HETEROCYCLES, Vol. 100, No. 4, 2020, pp. -. © 2020 The Japan Institute of Heterocyclic Chemistry
Received, 5th February, 2020, Accepted, 13th March, 2020, Published online, 24th March, 2020
DOI: 10.3987/COM-20-14228

IDENTIFICATION AND QUANTIFICATION OF ALKALOID COMPOUNDS FROM DIFFERENT PARTS AND PRODUCTION AREAS OF *DATURA METEL* L.

Yang Shi Hui,[1†] Liu Yan,[1†] Wang Qi,[2] Sun Yan Ping,[1] Guan Wei,[1] Liu Yuan,[1] Yang Bing You,[1*] and Kuang Hai Xue[1*]

[1] Key Laboratory of Chinese Materia, Heilongjiang University of Chinese Medicine, Ministry of Education, Harbin 150040, China. [2] Department of Medicinal Chemistry and Natural Medicine Chemistry, College of Pharmacy, Harbin Medical University, Harbin 150040, China. E-mail: ybywater@163.com (Yang Bing You), hxkuang@yahoo.com (Kuang Hai Xue).

Abstract – An ultra-high-performance liquid chromatography coupled with quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) method was used to identify alkaloids in six parts of *Datura metel* L. (*D. metel* L.). 65 Alkaloids were tentatively identified. Meanwhile, a rapid, sensitive and reliable ultra-high-performance liquid chromatography tandem triple quadrupole mass spectrometry (UPLC-Q-TRAP-MS/MS) technique had developed to determine the contents of 22 alkaloids in five minutes, with good precision and repeatability. This method provided a feasible solution for the content determination of alkaloid in *D. metel* L.

INTRODUCTION

The flowers of *Datura metel* L. (*D. metel* L.) were traditional Chinese medicine, known as *yangjinhua* for a long history.[1] Alkaloid was one of the pharmacologically active ingredients from *D. metel* flowers. It had many clinical applications, including relieving asthma and cough, curing the convulsion, antimycotics, easing the pain, and so on. The dried flowers of *D. metel* L. were the most commonly-used medicinal parts in clinic, and other parts, such as the seeds, roots, peels, stems and leaves had usually abandoned as the wastes. In fact, the non-flowering parts of *D. metel* L. also possessed diverse biological activities. The seeds and roots presented the great effect of anti-inflammatory and antispasmodic.[2-4] Besides medicinal value, the leaves and peels of *D. metel* L.

extracts were recommended as cheap and efficient insecticides with almost no pollution on the environment.[5-7]

Currently, our group also isolated many types of alkaloids from other parts of *D. metel* L. Combined the results of our isolation with the physiological activities of those different parts, we had a reasonable guess that the difference in these biological activities depended on the differences in the chemical composition and content of their various parts. Focused on some recent literatures, although some different levels and depths researches had been developed on the alkaloid of *D. metel* L., the results were unsatisfactory with the types and numbers of its qualitative and quantitative. In the latest report, only nine alkaloids were identified and two tropane alkaloids were merely determined the content from *D. metel* L.[8,9] In other words, neither the other alkaloids distribution nor the contents of six parts were completely investigated. Additionally, the geo-distribution factor extremely influenced the alkaloid contents and types in *D. metel* L. For the further study of different types of alkaloids in *D. metel* L., the selection of the best production areas was also an urgent problem.

Ultra-high-performance liquid chromatography tandem quadrupole time-of-flight mass spectrometry (UPLC-Q-TOF-MS/MS) technology, was usually used to identify a huge number of phytochemicals, providing the structure information of the compounds by the separation of complex samples rapidly. On the other hand, an efficient, sensitive and credible ultra-high performance liquid chromatography tandem triple quadrupole mass spectrometry (UPLC-Q-TRAP-MS/MS) method was developed to quantify 22 alkaloids in 66 samples of eleven production areas of *D. metel* L. Multiple reaction monitoring (MRM) mode in UPLC-Q-TRAP-MS/MS was a commonly used quantitative method. It only collected the mass spectrum signals of selected specific ion pairs, and effectively eliminated interference from complex matrices. It also had extremely low detection and quantitation limits. Finally, the linearity, accuracy and precision of method also were verified. This research was a groundbreaking progress in the determination of alkaloids in *D. metel* L., no matter in qualitative or quantitative. The effects of production areas on alkaloid levels of different parts were also firstly studied in this manuscript. It provided a reference for the content analysis of alkaloids and the producing areas selection in *D. metel* L.

RESULTS AND DISCUSSION

IDENTIFICATION AND DIFFERENCE ANALYSIS OF ALKALOIDS IN DIFFERENT PARTS OF *D. METEL* L.

The chemical profiles of six parts (flower, seed, root, peel, leaf and stem) were comprehensively analyzed by the developed UPLC-Q-TOF-MS/MS method in positive ion modes with a good decomposition. The

representative LC-MS base peak chromatograms (BPCs) for different parts of *D. metel* L. were shown in Figure 1. The BPCs indicated the significant difference and distinctions in composition and content of six parts. The results presented the consistent with the separation results of our groups separating from six parts. A total of 65 alkaloid chromatographic peaks were characterized with accurate molecular mass and fragment ions, and matched this data with our in-house databases, literatures and standards. Among the 65 alkaloids, there were mainly three types: indole alkaloids, amide alkaloids and tropane alkaloids. The fragmentation pathways of the three type alkaloids represented respective characteristics. For Indole alkaloids, peak 7 showed a molecular formula of $C_{13}H_{14}N_2O_2$ (*m/z* 231.1128 $[M+H]^+$). Its fragment ions were at 158 $[M+H^+-C_2H_3NO_2]^+$ and 132 $[M+H^+-C_2H_3NO_2-C_2H_2]^+$, identified as (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid according to the literature and standard.[10] For amide alkaloids, peak 31 provided $[M+Na]^+$ (*m/z* 336.1212) and $[M+H]^+$ (*m/z* 314.1393), demonstrated its molecular formula as $C_{18}H_{19}NO_4$. In the MS^2 spectrum of peak 31, *m/z* 179 $[M+H^+-C_8H_9NO]^+$, 164 $[M+H^+-C_8H_9NO-CH_3]^+$ were clearly observed in the positive ion mode. Based on this information, peak 31 was identified as *N-trans*-feruloyltyramine.[11] Unquestionably, it was further confirmed by comparison with a reference compound. As the tropane alkaloids, the fragmentation spectra of peak 18 indicated its hydrogen adduct at *m/z* 290.1751 ($C_{17}H_{23}NO_3$) and a series of common fragment ions at *m/z* 260 (loss of CH_2O, 30 Da), 142 (loss of C_8H_6O, 118 Da), 124 (loss of H_2O, 18 Da). Atropine was identified by comparing with the reported literature.[12] (See Supporting Information Figure S1 and Table 1).

In the BPCs of six parts, the numbers of alkaloid were determined as 48, 47, 45, 41, 39 and 37 corresponding to the seed, root, peel, flower, leaf and stem. Among them, 25 alkaloids (compound **3**, **5**, **8**, **9**, **12**, **14**, **17**, **18**, **20**, **23**, **24**, **25**, **26**, **27**, **29**, **30**, **31**, **36**, **38**, **47**, **49**, **56**, **61**, **62** and **65**) appeared on each part of *D. metel* L. The data matrices had imported into SIMCA software. The PCA analysis results illustrated the six parts of *D. metel* L. could be divided obviously by that constituent and content difference. The three parts (seeds, stems and flowers) exhibited the distinct difference compared with the parts of roots, peels and leaves (Figure 2a).

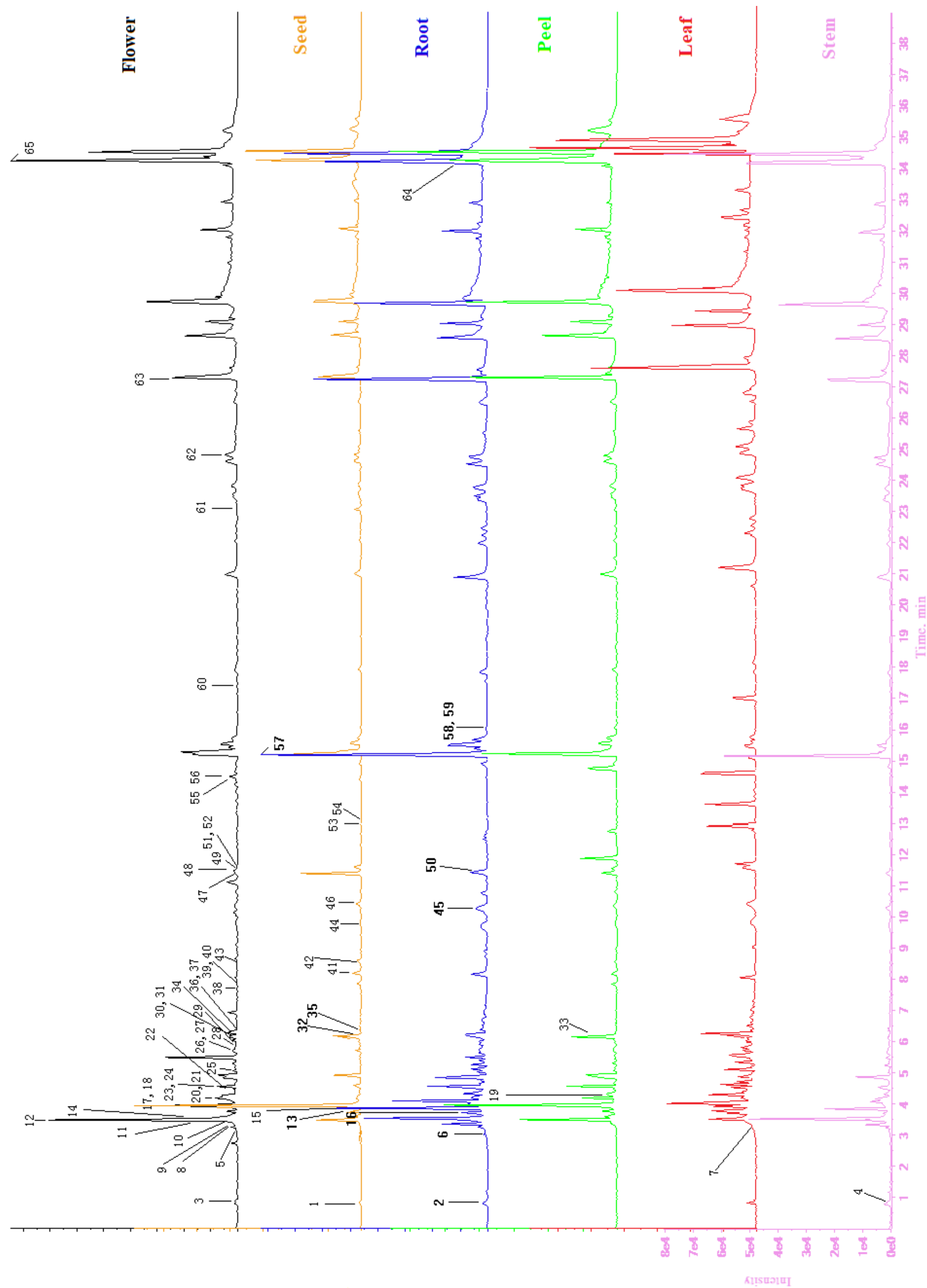

Figure 1. The base peak chromatogram (BPC) graphs of extract of *Datura metel* L (*D. metel* L.) in ESC^{+} mode

Table 1. UPLC-QTOF-MS/MS identified alkaloids in *Datura metel* L (*D. metel* L.)

Peak No.	Expected t_R (min)	Found at mass (Da)	Formula	Mass (Da)	Adduct	Error (ppm)	Compound [ref]	Fragment ions
1	0.79	160.0360	$C_4H_5N_3O_4$	159.0280	H	1.1	(2,5-dioxo-4-imidazolidinyl)-carbamic acid [13]	182 $[M+Na]^+$, 160 $[M+H]^+$, 138 $[M+Na-CO_2]^+$, 92 $[M+Na-CO_2-HCO_2H]^+$
2	0.81	278.1155	$C_{16}H_{17}NO_2$	255.1259	Na	-0.9	*N*-benzoyl-L-phenylalaninol [14]	278 $[M+Na]^+$, 260 $[M+Na-H_2O]^+$
3	0.85	225.0634	$C_{11}H_{10}N_2O_2$	202.0742	Na	-2.6	1H-pyrido[3,4-*b*] indol-1-one,2,3,4,9-tetrahydro-7-hydroxy	225 $[M+Na]^+$, 161 $[M+Na-H_2O-C_2H_6O]^+$
4	0.86	243.0990	$C_{10}H_{14}N_2O_5$	242.0903	H	3.8	deoxythymidine	243 $[M+H]^+$, 207 $[M+H-2H_2O]^+$
5	3.07	188.0696	$C_9H_{11}NO_2$	165.0790	Na	4.7	(S)-methyl-2-amino-2-phenylacetate	188 $[M+Na]^+$, 146 $[M+Na-C_2H_2O]^+$
6	3.09	188.0689	$C_9H_{11}NO_2$	165.0790	Na	0.9	*N*-(2-hydroxyl) benzamide	188 $[M+Na]^+$, 80 $[M+Na-C_5H_{16}O_2]^+$
7	3.18	231.1128	$C_{13}H_{14}N_2O_2$	230.1055	H	-2.3	(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid	231 $[M+H]^+$, 158 $[M+H-C_2H_3NO_2]^+$
8	3.22	322.1641	$C_{17}H_{23}NO_5$	321.1576	H	-4.2	dihydroanisodine [15]	322 $[M+H]^+$, 158 $[M+H-C_9H_{10}NO_2]^+$, 140 $[M+H-C_9H_{10}NO_2-H_2O]^+$
9	3.35	311.1325	$C_{18}H_{18}N_2O_3$	310.1390	H	-2	cyclo (PheTyr)	311 $[M+H]^+$, 257 $[M+H-3H_2O]^+$
10	3.36	320.1488	$C_{17}H_{21}NO_5$	319.1420	H	-3.2	anisodine [16]	320 $[M+H]^+$, 256 $[M+H-H_2O-HCO_2H]^+$, 156 $[M+H-H_2O-HCO_2H-C_4H_6NO_2]^+$
11	3.42	306.1695	$C_{17}H_{23}NO_4$	305.1627	H	-3.4	desoxyanisodine [16]	306 $[M+H]^+$, 156 $[M+H- C_9H_{10}O_2]^+$, 124 $[M+H- C_9H_{10}O_4]^+$
12	3.45	304.1541	$C_{17}H_{21}NO_4$	303.1471	H	-2.4	hyoscine [16]	304 $[M+H]^+$, 156 $[M+H-C_6H_{12}O_4]^+$, 138 $[M+H- C_6H_{12}O_4-H_2O]^+$
13	3.46	257.0920	$C_{14}H_{12}N_2O_3$	256.0848	H	-2.4	9-hydroxycanthin-6-one [13]	257 $[M+H]^+$, 161 $[M+H-C_6H_8O]^+$
14	3.54	306.1694	$C_{17}H_{23}NO_4$	305.1627	H	-3.7	anisodamine	306 $[M+H]^+$, 140 $[M+H-C_7H_{20}NO_3]^+$, 122 $[M+H-C_7H_{20}NO_3-H_2O]^+$
15	3.68	325.1407	$C_{15}H_{20}N_2O_6$	324.1321	H	2.2	1-ribityl-2,3-diketo-1,2,3,4-tetrahydro-6,7-dimethylquinoxaline [13]	325 $[M+H]^+$, 205 $[M+H-2MeCO_2H]^+$
16	3.79	359.1388	$C_{20}H_{20}N_2O_3$	336.1474	Na	4.6	*N*-*trans*-ferulyltryptamine [17]	359 $[M+Na]^+$, 194 $[M+Na-C_8H_{17}NO_2]^+$
17	3.91	290.1751	$C_{17}H_{23}NO_3$	289.1678	H	-1.8	littorine [18]	290 $[M+H]^+$, 142 $[M+H-C_8H_{10}NO_4]^+$
18	3.92	290.1751	$C_{17}H_{23}NO_3$	289.1678	H	-1.8	atropine	290 $[M+H]^+$, 260 $[M+H-CH_2O]^+$, 142 $[M+H-C_8H_6O]^+$
19	4.23	476.1921	$C_{24}H_{29}NO_9$	475.1842	H	1.7	*trans*-feruloyltyramine-4-O-β-D-glucopyranoside	476 $[M+H]^+$, 338 $[M+H-H_2O-2MeCO_2H]^+$
20	4.25	322.1053	$C_{17}H_{17}NO_4$	299.1158	Na	-0.8	*N*-*trans*-*p*-coumaroyloctopamine	322 $[M+Na]^+$, 148 $[M+Na-C_7H_{12}NO_4]^+$

21	4.26	322.1053	$C_{17}H_{17}NO_4$	299.1158	Na	-0.8	*N-cis-p*-coumaroyloctopamine [14]	322 $[M+Na]^+$, 224 $[M+Na-3H_2O-C_2H_4O]^+$
22	4.48	780.4572	$C_{41}H_{65}NO_{13}$	779.4456	H	4.8	meteloside H	780 $[M+H]^+$, 636 $[M+H-8H_2O]^+$
23	4.66	352.1172	$C_{18}H_{19}NO_5$	329.1263	Na	3	*N-trans*-feruloyloctopamine	352 $[M+Na]^+$, 290 $[M+Na-H_2O-CO_2]^+$
24	4.66	352.1157	$C_{18}H_{19}NO_5$	329.1263	Na	-1.1	*N-cis*-feruloyloctopamine [14]	352 $[M+Na]^+$, 290 $[M+Na-H_2O-CO_2]^+$
25	5.13	330.1346	$C_{18}H_{19}NO_5$	329.1263	H	1.4	*N-trans*-feruloyl-3',4'-dihydroxyphenylethylamine	352 $[M+Na]^+$, 330 $[M+H]^+$, 210 $[M+H-2MeCO_2H]^+$
26	5.77	322.0843	$C_{17}H_{17}NO_3$	283.1208	K	-0.8	*N-cis*-coumaroyltyramine	322 $[M+K]^+$, 230 $[M+K-2C_2H_6O]^+$
27	5.78	284.1286	$C_{17}H_{17}NO_3$	283.1208	H	-0.2	*N-trans*-coumaroyltyramine [8]	284 $[M+H]^+$, 147 $[M+H-C_7H_{21}O_2]^+$
28	5.9	510.2122	$C_{28}H_{31}NO_8$	509.2050	H	-1.1	canabisine H [14]	510 $[M+H]^+$, 492 $[M+H-H_2O]^+$, 312 $[M+H-H_2O-3MeCO_2H]^+$
29	6.06	272.1641	$C_{17}H_{21}NO_2$	271.1572	H	-3.4	apoatropine [19]	272 $[M+H]^+$, 124 $[M+H-C_8H_{20}O_2]^+$
30	6.11	314.1393	$C_{18}H_{19}NO_4$	313.1314	H	0.1	*N-cis*-feruloyltyramine	314 $[M+H]^+$, 178 $[M+H-C_5H_{12}O_4]^+$
31	6.12	314.1393	$C_{18}H_{19}NO_4$	313.1314	H	0.1	*N-trans*-feruloyltyramine	314 $[M+H]^+$, 179 $[M+H-C_8H_9NO]^+$ 164 $[M+H-C_8H_9NO-Me]^+$
32	6.13	314.1393	$C_{18}H_{19}NO_4$	313.1314	H	0.1	isocephalotaxinone [20]	314 $[M+H]^+$, 276 $[M+H-C_3H_2]^+$
33	6.15	490.1849	$C_{28}H_{27}NO_7$	489.1788	H	-3.4	hibiscuwanin B	490 $[M+H]^+$, 316 $[M+H-3H_2O-2MeCO_2H]^+$
34	6.2	634.3958	$C_{35}H_{55}NO_9$	633.3877	H	0.4	meteloside F [21]	634 $[M+H]^+$, 536 $[M+H-3H_2O-CO_2]^+$
35	6.22	338.1971	$C_{18}H_{27}NO_5$	337.1889	H	1.1	3α,6β-ditigloyloxytropan-7β-ol	338 $[M+H]^+$, 238 $[M+H-3H_2O-HCO_2H]^+$
36	6.4	344.1498	$C_{19}H_{21}NO_5$	343.1420	H	-0.1	3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-methoxy ethyl]acrylamide	344 $[M+H]^+$, 281 $[M+H-C_2H_7O_2]^+$
37	6.42	344.1498	$C_{19}H_{21}NO_5$	343.1420	H	-0.1	*N-cis*-Feruloyl-3'-methoxytyramine [22]	344 $[M+H]^+$, 192 $[M+H-C_6H_6NO_4]^+$
38	7.83	643.2648	$C_{36}H_{38}N_2O_9$	642.2577	H	-1.2	cannabisin E	643 $[M+H]^+$, 431 $[M+H-2HCO_2H-2MeCO_2H]^+$
39	7.85	308.2227	$C_{18}H_{29}NO_3$	307.2147	H	0.6	fawcettiine [20]	308 $[M+H]^+$, 262 $[M+H-HCO_2H]^+$
40	7.87	1058.5544	$C_{52}H_{83}NO_{21}$	1057.5458	H	0.7	meteloside B [21]	1058 $[M+H]^+$, 988 $[M+H-5H_2O]^+$
41	8.12	643.2654	$C_{36}H_{38}N_2O_9$	642.2577	H	-0.3	lyciumamide K [23]	643 $[M+H]^+$, 463 $[M+H-3MeCO_2H]^+$
42	8.47	926.5118	$C_{47}H_{75}NO_{17}$	925.5035	H	0.5	meteloside A [21]	926 $[M+H]^+$, 812 $[M+H-3H_2O-MeCO_2H]^+$
43	8.5	492.2011	$C_{28}H_{29}NO_7$	491.1944	H	-2.3	grossamide K [24]	492 $[M+H]^+$, 462 $[M+H-CH_2O]^+$
44	9.75	268.1330	$C_{17}H_{17}NO_2$	267.1259	H	-2.7	*N-trans*-cinnamoyltyramine	268 $[M+H]^+$, 136 $[M+H-C_7H_{16}O_3]^+$
45	10.2	492.2005	$C_{28}H_{29}NO_7$	491.1944	H	-3.5	lyciumamide C	530 $[M+K]^+$, 492 $[M+H]^+$, 432 $[M+H-MeCO_2H]^+$
46	10.45	641.2555	$C_{36}H_{36}N_2O_8$	624.2472	H	0.9	cannabisin F [11]	625 $[M+H]^+$, 611 $[M+H-CH_3]^+$, 474 $[M+H- C_8H_{11}NO]^+$

47	11.34	625.2546	$C_{36}H_{36}N_2O_8$	624.2472	H	-0.7	lyciumamide B	625 $[M+H]^+$, 429 $[M+H-2HCO_2H-2MeCO_2H]^+$
48	11.34	625.2555	$C_{36}H_{36}N_2O_8$	624.2471	H	0.9	(2a*S*,3a*S*) lyciumamide D	625 $[M+H]^+$, 429 $[M+H-5H_2O-C_2H_6O-MeCO_2H]^+$
49	11.35	625.2553	$C_{36}H_{36}N_2O_8$	624.2472	H	0.4	grossamide	625 $[M+H]^+$, 325 $[M+H-C_{18}H_{22}NO_3]^+$
50	11.36	664.2171	$C_{36}H_{37}N_2O_8$	625.2550	K	-2.5	tribulusamide A [20]	664 $[M+K]^+$, 610 $[M+K-3H_2O]^+$
51	11.38	625.2546	$C_{36}H_{36}N_2O_8$	624.2472	H	-0.7	cannabisin G [14]	625 $[M+H]^+$, 463 $[M+H-4H_2O-CO_2-HCO_2H]^+$
52	11.41	625.2523	$C_{36}H_{36}N_2O_8$	624.2472	H	-4.3	cannabisin D	625 $[M+H]^+$, 463 $[M+H-4H_2O-CO_2-HCO_2H]^+$
53	12.91	964.4842	$C_{47}H_{75}NO_{18}$	941.4984	Na	-4.1	meteloside G [21]	964 $[M+Na]^+$,826 $[M+Na-H_2O-2MeCO_2H]^+$
54	13.05	687.7992	$C_{36}H_{34}N_2O_8S_2$	686.7937	H	-0.2	benzhydryl[2R-[1(*Z*),2α,3α]]-α-(1-methoxyethylidene)-4-oxo-3-[(phe noxyacetyl)amino]-2-[[(p-tolyl)sulphonyl]thio]azetidine-1-acetate	687 $[M+H]^+$, 551 $[M+H-5H_2O-HCO_2H]^+$
55	14.32	936.3708	$C_{54}H_{53}N_3O_{12}$	935.3629	H	1.7	thoreliamide C	936 $[M+H]^+$, 788 $[M+H-2CO_2-MeCO_2H]^+$
56	14.43	641.2493	$C_{36}H_{36}N_2O_9$	640.2421	H	-1	melongenaminde B	641 $[M+H]^+$, 537 $[M+H-3H_2O-MeCO_2H]^+$
57	15.2	275.1078	$C_{14}H_{14}N_2O_3$	258.1004	H	-1.7	daturametelindole B	259 $[M+H]^+$, 162 $[M+H-C_5H_7NO]^+$
58	16.12	316.0958	$C_{15}H_{19}NO_4$	277.1314	K	2.2	baimantuoluoamide B	316 $[M+K]^+$, 272 $[M+K-CO_2]^+$
59	16.78	316.0958	$C_{15}H_{19}NO_4$	277.1314	K	2.1	baimantuoluoamide A	316 $[M+K]^+$, 272 $[M+K-CO_2]^+$
60	17.34	322.0843	$C_{17}H_{17}NO_3$	283.1208	K	-0.8	paprazine [25]	322 $[M+K]^+$, 284 $[M+H]^+$, 230 $[M+H-3H_2O]^+$
61	23.02	677.2120	$C_{36}H_{34}N_2O_{10}$	654.2214	Na	1.3	cannabisin L	677 $[M+Na]^+$, 521 $[M+Na-2H_2O-2MeCO_2H]^+$
62	24.77	368.0902	$C_{18}H_{19}NO_5$	329.1263	K	0.4	*N*-[2-(3,4-dihydroxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl) prop-2-enamide	368 $[M+K]^+$, 330 $[M+H]^+$, 206 $[M+H-H_2O-HCO_2H-MeCO_2H]^+$
63	27.27	331.1642	$C_{18}H_{22}N_2O_4$	330.1580	H	-4.7	daturametelindole C	331 $[M+H]^+$, 223 $[M+H-C_2H_8N_2O_3]^+$
64	34.21	483.1697	$C_{27}H_{28}N_2O_4$	444.2049	K	2.2	aurantiamide [26]	483 $[M+K]^+$, 403 $[M+K-C_2H_8O_3]^+$
65	34.2	645.1985	$C_{36}H_{34}N_2O_7$	606.2366	K	-2.7	chenoalbicin	645 $[M+K]^+$, 599 $[M+K-HCO_2H]^+$

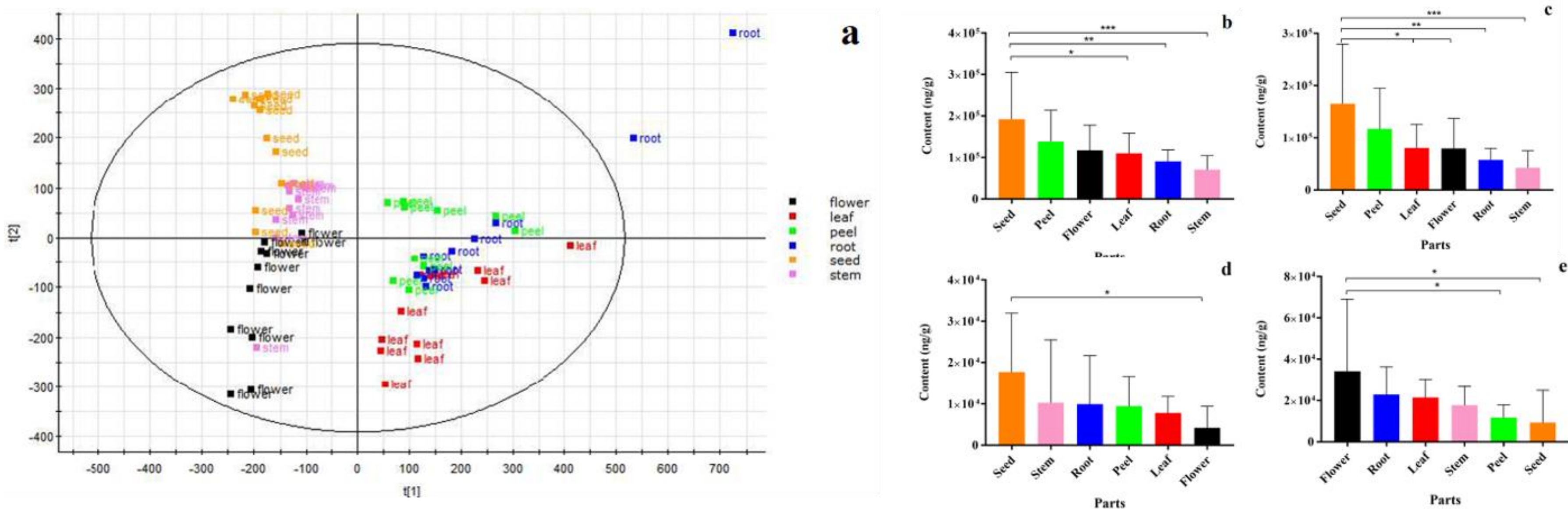

Figure 2. PCA based on the holistic chemical profiling of six parts from eleven production areas of *D. metel* L. (**a**) and the distribution of total 22 alkaloids (**b**), amide alkaloids (**c**), tropine alkaloids (**d**) and indole alkaloids (**e**) in six parts of *D. metel* L.

^{b} *Compare to seed*; ^{c} *Compare to seed*; ^{d} *Compare to seed*; ^{e} *Compare to flower*; * *(P<0.05)*, ** *(P<0.005)*, *** *(P<0.0005)*

QUANTITATIVE ANALYSIS OF 22 ALKALOIDS BY UPLC-Q-TRAP-MS

The content of 22 alkaloids from the eleven production areas of *D. metel* L. were investigated by the developed UPLC-Q-TRAP-MS/MS method of MRM mode. The contents of the analyte were calculated by the constructed calibration curves. The GraphPad software was used to analyze the quantitative data. For the total accumulation of 22 alkaloids, in terms of parts, the total alkaloid content of seed ($\overline{x}$=192348.0 ng/g) was higher than other parts (P<0.05) (Figure 2b). As the secondary plant metabolites, alkaloid was regarded as promising sources of plant-protecting substances. The increase in the content of secondary metabolites in plant organs was a common effect of pest invasion, and you could even say it could be described as a chemical weapon of the plant itself. It was not difficult to infer that the reason for the highest alkaloid content in seeds might be closely related to the protection mechanism for successfully resisting insect damage. Some reports also confirmed this speculation. They explored the insecticidal effects of different parts extracts from *D. metel* L. on aphids, nematodes and slime worms. The results showed that the insecticidal effect of the seeds was the most prominent (Figure 2c).[27] Meanwhile, many of the 22 alkaloids selected were amide alkaloids and tropine alkaloids. Compared with the other four parts, the content of these alkaloids in peels and seeds of plant performed a huge advantage than others (Figure 2d). According to relevant literature reports, we could make a bold guess: Seeds were affected by the content of amide alkaloids and tropine alkaloids, which increased the probability of developing resistance to all pests. However, for indole alkaloids, flower was a part with the most accumulation, followed by root and lowest was peel (Figure 2e). This situation was completely opposite to the others types alkaloid distribution. We had also collected some interest information about the plant defense ability of *D. metel* flowers and leaves. Unfortunately, there were fewer indole alkaloids analyzed in this experiment. So, whether indole alkaloids were the main components of contact toxicity to red ants in flowers and leaves need further study.[28]

Then, for the total content of 22 alkaloids from each production area, the highest was Taizhou (899277.9 ng/g), followed by Bozhou (887969.7 ng/g), and the lowest was Luoyang (449155.0 ng/g) (Figure S2a). However, the results also showed significant differences in the distribution of three types alkaloids across different production areas. For amide alkaloids, Taizhou ranked the first production area with content came up 741015.4 ng/g, Bozhou (703964.8 ng/g) and Nanning (587508.3 ng/g) attained second and third respectively. The bottom was Luoyang (331715.8 ng/g) (Figure S2b). The total amount of tropine alkaloids in Cangzhou (22908.7 ng/g) was the largest, followed by Bozhou (14350.8 ng/g) and Xianyang (13466.2 ng/g) (Figure S2c). Not to be ignored, for the indole alkaloids, Nanning (36038.2 ng/g) was far ahead of other areas (Figure S3d), but not the optimization area for two other alkaloids. This illustrated an interesting phenomenon that the production area had great influence on the type and content of alkaloids in *D. metel* L. It could also provide us with some important tips and references while conducting in-depth

research on different types alkaloids in *D. metel* L.

Among the 22 analytes, 19 compounds (**3**, **5**, **9**, **14**, **18**, **20**, **23**, **25**, **26**, **30**, **31**, **36**, **38**, **47**, **49**, **56**, **61**, **62** and **65**) were distributed in each part (Figure 1). Interestingly, the distributions of these compounds' contents in the six parts of *D. metel* L. were different evidently. Although seed was a part with the highest total 22 alkaloids amount in plant, not all compounds were most abundant in seeds. Compound **5** as an example, its famous accumulation was in flowers, and the lower were peels and seeds. This situation was completely opposite to the distribution situation of total 22 alkaloids. Only compounds **9**, **18**, **25**, **26**, **36**, **62** and **65** were richest in seeds. For other compounds, **14**, **23**, **38**, **49**, and **56** were the most abundant in the roots. Compounds **5**, **31** and **47** had the highest level in flowers, while compounds **3** and **20** were richest in the leaves. **30** and **61** were richest in stems and peels (Table S1). The uneven distribution of certain compounds in different parts might also be the result of their mutual transformation during plant growth. For example, during plant growth, the levels of compounds **30** and **52** in the peels and seeds increased rapidly with **55** content increased very slowly. Based on their structural connection, we speculated the reason for the abrupt increase in compounds **30** and **52** might be caused by the cracking of **55** during plant reproductive growth of *D. metel* L. (Figure 3).

On the other hand, we harvested the different plant parts based on the optimal harvesting period, in order to obtain more of the ingredients. The flowers were measured during their flourishing florescence, and the leaves, stems and roots collected in the vegetative growth stage. The peels and seeds were harvested at fruit maturity.[1,29] Additionally, due to the wide geographical differences in the areas of harvest, there were some differences in harvest time (Table S3). Whether seasonal effects were also an important interferes factor for alkaloid content in different parts of the plants would be the topic of our next research.

EXPERIMENTAL

PLANT MATERIALS AND SAMPLE PREPARATION

66 Batches of eleven production areas (**S01**-**S11**) of *D. metel* L. were gathered for this study (Figure S3 and Table S2). The herbs authenticated by Prof. Rui-feng Fan from the School of Pharmacy, Heilongjiang University of Chinese Medicine. Voucher specimens were retained in the Chinese Medicine Chemistry Laboratory of Heilongjiang University of Chinese Medicine.

All of these batches were lyophilized, ground, then sifted through a 50-mesh sieve. 2 g of each batch samples accurately weighed, heated circumfluence extraction with 100 mL of EtOH (EtOH : water = 70: 30) for 120 min 2 times. Combined the extract and then centrifuged at 10000 r/ min for 10 min. The extract was filtered through a 0.22 μm filter, and the supernatant was dried at a low temperature vacuum, accurately weighed. MeOH was added to dissolve the sample at 500 μg/mL and then stored at – 20 °C

after sealing.

CHEMICAL REAGENTS AND STANDARD SOLUTIONS

The 1,2,3,4-tetrahydro-7-hydroxy-1-oxo-β-carboline (**3**), (*S*)-methyl 2-amino-2-phenylacetate (**5**), (1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid (**7**), cyclo (PheTyr) (**9**), anisodamine (**14**), atropine (**18**), *N-trans-p*-coumaroyloctopamine (**20**), *N-trans*-feruloyloctopamine (**23**), *N-trans*-feruloyl-3',4'-dihydroxyphenylethylamine (**25**), *N-cis*-coumaroyltyramine (**26**), *N-cis*-feruloyl-tyramine (**30**), *N-trans*-feruloyltyramine (**31**), 3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-methoxyethyl]acrylamide(**36**), cannabisin E (**38**), lyciumamide B (**47**), grossamide (**49**), cannabisin D (**52**), thoreliamide C (**55**), melongenaminde B (**56**), cannabisin L (**61**), *N*-[2-(3,4-dihydroxy-phenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl)prop-2-enamide(**62**), chenoalbicin (**65**) were isolated and purified by the laboratory. Their structures were characterized using nuclear magnetic resonance (Figure 3, S4–S25 and Table S3). The purity of each chemical was above 98%, as determined by UPLC analysis. MeOH for UPLC-MS was obtained from Merck (Merck KGaA, Darmstadt, Germany). MeCN for UPLC-MS was supplied from Fisher Chemical (Thermo Fisher Scientific, MA, United States). Deionized water was purified by a Milli-Q water purification system (Merck KGaA, Darmstadt, Germany). Individual standard stock solutions were processed at 1000 μg/mL; each standard was dispersed in methanol. All stock standard solutions were sealed in a dark place at – 20 °C for 6 months. The standard solutions were obtained by diluting in methanol and stored at 4 °C for 1 month.

QUALITATIVE FOR UPLC-Q-TOF-MS/MS ANALYSIS

Qualitative analyst was acquired using ACQUITY UPLC^{TM} (Waters Corp., MA, USA) system connected to the Triple TOF^{TM} 5600^{+} mass spectrometer (ABsciex, MA, USA). The chromatography separation was carried out with a UPLC C_{18} analytical column (2.1 mm×100 mm, I. D. 1.7 μm, ACQUITY UPLC CSH, Waters Corp., MA, USA) at ambient temperature of 25 °C. The mobile phase for both identification and quantitation included of water (A) and MeCN (B), both of which contained 0.1% formic acid. Separation for identification was accomplished using the following optimized linear gradient elution program: 0-1 min, 5% B; 1-3 min, 5-23% B; 3-8 min, 23-30% B; 8-15 min, 30-44% B, 15-19 min, 44-48% B; 19-34 min, 48-95% B; 34-39 min, 95-5% B. The flow rate was set at 0.3 mL/min, and the injection volume was 3 μL. The MS/MS parameters were optimized as follows: Scan Type: Positive TOF-MS, GS1: 50, GS2: 50, CUR: 30, TEM: 550, InoSpray Voltage Floating: 4500, DP: 100, CE: 15; Positive Product Ion, GS1: 50, GS2: 50, CUR: 30, TEM: 550, InoSpray Voltage Floating: 4500, DP: 100, CE: 25. The Analyst 1.6.4 (ABsciex, USA) software was controlled the UPLC-MS/MS system and processed the data.

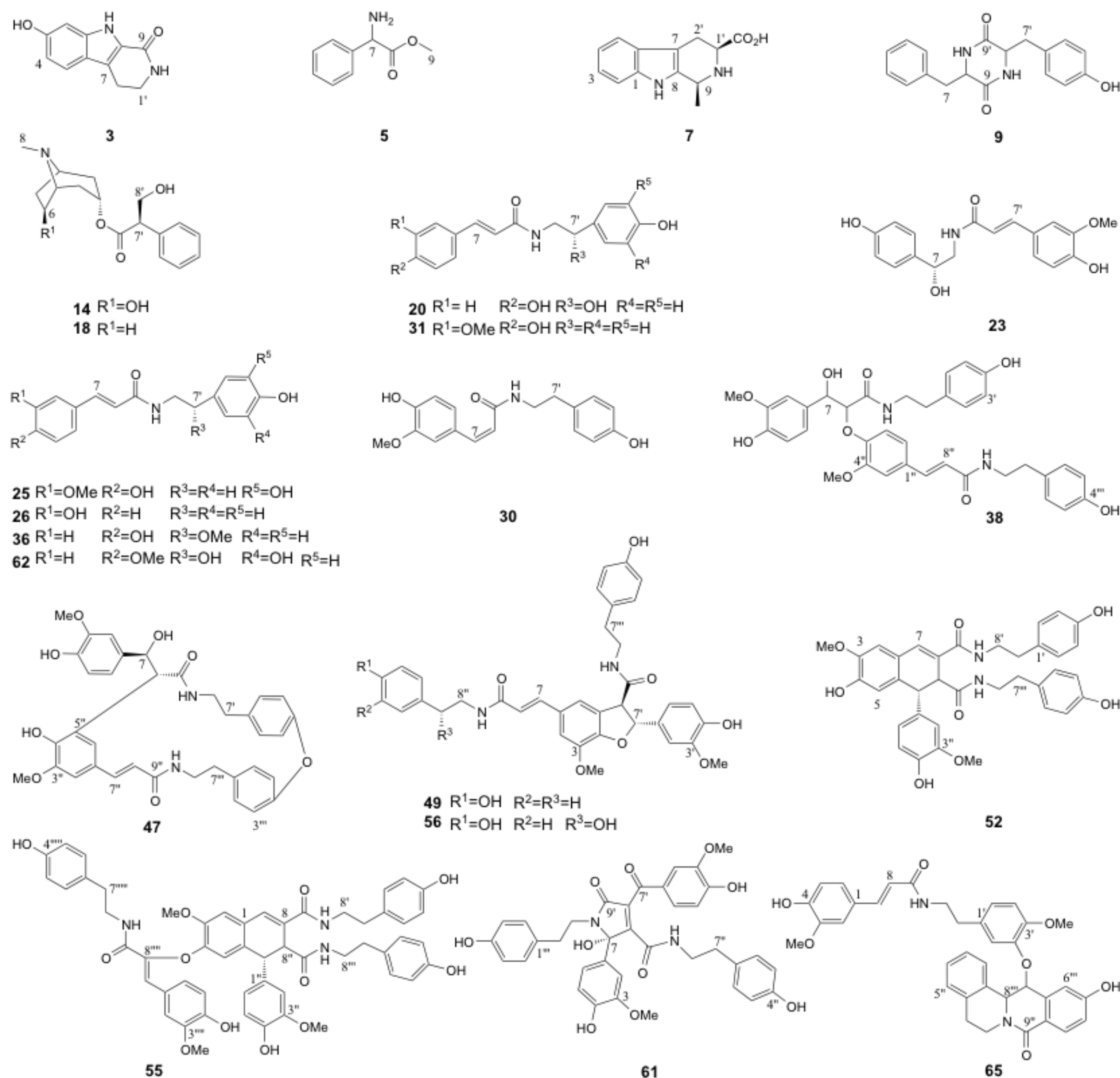

Figure 3. Chemical structures of 22 quantitative alkaloids

METHOD VALIDATION

Method validation for the quantitative UPLC-Q-TRAP-MS/MS analysis included linearity, precision (intraday, daytime), limits of detection (LODs), limits of quantitation (LOQs), recovery, repeatability and stability. The calibration curve of 22 standards were established by quantitatively diluting the standard solution with methanol in 6 different multiples. Each standard solution was analyzed for six replicates within the same day and additionally on three consecutive days for evaluating intra- and inter-day precision. The LODs and LOQs were experimentally taken as the concentration, which produced a detector signal clearly distinguishable from the baseline noise (3 and 10 times the baseline noise). Because the flowers of *D. metel* L. were the commonly-used medicinal parts in Chinese Pharmacopoeia, the inspection of stability, repeatability and recovery should focus on the flowers. To assess stability, **S01**-flower extract was analyzed at 0, 2, 4, 8, 12, 24, and 48 h at room temperature. Repeatability was

evaluated by analyzing six sample solutions (**S01**-flower) prepared in parallel. Recovery test was executed by spiking approximately 50%, 100% and 150% of contents of known chemical markers in the sample (**S01**-flower), with six independent spiked analyses. The recoveries were calculated by the formula:

$$Recovery\,(\%) = \frac{Amount\ Found - Original\ Amount}{Amount\ Spiked} \times 100\%$$

All of the correlation coefficient values ($r^2 > 0.992$) indicated a good linear relationship between the analyte concentrations and their peak areas within the relatively wide test ranges. The RSDs of intra- and interday variations of 22 analytes were within 0.48%-3.44% and 1.04%-4.39%, respectively. The RSDs for stability were lower than 3.08%. As for repeatability, the RSDs were less than 4.90%. The recoveries for 22 alkaloids also ranged in Table S4. All these results distinctly demonstrated that the developed quantitative UPLC-Q-TRAP-MS/MS method was linearity, precision, stability, sensitivity, repeatability and accurately enough for determination of 22 reference compounds in different parts of *D. metel* L.

QUANTITATION FOR UPLC-Q-TRAP-MS/MS ANALYSIS

Our group was engaged in the related research on chemical compositions of *D. metel* L. for a long time. From the large number of alkaloids isolated, we selected 22 alkaloids for the quantitative detection with considerable amount and certain biological activity as the indicators. For example, atropine was performed to treat iridocyclitis, ophthalmology diagnostics and mydriasis.[30] Anisodamine was mainly used to dilate vessels, remove vasospasm, improve blood circulation, increase tolerance of ischemia hypoxia and reduce the probability of the surrounding tissue necrosis.[31] In the field of medical defense, (1*S*,3*S*)-1-methyl-1,2,3,4- tetrahydro-β-carboline-3-carboxylic acid was proved to be a good antioxidant.[32] Grossamide significantly inhibited the secretion of pro-inflammatory mediators such as interleukin 6 (IL-6) and tumor necrosis factor α (TNF-α), and decreased the level of LPS-mediated IL-6 and TNF-α mRNA.[33] Cannabisin D-E, melongenaminde B, melongenaminde D were exhibited inhibition of NO production.[34]

The target analytes were examined in positive ionization mode by multiple reaction monitoring (MRM) method. All the compounds were then determined respectively in direct infusio mode, and $[M+H]^+$ was selected as precursor ion for all alkaloids and determination the cone energy and declustering potential (Table 2 and Figure S26). This method did not require complete separation of the chromatographic peaks of multiple components, as long as the target analysis were non-interfering with each other.

Figure 2 showed that isomers present existed in the 22 target alkaloids we selected. Separating these isomers by UPLC-Q-TRAP-MS/MS system was a difficult challenge, especially those with the same precursor or product ion pairs. In our work, different elution conditions further investigated to achieve

good resolution of 22 alkaloids. Ultimately, MeCN-0.1% formic acid and water-0.1% formic acid (63: 37 v/v) was the best elution system.

Table 2. The information for MRM parameters of 22 quantitative alkaloids in *D. metel* L.

No.	Compound	Ion transition 1	Ion transition 2	CE	DP	t_R (min)
3	1,2,3,4-tetrahydro-7-hydroxy-1-oxo-β-carboline	203/143	203/88	16	92	0.65
5	(S)-methyl 2-amino-2-phenylacetate	166/124	166/121	62	15	1.05
7	(1*S*,3*S*)-1-methyl-1,2,3,4-tetrahydro-β-carboline-3-carboxylic acid	231/158	231/124	21	76	1.93
9	cyclo (PheTyr)	311/136	311/120	27	78	4.03
14	anisodamine	306/140	306/93	32	69	1.63
18	atropine	290/124	290/93	12	40	0.61
20	*N-trans-p*-coumaroyloctopamine	300/282	300/147	10	40	1.84
23	*N-trans*-feruloyloctopamine	330/177	330/145	23	45	2.54
25	*N-trans*-feruloyl-3',4'-dihydroxyphenyl-ethylamine	330/312	330/289	21	98	2.62
26	*N-cis*-coumaroyltyramine	284/147	284/121	20	88	2.07
30	*N-cis*-feruloyltyramine	314/177	314/145	22	83	3.41
31	*N-trans*-feruloyltyramine	314/177	314/145	24	93	2.56
36	3-(4-hydroxy-3-methoxyphenyl)-*N*-[2-(4-hydroxyphenyl)-2-methoxyethyl]acrylamide	344/312	344/177	13	37	4.73
38	cannabisin E	643/462	643/281	22	82	2.15
47	lyciumamide B	625/488	625/535	25	120	0.83
49	grossamide	625/462	625/177	19	98	4.29
52	cannabisin D	625/460	625/488	25	100	1.32
55	thoreliamide C	936/774	936/488	34	150	1.71
56	melongenaminde B	641/351	641/281	17	44	3.78
61	cannabisin L	655/637	655/354	11	49	3.19
62	*N*-[2-(3,4-dihydroxyphenyl)-2-hydroxyethyl]-3-(4-methoxyphenyl)prop-2-enamide	330/312	330/177	11	37	2.84
65	chenoalbicin	607/353	607/277	20	56	3.10

Quantitation analyst was obtained using ACQUITY UPLC^{TM} (Waters Corp., MA, USA) system connected to the 4000 Q-TRAP mass spectrometer (ABsciex, MA, USA). An UPLC C_{18} analytical column (2.1 mm×100 mm, I. D. 1.7 μm, ACQUITY UPLC CSH, Waters Corp., MA, USA) was applied for separation at ambient temperature of 25 °C. Isocratic elution was performed as follow: 0-5 min, 63% B, and an aliquot of 2 μL sample solution were injected for quantitative analysis. Detection was performed in positive electrospray ionization mode (ESI^+) in the multiple-reaction monitoring mode (MRM). The data processing software Analyst 1.6.4 software controlled the UPLC-MS/MS system and the target compound content was measured by substituting the peak area into a standard curve.

ANALYSIS OF UPLC-MS/MS DATA

The raw mass spectrometry and chromatographic data were imported into the PeakView 2.1 (ABsciex, MA, USA) software for peak picking, peak alignment, peak identification and another pretreatment. The following settings: the range of t_R was 0.5-35 min; *m/z* 100-1200; the range of the deviation of t_R and quality error were 0.1 min, 5×10^{-6}. Then, import the data into SIMCA 14.1 (Umetrics, Umea, Sweden) software for PCA analysis. The differences in various parts of *D. metel* L. were comprehensively and deeply analyzed from the whole to the partial. The GraphPad Prism 7 (GraphPad, CA, USA) software was used to perform significant analysis of differential indicator components and drawing.

ACKNOWLEDGEMENTS

This work was financially supported by the National Natural Science Foundation of China (NSFC) (81773883; 81903781); China Postdoctoral Science Foundation (2018M631978); Excellent Youth Project of Heilongjiang Natural Science Foundation (YQ2019H029); Heilongjiang Postdoctoral Science Foundation (LBH-Z18245); and Heilongjiang University of Chinese Medicine Founds (2018pt01, 2018bs03).

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