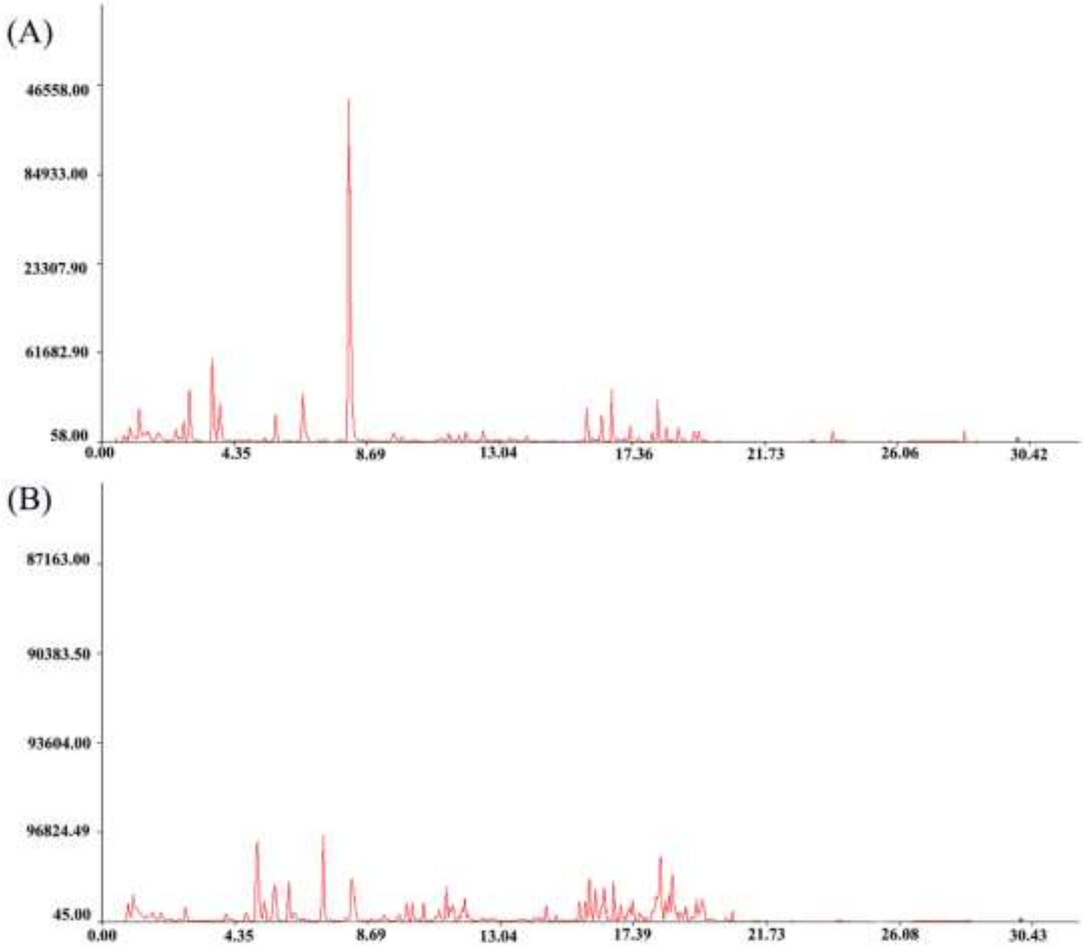
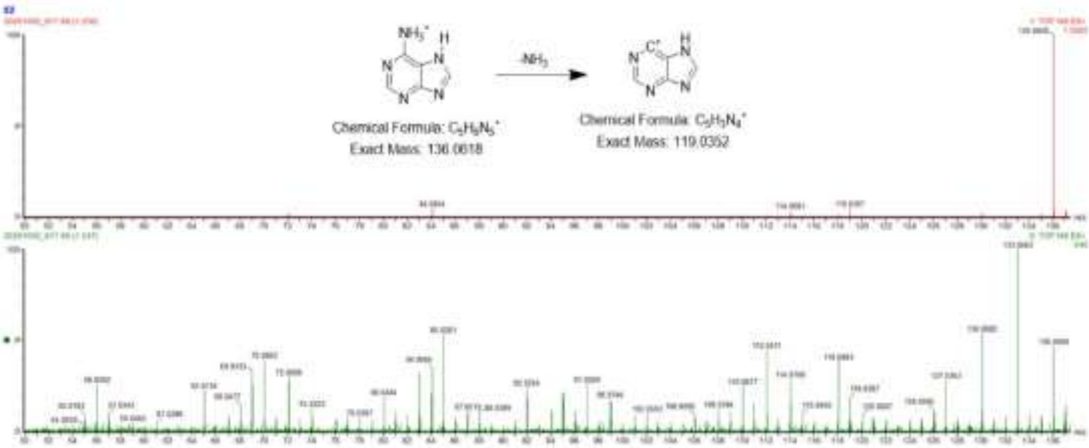
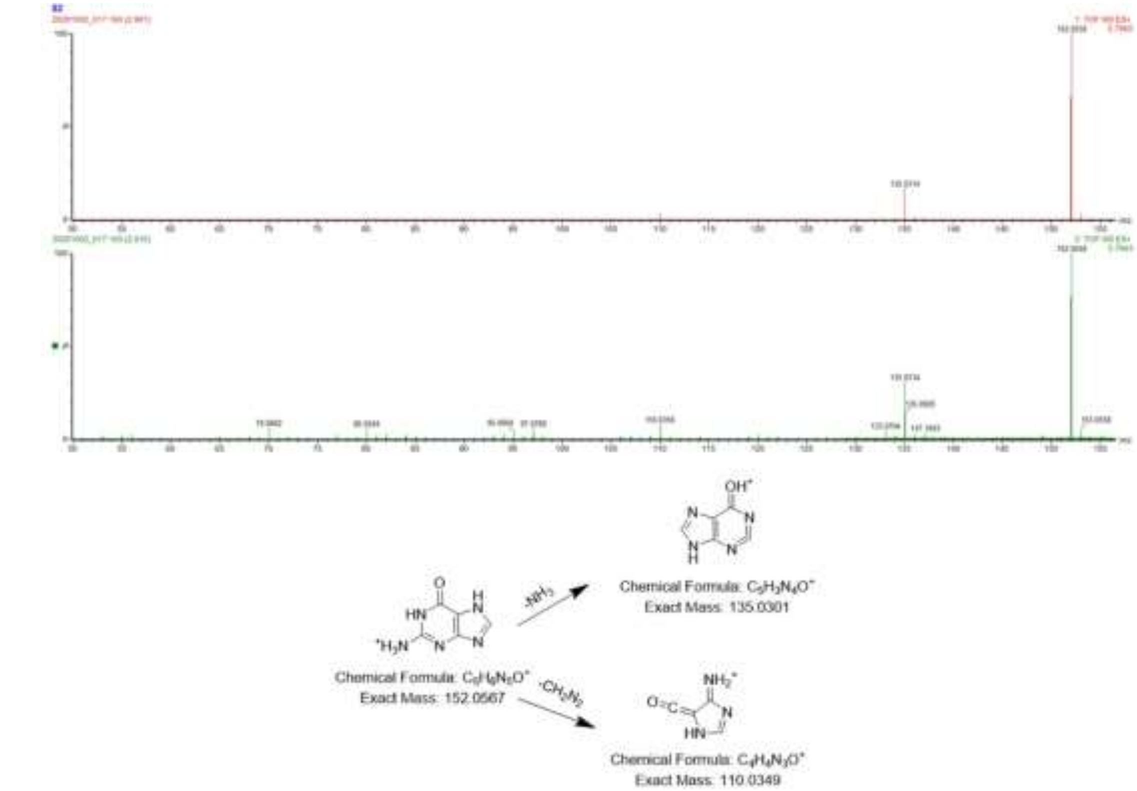


Fig S2 Referential fingerprint in (A) positive ion mode; (B) negative ion mode

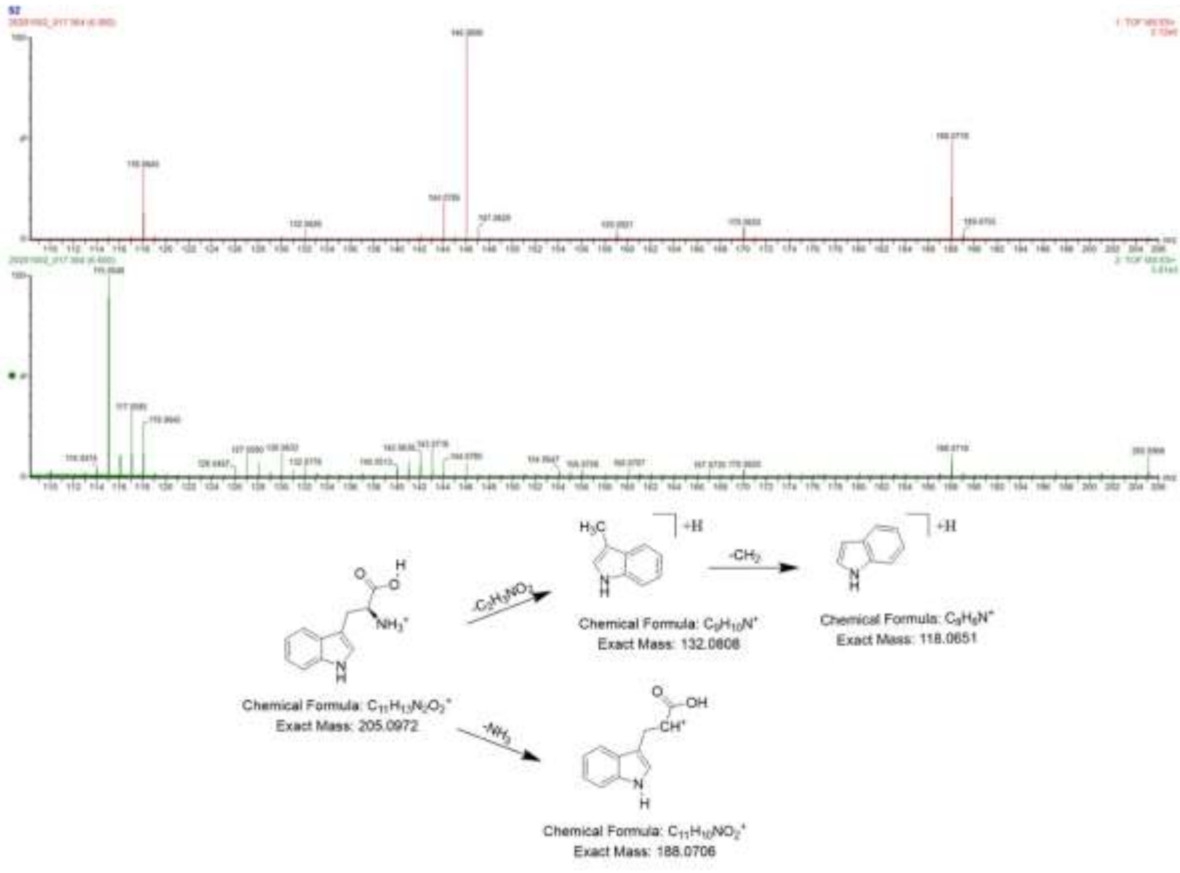
1. Adenin

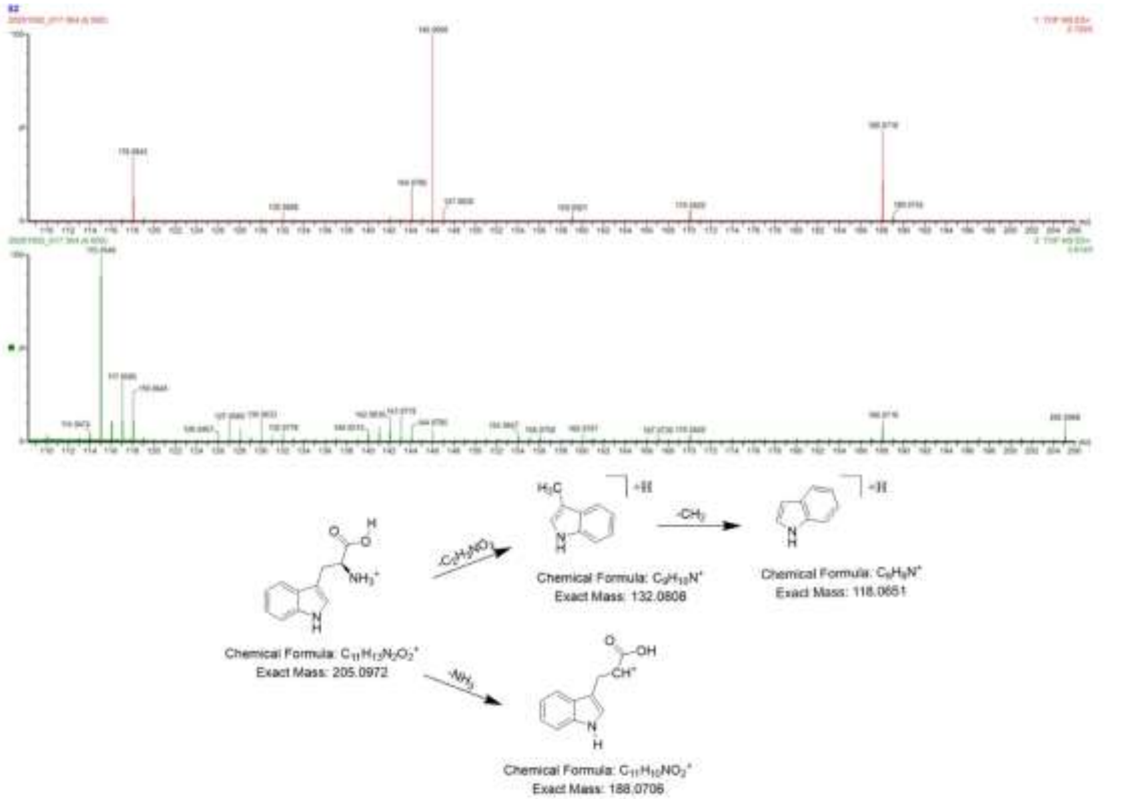


2. Guanine

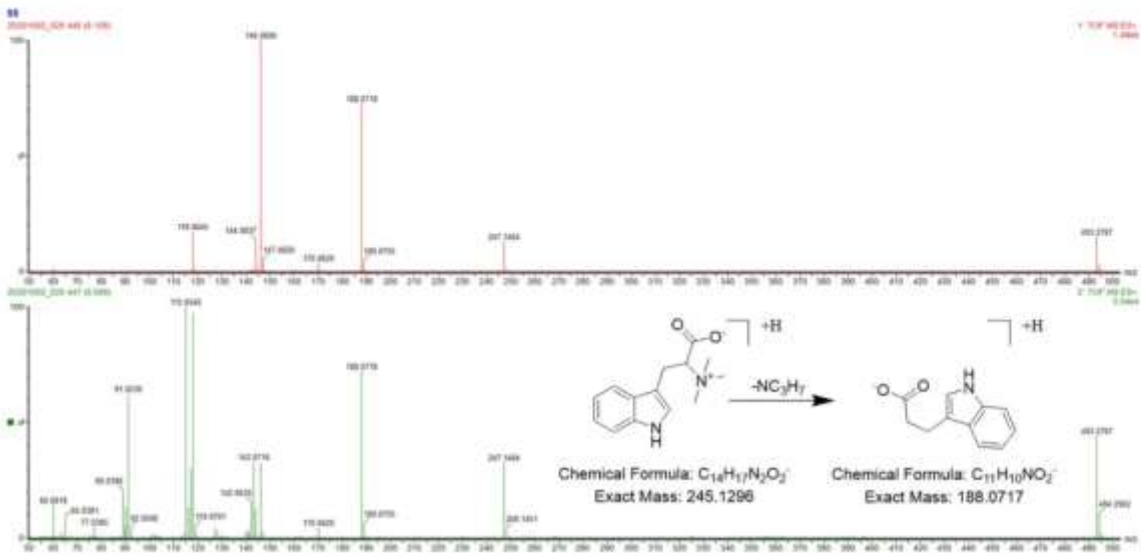


3. Tryptophan

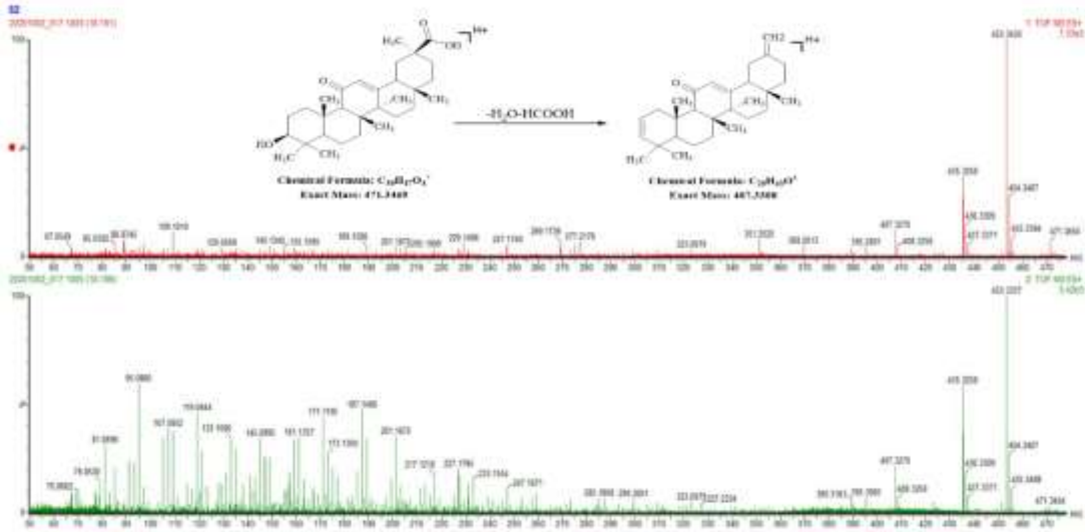




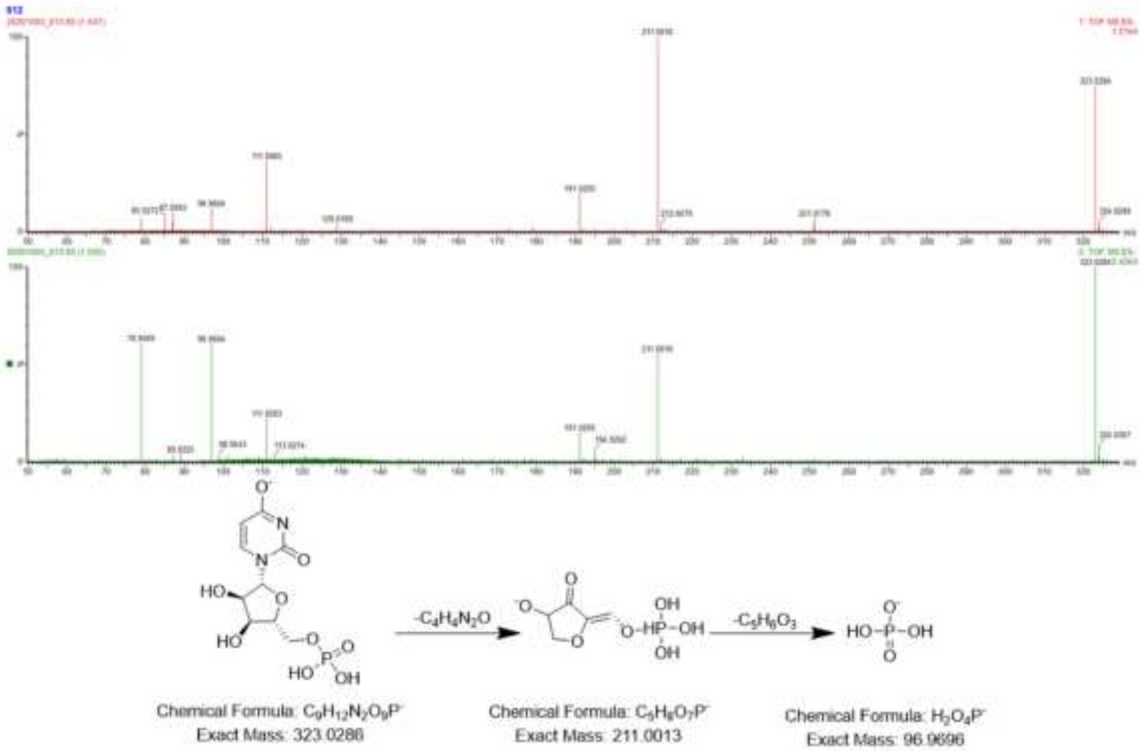
4. Lenticin



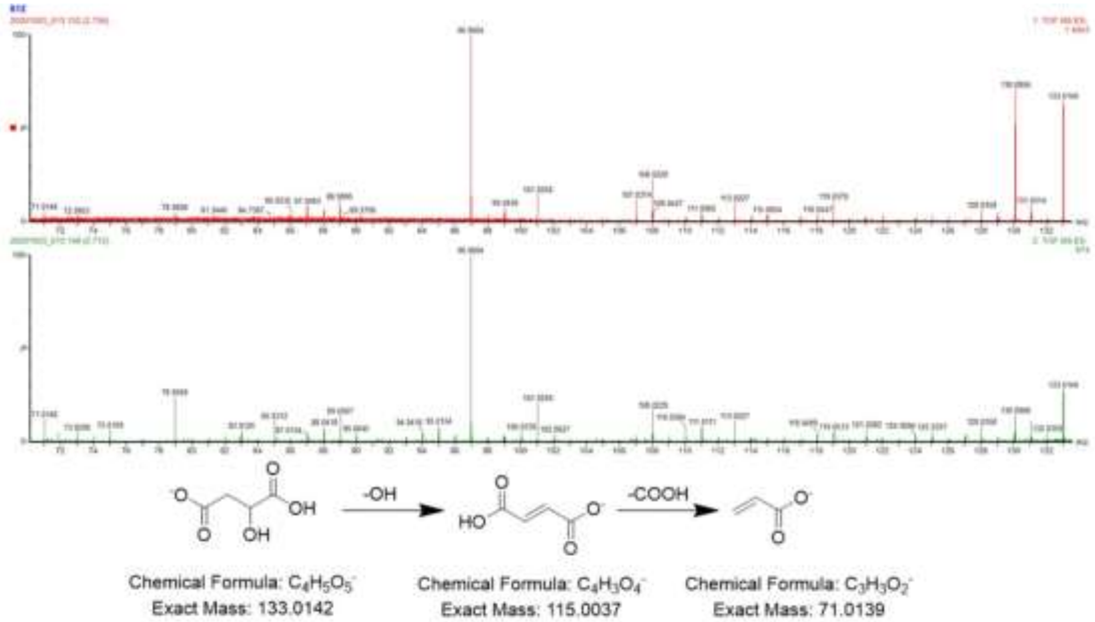
5. Glycyrrhetinic acid



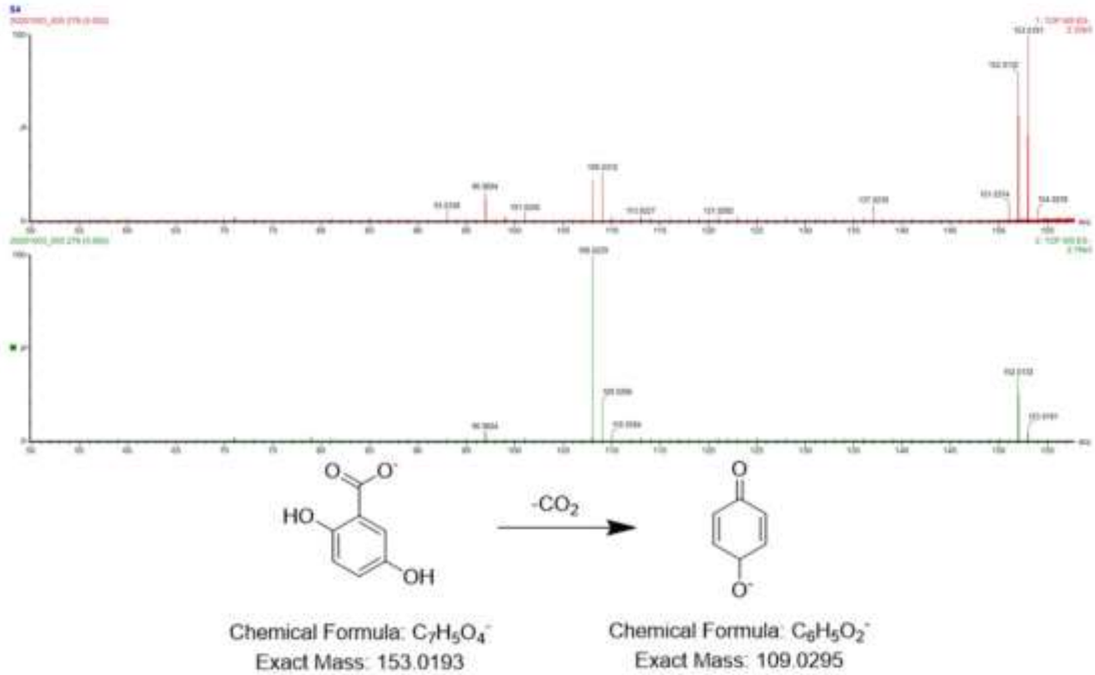
6. Uridine 5’-monophosphate



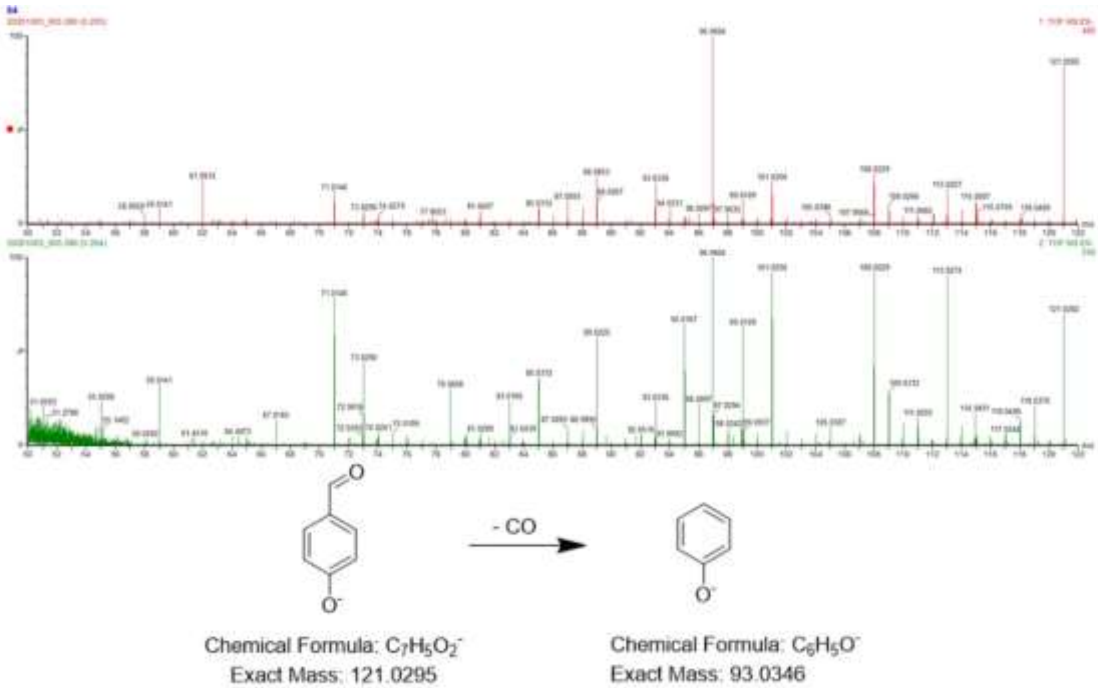
7. Malic acid



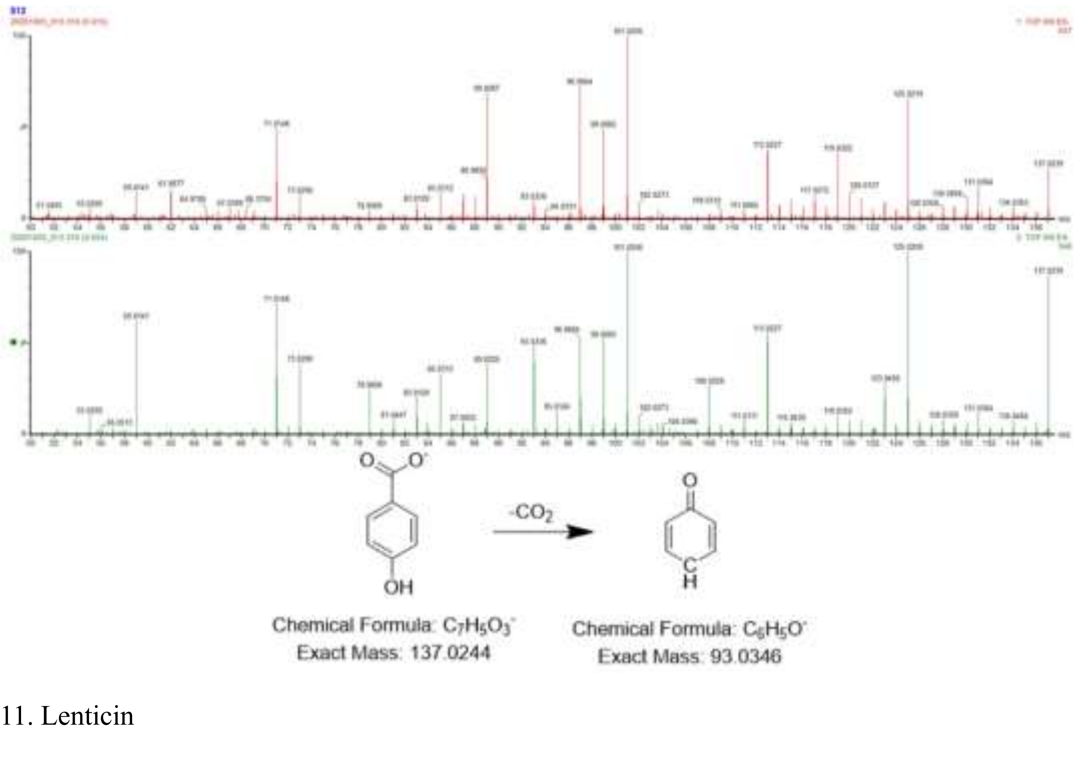
8. Gentisic acid



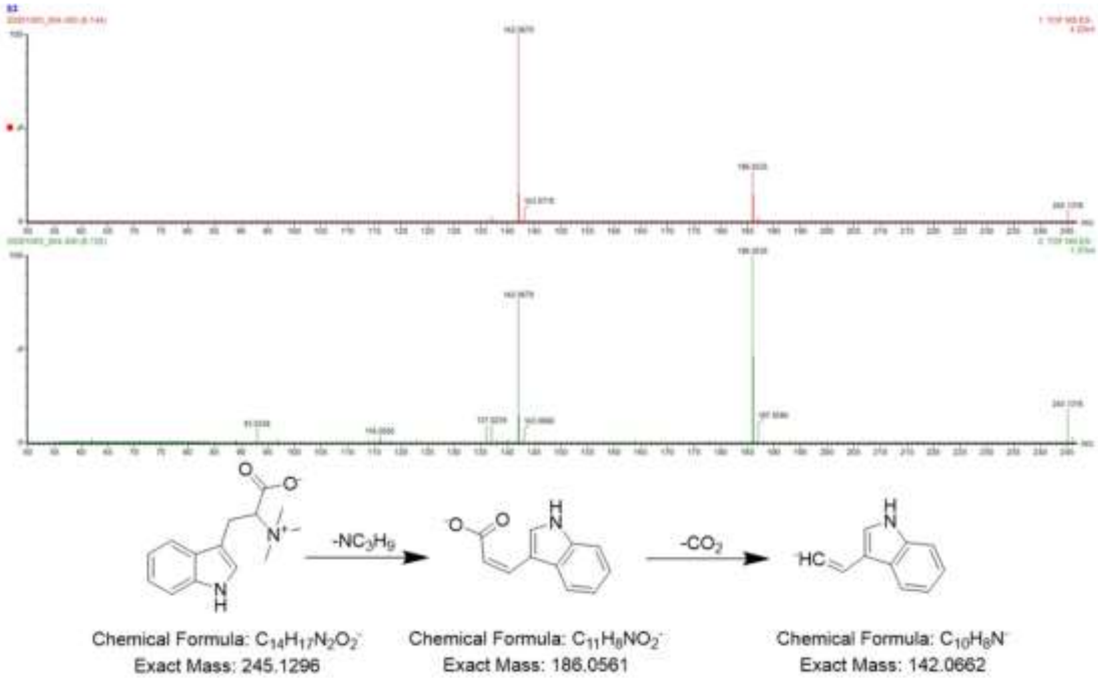
9. 4-Hydroxybenzaldehyde



10. 4-Hydroxybenzoic



11. Lenticin



12. L-aspartic acid

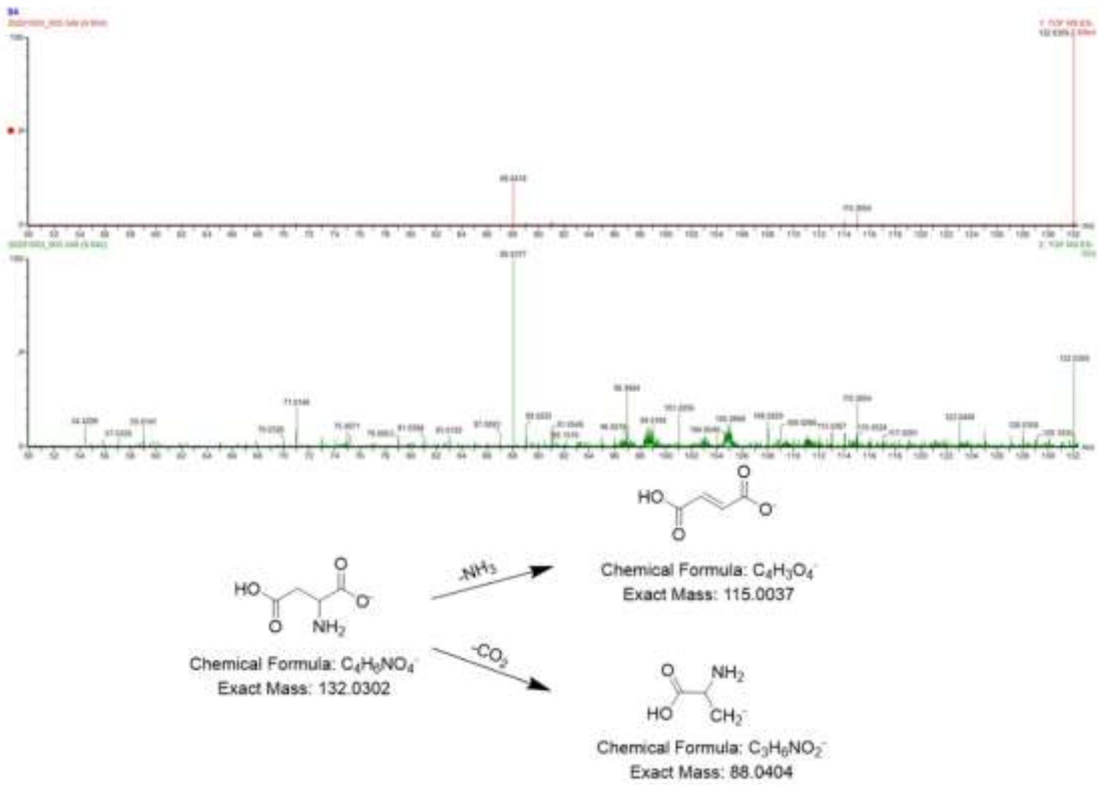


Fig S3 Mass spectrometry information and fragmentation patterns of common components of MSC

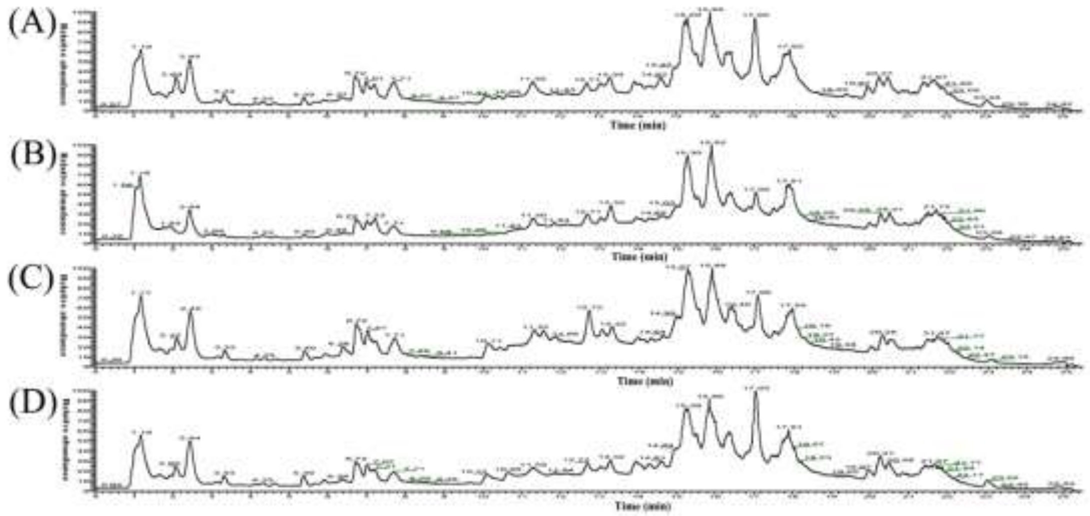


Fig S4 Total ion chromatogram of different dose groups. (A) : 6.25 g/kg; (B) : 15.625 g/kg; (C) : 25.0 g/kg; (D) : 31.25 g/kg

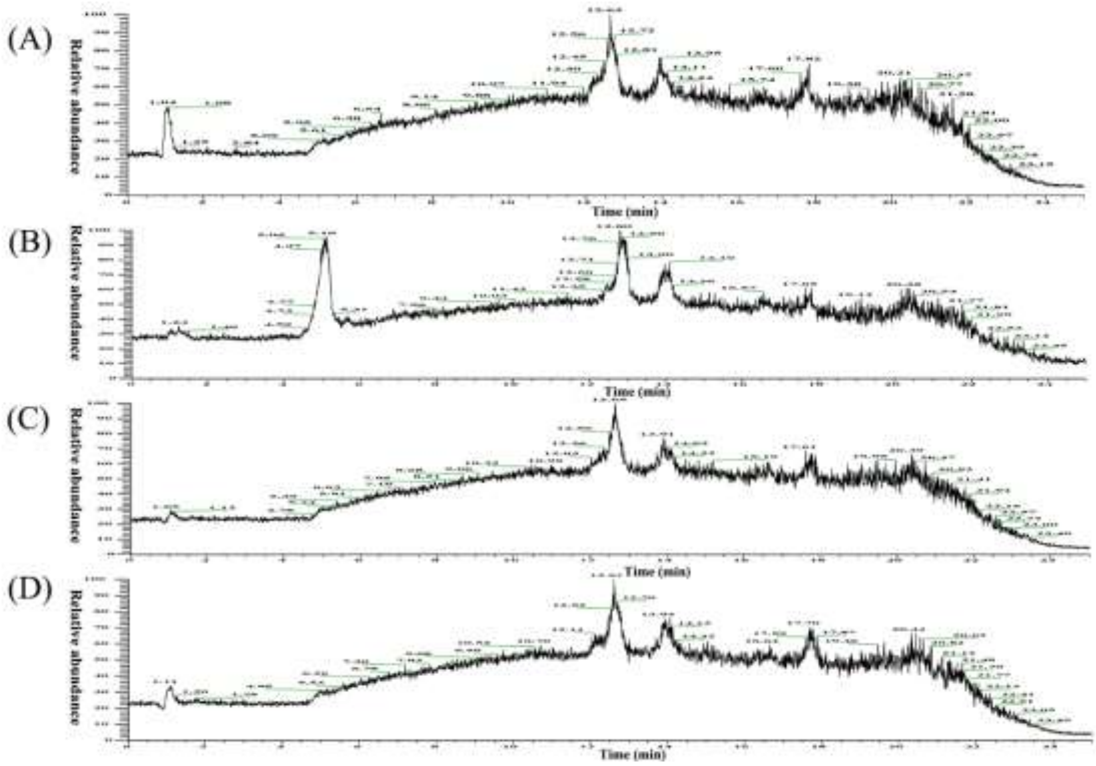


Fig S5 Total ion chromatogram at different blood sampling times. (A) 1h; (B) : 3h; (C) : 6h; (D) : 12h

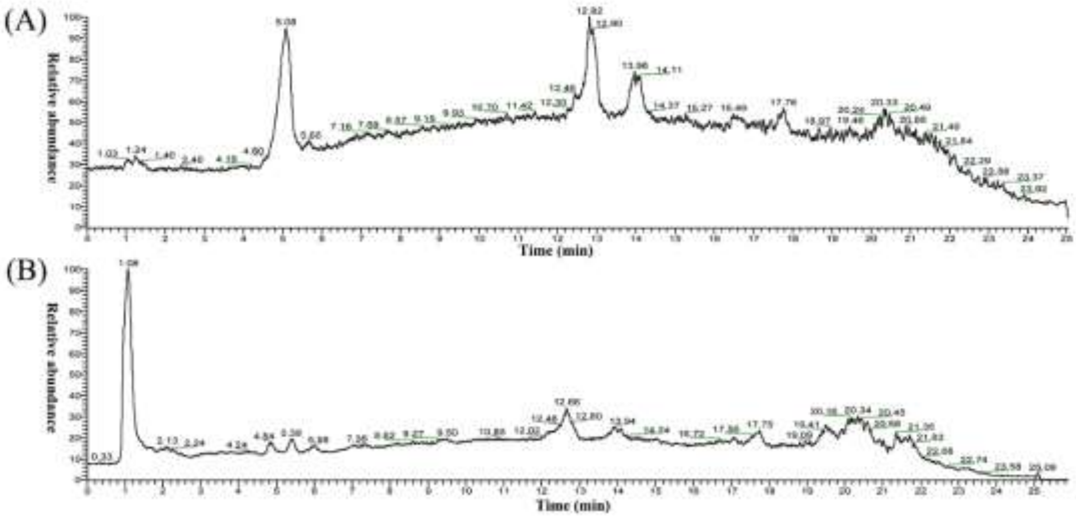
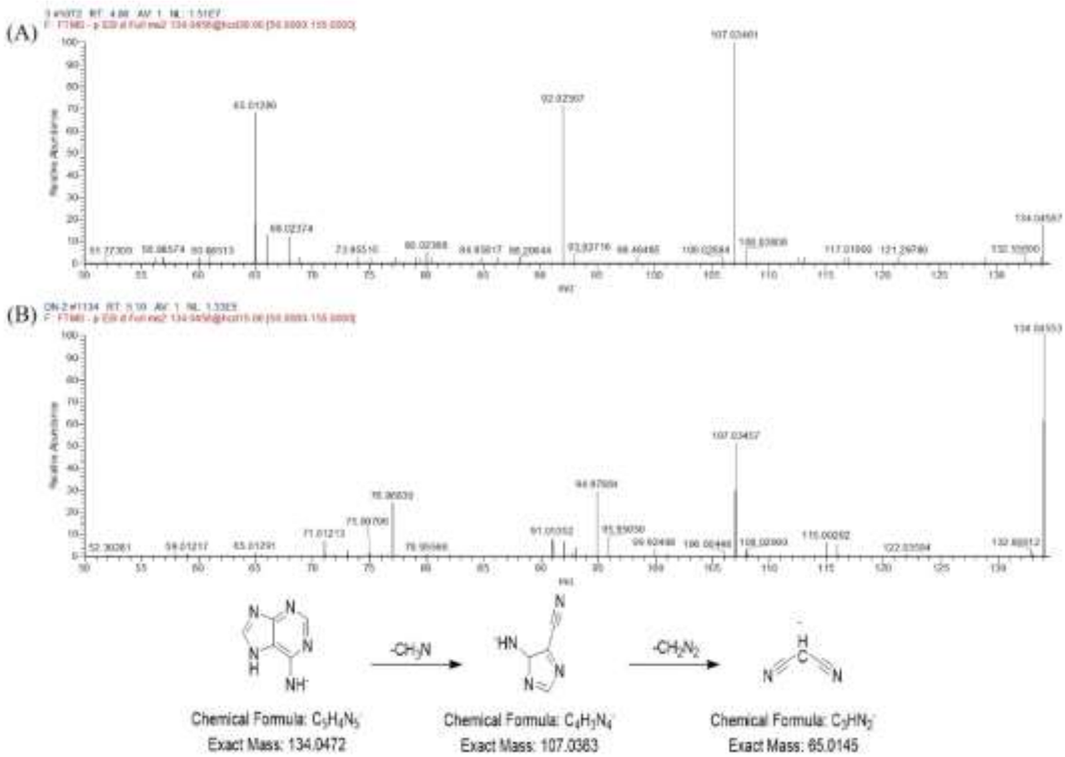
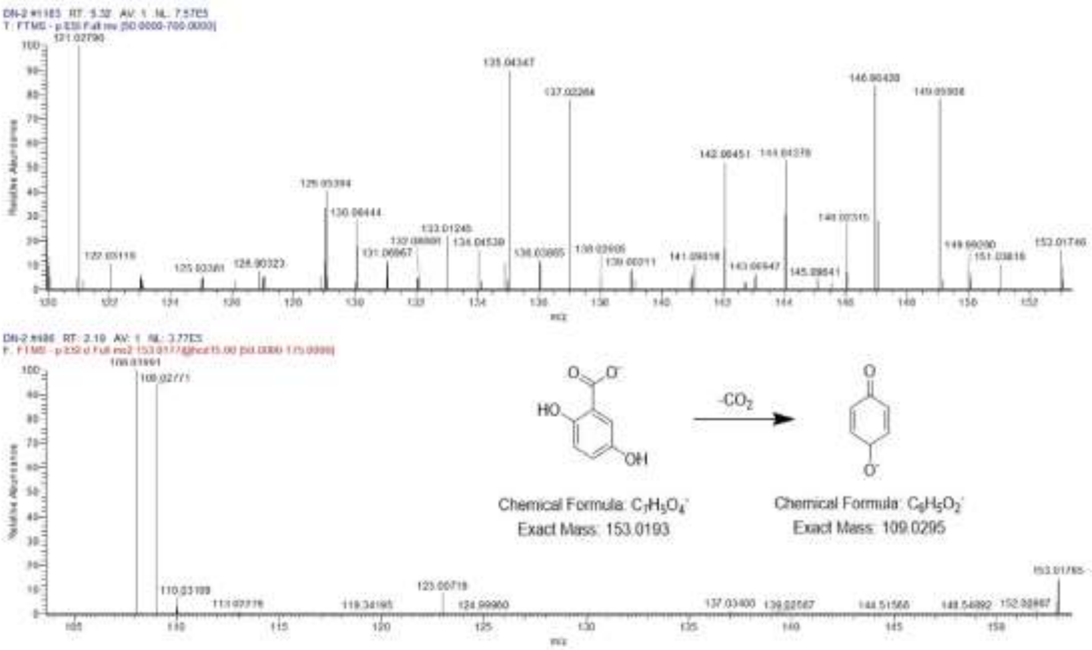


Fig S6 Total ion chromatogram of different precipitated solvents. (A) Methanol; (B) Acetonitrile

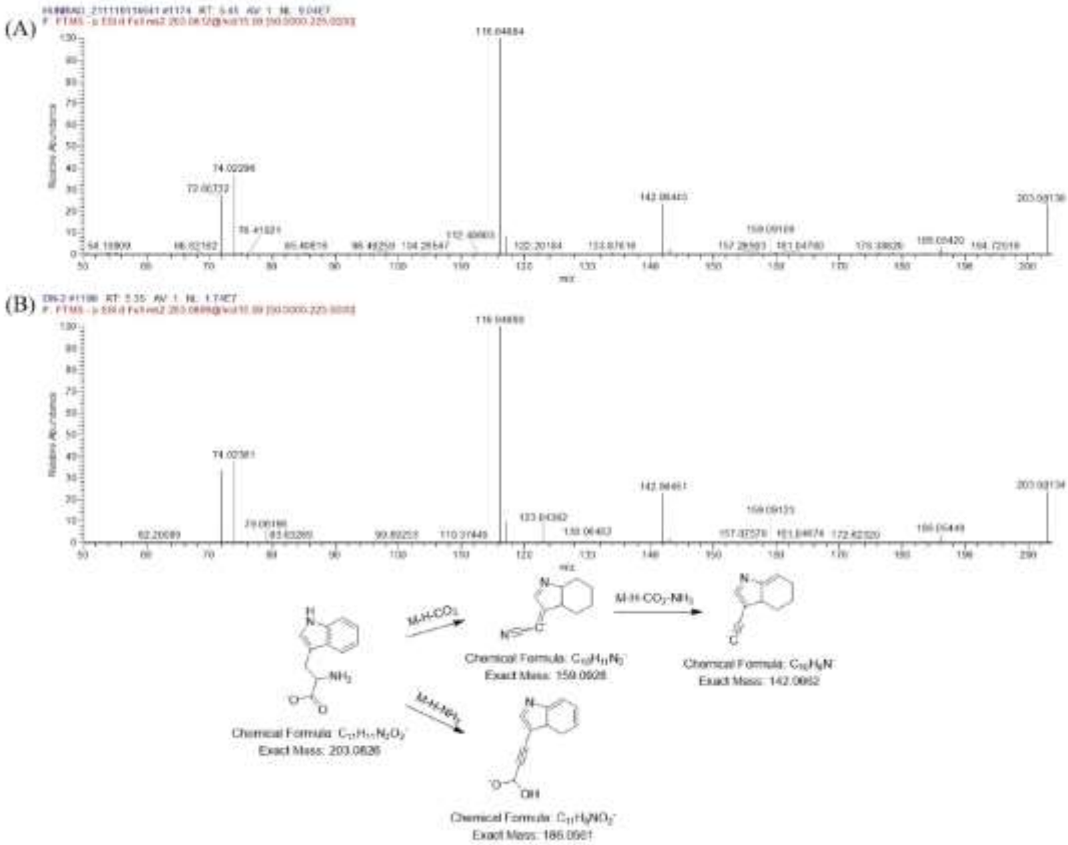
1. Adenine



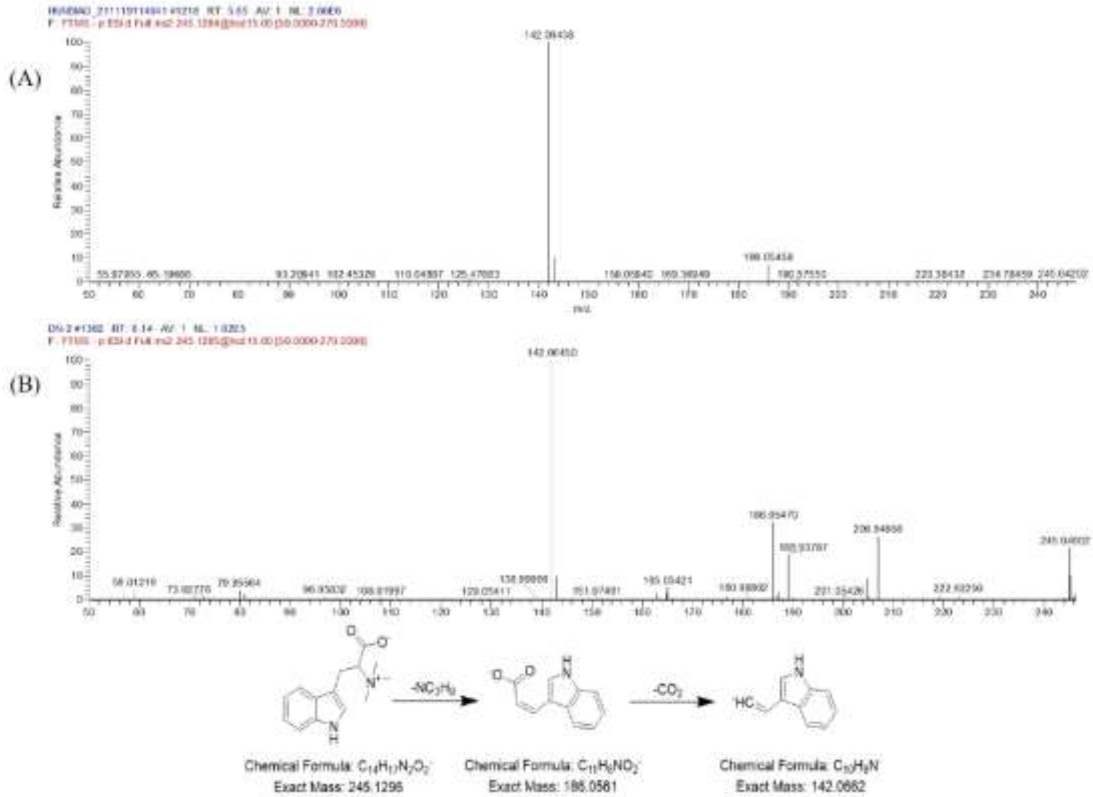
2. Gentisic acid



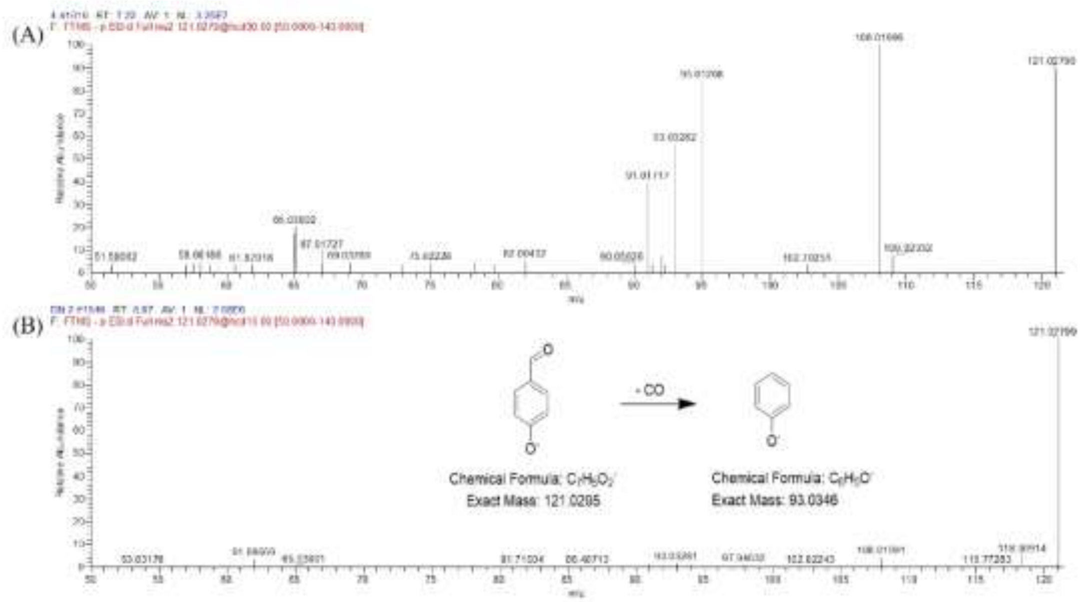
3. Tryptophan



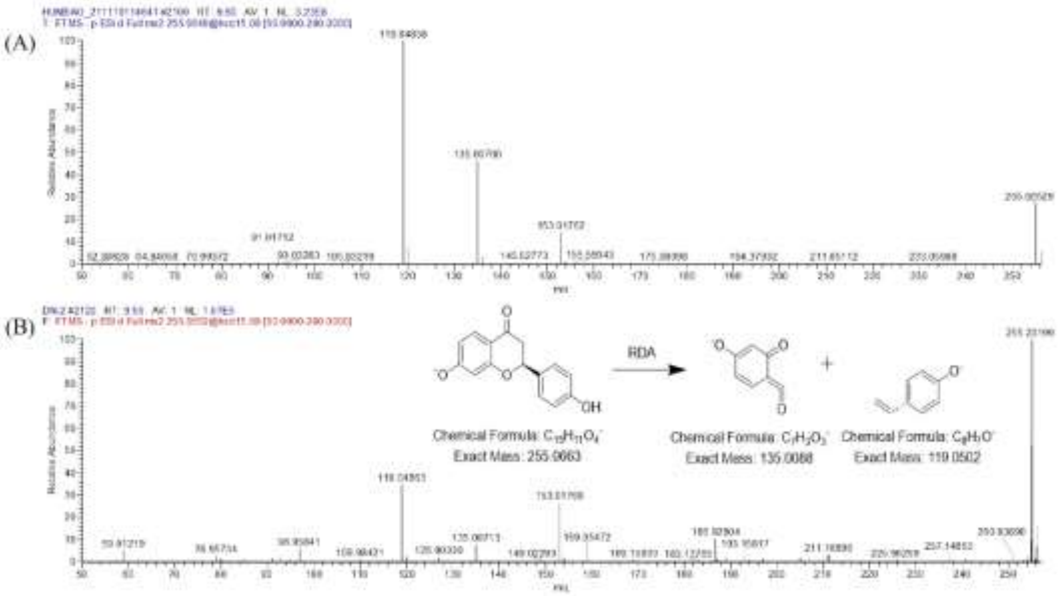
4. Lenticin



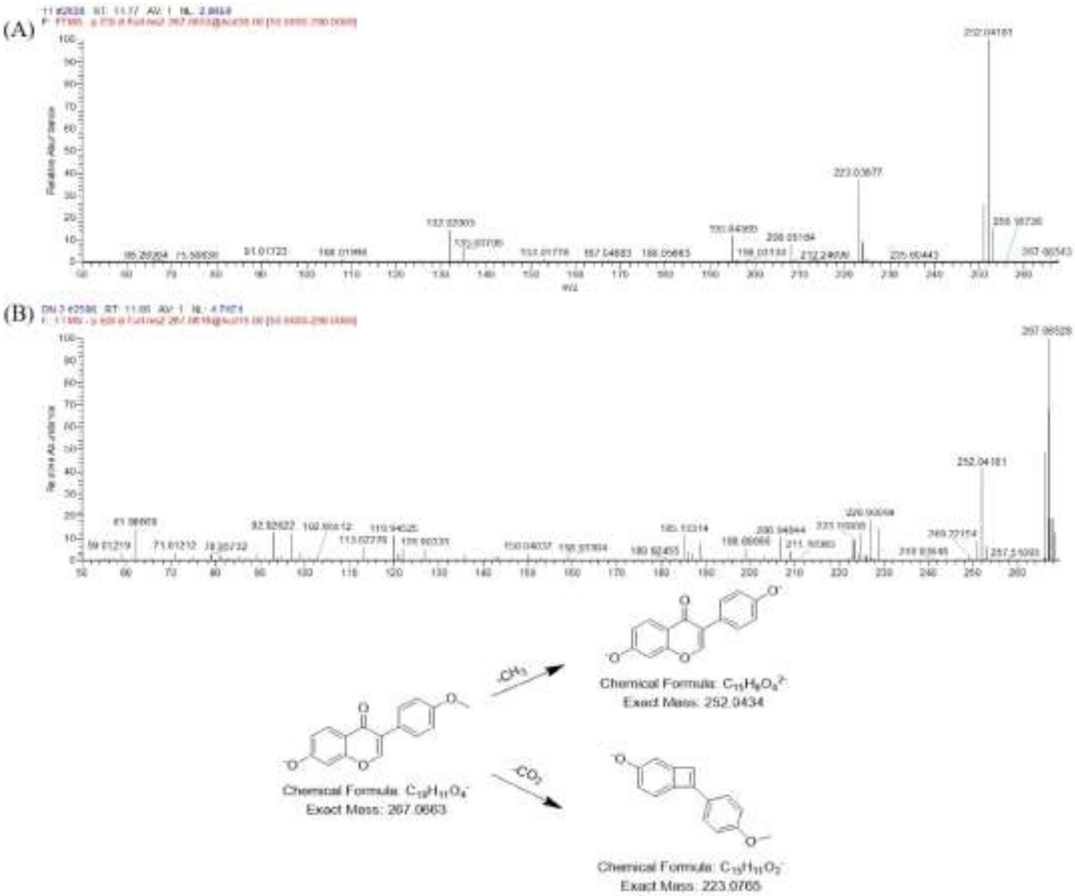
5. 4-Hydroxybenzaldehyde



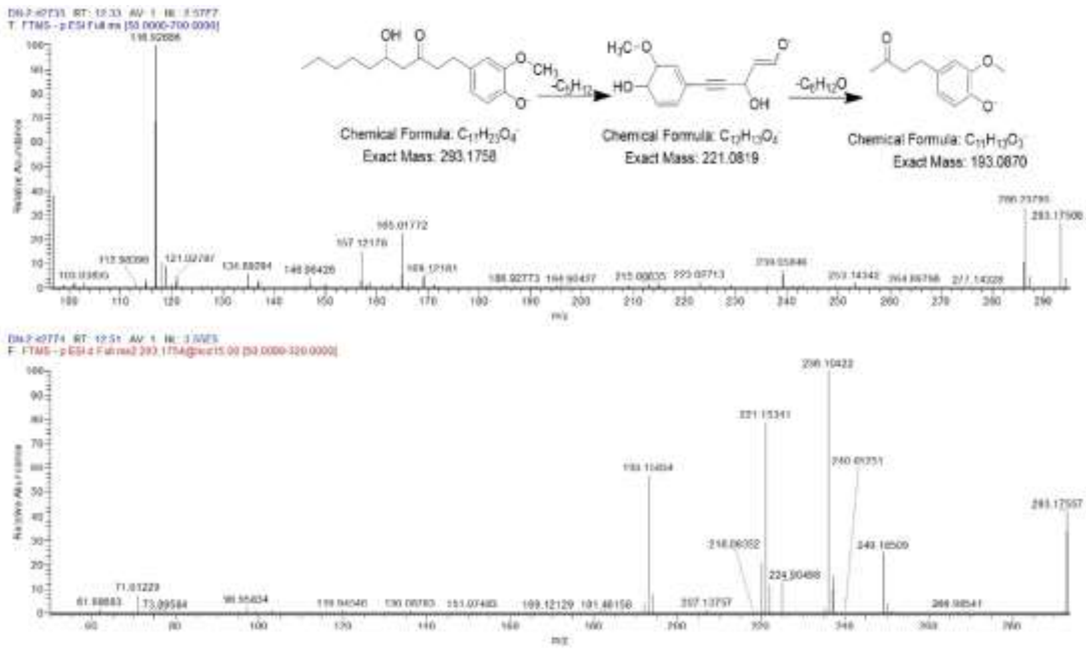
6. Liquiritigenin



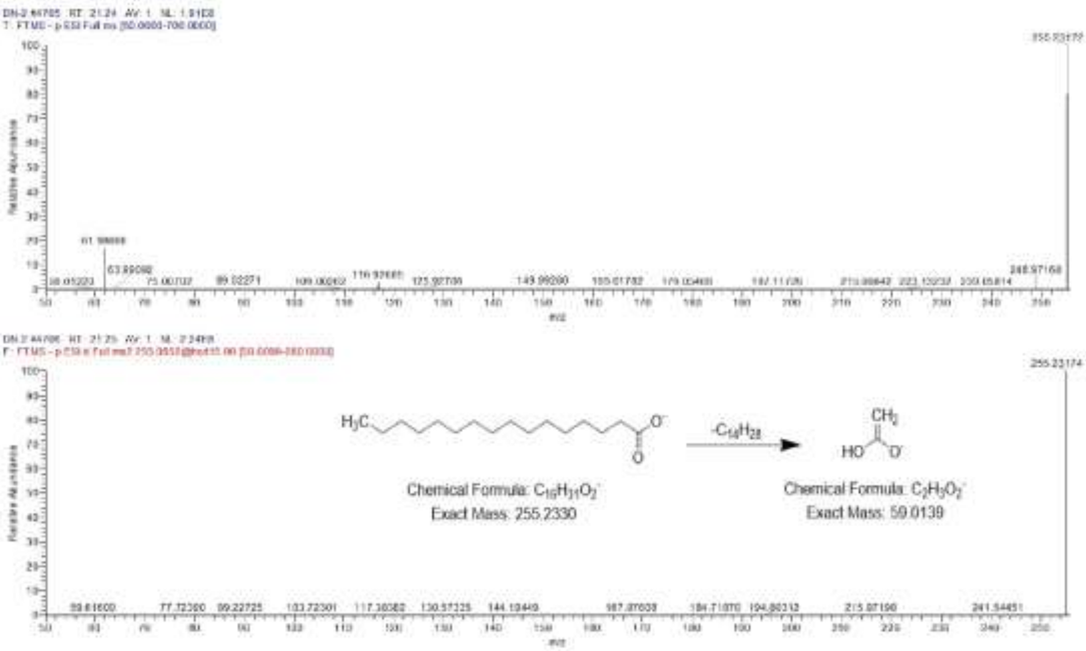
7. Formononetin



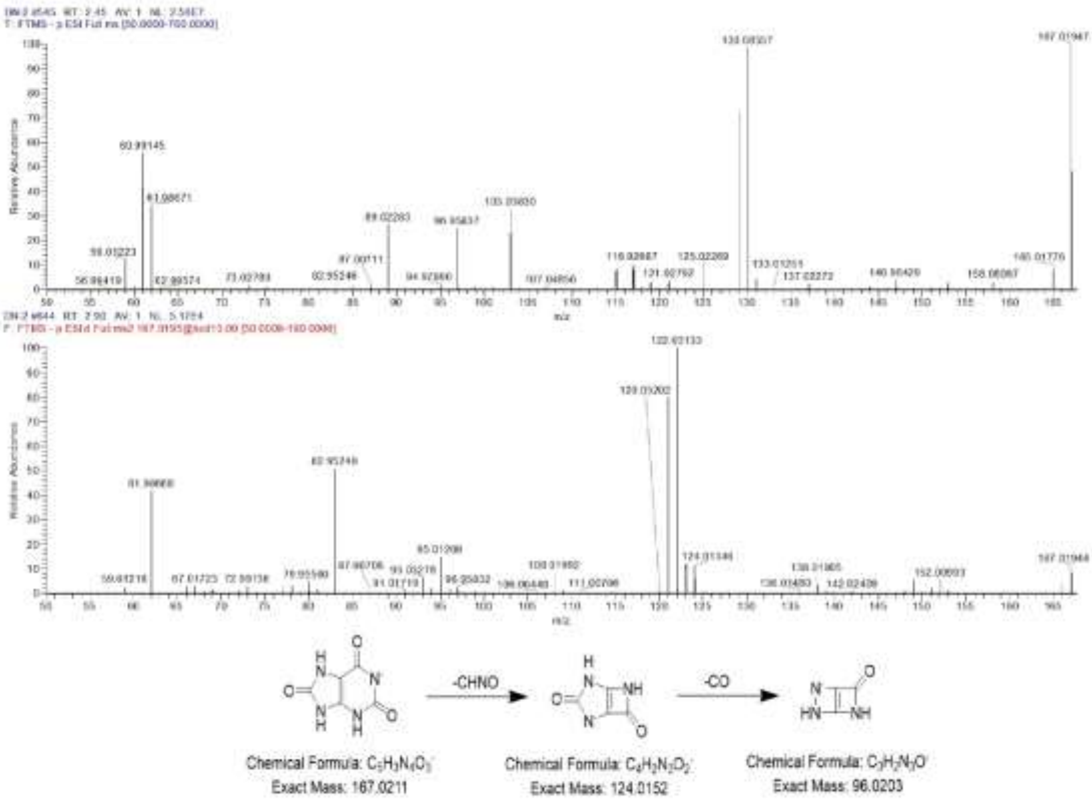
8. 6-gingerol



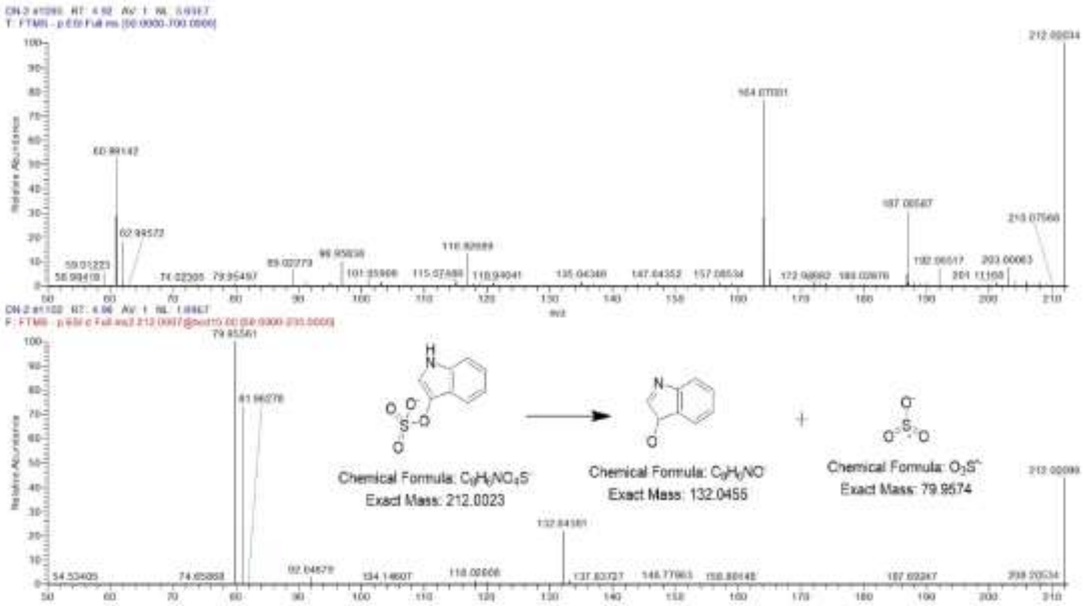
9. Palmitic acid



10. Uric acid



11. 3-indoxyl sulfate



12. Indole-3-propionic

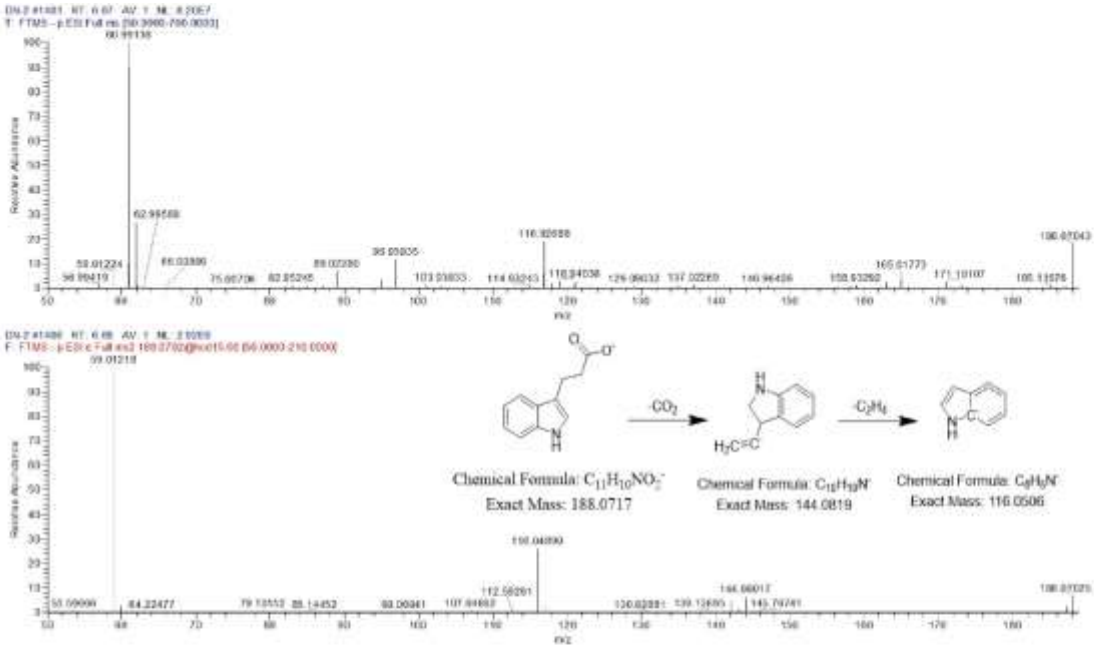


Fig S7 Mass spectrometry information and fragmentation patterns of the components absorbed into serum of MSC

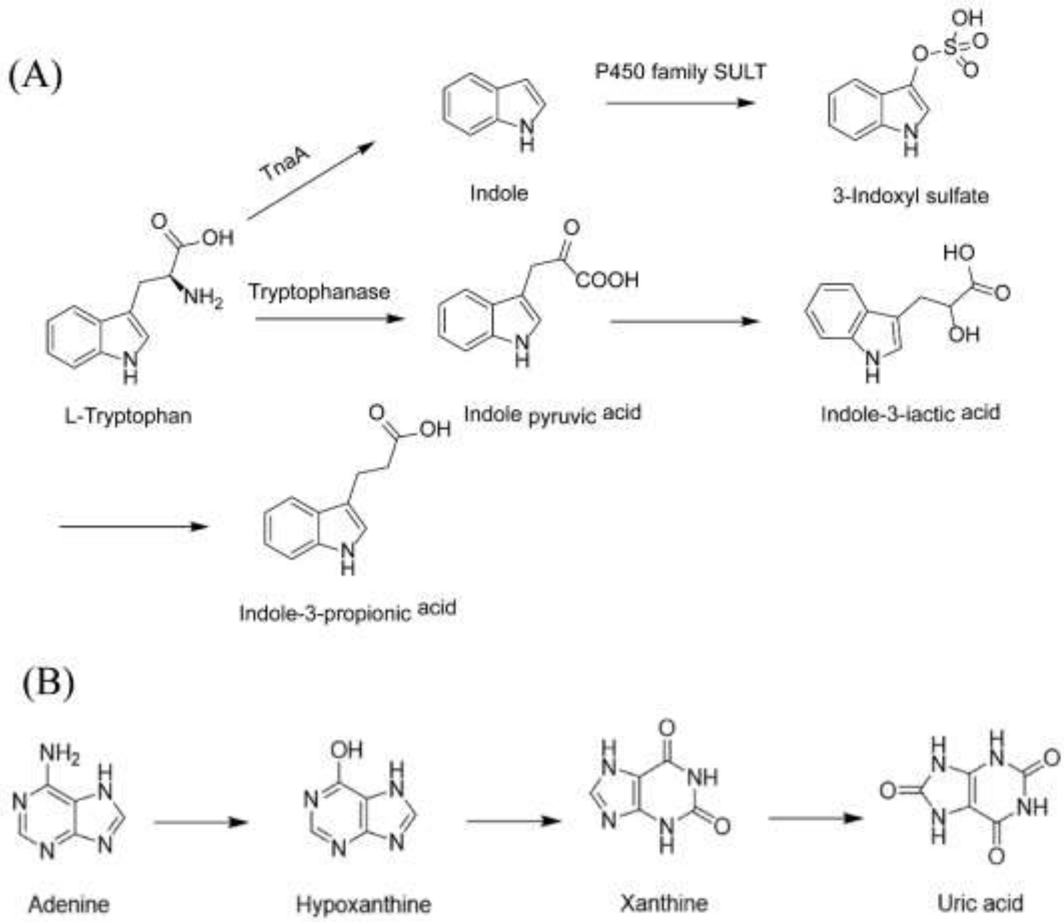


Fig S8 The metabolism pathway of (A) Tryptophan and (B) Adenine

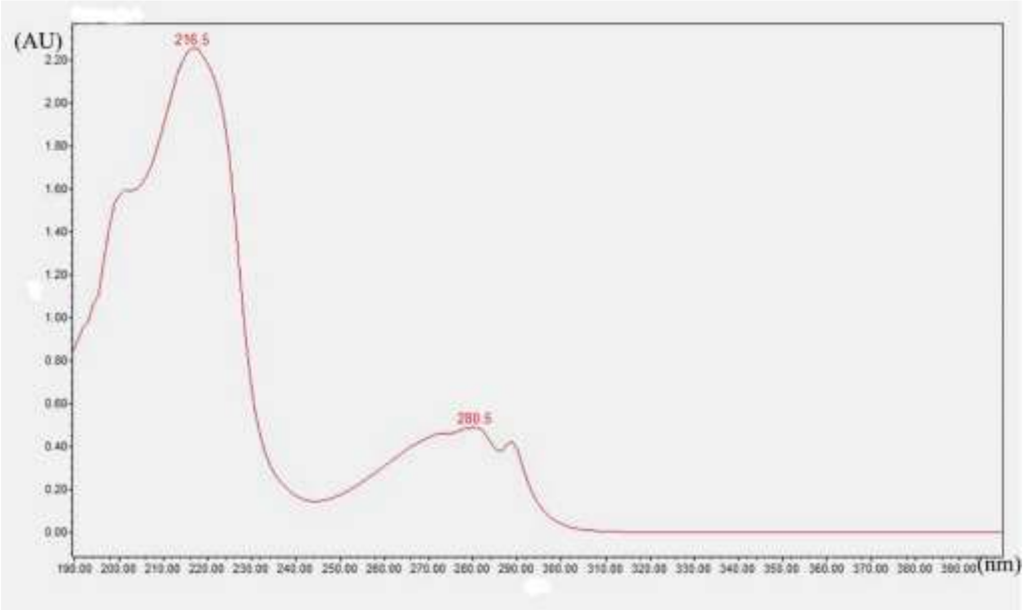


Fig S9 Full wavelength scanning of lenticin

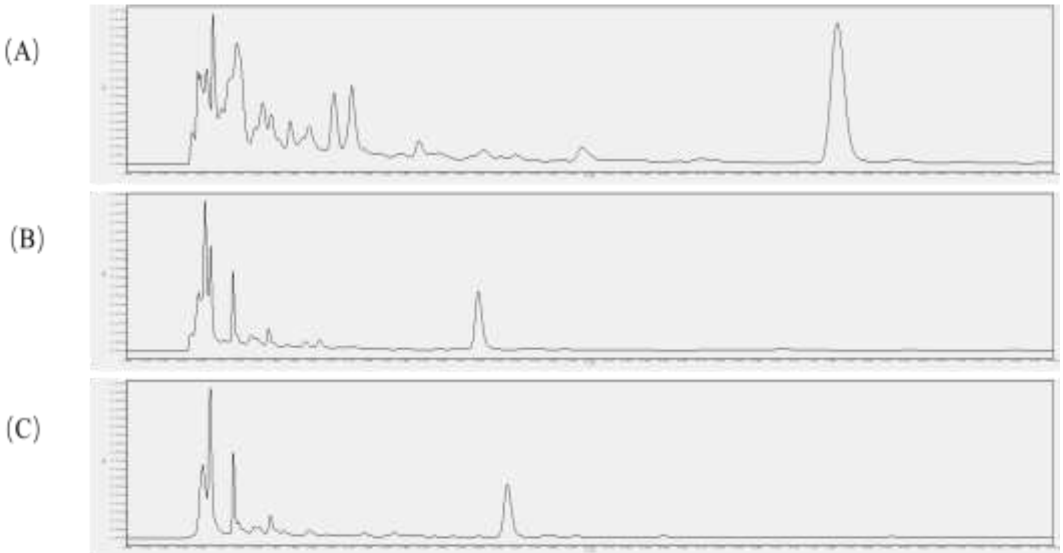


Fig S10 UPLC diagram of different mobile phase. (A) Methanol water; (B) Acetonitrile water; (C) Acetonitrile-0.1% formic acid

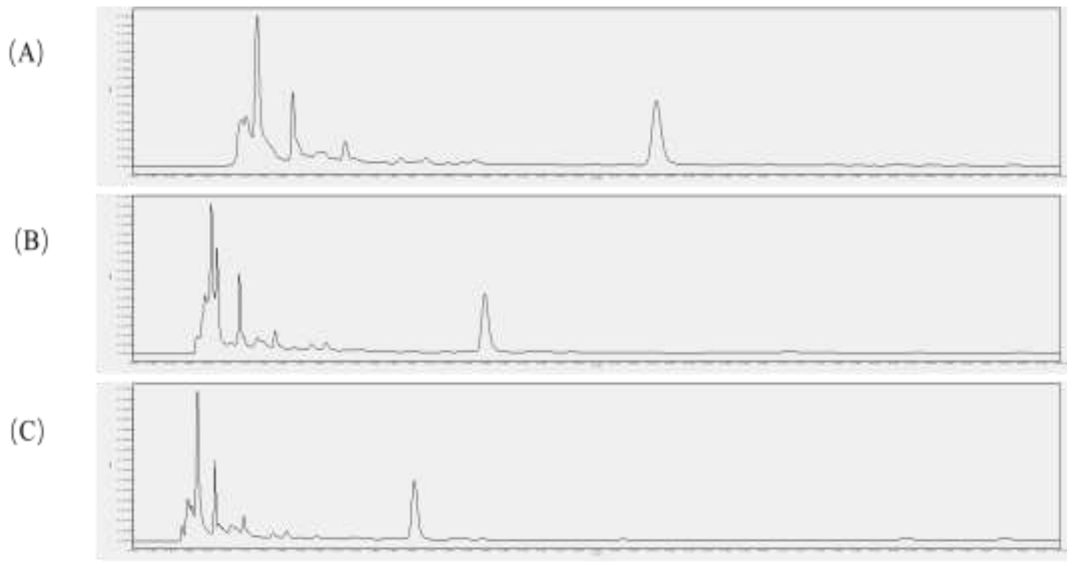


Fig S11 UPLC diagram of different flow rates. (A) Flow rate 0.2 ml/min; (B) Flow rate 0.3 ml/min; (C) Flow rate 0.4 ml/min

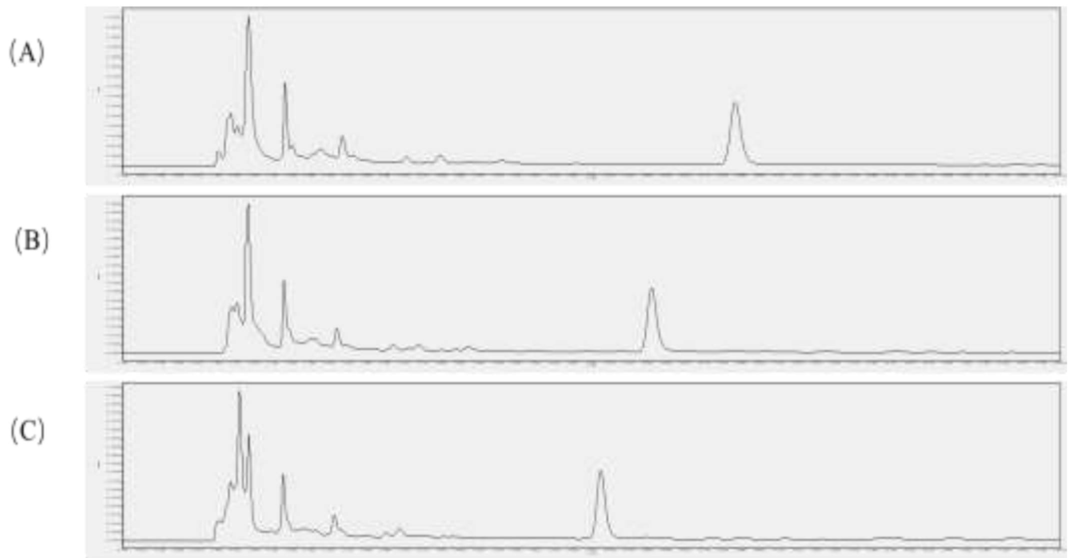


Fig S12 UPLC diagram of different column temperate. (A) Column temperature 20℃; (B) Column temperature 30℃; (C) Column temperature 40℃