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Research Article

RAPID SEPARATION AND IDENTIFICATION OF MULTIPLE CONSTITUENTS IN VINE TEA BY UFLC SYSTEM COUPLED WITH Q-TOF-MS/MS

Wang Xi-long[1], Zhou Ye[1], Wang Ping[2], Xiao Wei-zhen[1], Wang Jian-zhuang[1], Lv Hua-chong[1], Liu Hui-qiong[1]*
[1]College of Pharmacy, Guangdong Pharmaceutical University, Guangzhou, P.R. China
[2]Guangzhou Accurate and Correct Test Co., Ltd. China
*Corresponding Author Email: moonlhq@163.com

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ABSTRACT

Vine Tea (VT), as a well-known Chinese traditional liana medicine and functional tea, is a special plant which is rich in flavonoids; even though some ingredients were isolated via the conventional approach. Nevertheless, there have been no reports about chemical profile through LC-Q-TOF-MS/MS. In this study, an method has been established to rapid separation and structural identification the chemical constituents of Vine Tea for the first time by ultra-fast liquid chromatography coupled with quadrupole/time-of-flight mass spectra (UFLC-Q-TOF-MS/MS). Based on the accurate mass, retention time and MS spectra, 29 compounds in crude extract, mainly belonging to flavonoids and organic acids, were explicitly identified or tentatively characterized by comparing with reference standards or literatures. The possible fragmentation pathways were proposed and fragmentation rules of the major types of compounds were concluded. The results indicated that UFLC-Q-TOF-MS/MS based chemical profiling is a highly efficient approach for the original discrimination of Vine Tea as well as other traditional Chinese medicines.

Keywords: Vine Tea; Chemical constituents; UFLC-Q-TOF-MS/MS

INTRODUCTION

Vine Tea, cauline leaf of *Ampelopsis grossedentata* (Hand.-Mazz) W. T. Wang, is unique tea, medicinal plant which is particularly rich in flavonoids[1]. As a health protection tea, Pharmacological experiments show that total flavonoids of Vine Tea exhibits activities of easing pain[2], decreasing blood sugar[3], anticancer[4], antioxdation[5]. It is well accepted that the efficacy of liana medicines is contributed by the synergistic effects of their multi-components and multi-targets. Nevertheless, up to now, a few compounds were isolated from the crude extract using conventional means[6-8]; little attention was paid to the chemical profile. Therefore, getting a useful chemical profile consisting of a great number of constituents from Vine Tea is of significant importance for the understanding of its biological and medical significance and such a profile may also prove useful to assess the quality[9]. As an emerging technology, UFLC-Q-TOF-MS/MS achieved multidimensional separation, rapid and high sensitive detection as well as on-line structure elucidation in analyzing complex constituents from plant extract. In this paper; we developed a UFLC-Q-TOF/MS method for analysis of the chemical profile of Vine Tea. Separation was performed on a C_{18} reversed-phase column (2.1 mm × 100 mm, 1.8 μm) with 0.1 % formic acid aqueous solution and acetonitrile as the mobile phase under gradient elution. Quadrupole/time-of-flight (Q-TOF) MS can provide excellent mass accuracy over a wide dynamicrange, allowing measurements of the isotopic pattern and may be used in MS/MS experiments to provide the elemental composition of the parent and fragment ions. With this method, a total of 29 compounds in the formula were identified.

MATERIALS AND METHODS

Reagents and standards

Acetonitrile (HPLC grade) was purchased from Fisher Scientific (Pittsburgh, PA, USA) and HPLC-grade Formic acid (analytical grade) was purchased from Sigma (St. Louis, USA). All water used was purified by a Simplicity 185 personal water purification system (Millipore, Bedford, MA, USA). Dihydromycicetin, Myricetin, Apigenin, Kaempferol, Luteolin, Morin hydrate, Rutin, Quercetin, Catechin were obtained from Guangzhou Qiyun Biological Technology Co., Ltd. (Guangzhou, China). The purities of all standards were above 98 % detected by HPLC.

Plant materials and sample preparation

The raw material was purchased from Guangxi province, China. Vine Tea (30 g) were extracted with 60 % Methanol (1 L) for 2 h, repeated twice. The extract solution was filtered and evaporated with a rotary evaporator and then made a final concentration of 0.5 g/ml (equivalent to dry weight of raw materials). The solution was centrifuged at 12000 rpm for 10 min; the supernatants were transferred to auto sampler vial for UPLC-TOF/MS analysis.

Instrument conditions

Chromatographic analysis was carried out on a SHIMADZU UFLC XR system (Shimadzu Corporation, Kyoto, Japan) equipped with a LC-20AD-XR binary pump, SIL-20AD-XR auto sampler and a CTO-20A column oven. Chromatographic separation was performed on an Agilent Eclipse Plus C_{18} column (2.1 mm × 100 mm, 1.8 μm, Agilent Technologies, CA, USA) with the column temperature maintained at 25 . The mobile phases were composed of acetonitrile (A) and water with 0.1 % formic acid (B). The linear elution gradient program was as follows: 0-8 min 5 %-32 % A, 8-15 min 32 %-55 % A, 15-20 min 55 %-100 % A. The flow rate was set at 0.3 mL min^{-1} and the injection volume was 5 μL. Mass spectrometry was performed on the Triple TOF™ 5600 (AB SCIEX, Foster City, CA) a hybrid triple quadrupole time-of-flight mass spectrometer equipped with ESI source and mass range was set at m/z 100-1200. The condition of MS/MS detector were as follows: ion spray voltage 1500V; ion source gas 1 50 psi; ion source gas 2

60 psi; temperature 550 ; curtain gas 15 psi; collision gas pressure 8 psi; entrance potential 10V. Nitrogen was used as nebulizer and auxiliary gas. All the acquisition and analysis of data were controlled by the Peak View Software ^{TM}V. 1.1 (AB SCIEX, Foster City, CA)

RESULTS AND DISCUSSION

Optimization of chromatographic conditions

In order to achieve a better separation, a comparison of different kinds of mobile phase system including acetonitrile-water, methanol-water, acetonitrile-water with 0.1 % formic acid and methanol-water with 0.1 % formic acid were carried out. It was found that acetonitrile-water with 0.1 % formic acid was the optimum choice. The total ion current (TIC) chromatogram of vine tea extract in negative ion mode was illustrated in Figure 1.

Analysis of chemical constituents of vine tea by UFLC-Q-TOF-MS/MS

When a pure standard was available, the compound was identified by comparing its UFLC retention time, mass spectra with those of the standard. When no standard was available, the structures were proposed mainly based on the mass spectra. The mass error for molecular ions of all compounds identified in this study was within ±5 ppm. Under the aforementioned conditions, a total of 29 compounds were identified from vine tea sample, six of which were unambiguously identified by comparing their retention times, the accurate masses and fragment ions with those of reference compounds and 19 compounds were tentatively assigned by matching the empirical molecular formula with that of the known compounds previously reported in the literature. The structures of all the identified compounds were shown in Figure 2 and the detailed information were summarized in Table 1.

Characterization of organic acid

There are eight organic acids were identified from the vine tea crude extract. Peak 2 presented the $[M-H]^-$ ion at m/z 169.0135, corresponding to the molecular of $C_7H_6O_5$, It generated fragment ions at m/z 151.0040 and 125.0245, originated from successive losses of a H_2O and a CO_2 molecule. It was deduced as Gallic acid by comparison with the literature information[10]. Peak 3 displayed the $[M-H]^-$ ion at m/z 321.0235($C_{14}H_{10}O_9$), in the MS^2 spectra, the fragment ion at m/z 303.0146 and 277.034 were yielded by loss a H_2O and a CO_2. It was tentatively characterized as the gallic acid dimer which was reported in previous reference. Peak 1 exhibited an $[M-H]^-$ ion at m/z 191.0550,corresponding to a elemental composition $C_7H_{12}O_6$, On the basis of the available reference data[11], it was tentatively deduced as Quinic acid. The major fragment ion at m/z 173.0447 was yielded by the loss of H_2O. Peak 7, 8, 12, 13, 16 showed the $[M-H]^-$ ion at 181.0495 ($C_9H_{10}O_4$), 153.0189 ($C_7H_6O_4$),179.0340 ($C_9H_8O_4$), 167.0354 ($C_8H_8O_4$), 163.0396 ($C_9H_8O_3$), respectively. And these peaks often produced fragment ions by losses of H_2O, CO_2, CO or HCOOH residues suggesting the presence of $^-$OH and $^-$COOH group. According to the reference information[12-14], there were tentatively identified as Veratric acid, Protocatechuic acid, Caffeic acid, Vanillic acid and p-Coumaric acid.

Characterization of flavonoids

To the best of our knowledge, dehydration, loss of CO, CO_2 and the reprentative RDA fragments ($^{0,2}A^-$, $^{0,2}B^-$ and $^{1,3}A^-$, $^{1,3}B^-$ shown in Figure 3); which degraded by cleavage of bonds 0, 2 and 1, 3 of the C-ring were the characteristic fragmentation behaviour to most flavonoids and flavonols[15]. In the ESI multi-stage mass, loss of CO, CO_2, C_2H_2O and other neutral fragments first occurred in the C ring, then occurred in the A ring, B ring lost no neutral fragments, this rule also applies to the flavonol and isoflavone[16].

Peak 10 and 11 were a pair of enantiomers, both of them generated the identical $[M-H]^-$ ion at m/z 289.0704 ($C_{15}H_{14}O_6$) and fragmentation pattern, as shown in Table 1, peak 10 was undoubtedly identified as Catechin by comparison with an authentic standard and peak 11 was tentatively assigned as Epicatechin. Peak 15 exhibited an $[M-H]^-$ ion with high intensity at m/z 319.0450 ($C_{15}H_{12}O_8$), was explicitly identified as Dihydromyricetin by comparing its retention time, MS spectrum with the authentic standard, In the MS/MS spectrum (Figure 4), the fragment ion at m/z 301.0361 was formed by neutral losses of H_2O (18Da) from the deprotonated molecule $[M-H]^-$. The major fragment ion 193.0151 ($^{1,4}B^-$), 151.0039 ($^{1,3}A^-$) were observed in the MS^2 spectrum. The proposed fragmentation pathway was illustrated in Figure 5. With an $[M-H]^-$ ion at m/z 317.0296, peak 26, showed UV_{max} at 257, 368 nm indicated that it belong to flavonol. Fragment ions at m/z 271.0227 by successive losses of one H_2O and a CO. Minor fragment ions at m/z 137.0271 and 151.0061 were also observed by the A-ring RDA fragment. It was identified Myricetin according to its MS^2 fragments and further confirmed by comparison with the authentic standard. Peak 29 displayed an $[M-H]^-$ ions at m/z301.0348 ($C_{15}H_{10}O_7$) and yielded an ion at m/z 273.0374 by the losses of one CO (28Da); the fragment ions at m/z 178.9990 ($^{1,2}A^-$), 151.0039 ($^{1,2}A^-$-CO), 121.0322 ($^{1,2}B^-$) and 107.0154 ($^{1,2}A^-$-CO-CO_2) also definitely confirmed as Quercetin by comparing with the authentic standards. Peak 30 gave $[M-H]^-$ ion at m/z 271.0591, The TOF/MS spectra showed fragment ions at m/z 177.0169 was generated by the losses of B-ring, other fragment ions at 151.0038, 119.0520, resulting from Retro-Diels–Alder (RDA) fragmentation of the C ring. It was deduced as Naringenin with the literature data[12]. Peak 25 presented $[M-H]^-$ ion with mass accuracy at m/z 287.0541, the fragment ion at m/z 259.0596 and 243.0692 corresponded to loss of CO (28Da) and CO_2 (44Da). The RDA fragment ion 151.0049 was observed in the MS^2 spectrum, it was tentatively identified as Eriodictyol according to the reference[17]. Peak 31 had the molecular formula $C_{15}H_{10}O_5$ and showed the fragment ions at m/z 225.0531, 151.0024 and 117.0336, corresponding to the loss of CO_2 (44Da) and retrocyclization cleavages of C-ring. It was assigned as Apigenin by comparison with an authentic standard. Peak 22 produced $[M-H]^-$ ion at m/z 303.0501 and displayed the elemental composition of $C_{15}H_{12}O_7$, characteristic fragment ions at m/z 151.0396 originated from the cleavage of C-ring, other product ion at m/z 125.0627 ($^{1,3}A^-$-CO), 107.0155 ($^{1,2}A^-$-CO-CO_2) were also observed in the MS^2 spectra. According to the reference standard[18], peak 22 was identified as Taxifolin. Peak 27 was strongly confirmed as Morin in accordance with the authentic standard, $[M-H]^-$ ion at m/z 301.0339 ($C_{15}H_{10}O_7$) and displayed the secondary ion at m/z 255.0317 by loss of one H_2O and one CO. The RDA fragmentation ion at m/z 151.0054 ($^{1,3}A^-$) and 149.0264 ($^{1,3}B^-$) were also obtained in the MS spectrum. Peak 17, with the $[M-H]^-$ ion at m/z 333.0244, corresponding to the molecular composition of $C_{15}H_{10}O_9$. From the MS^2 spectrum, secondly ions at m/z 289.0348, 271.0211 and 227.0333 were produced by the loss of one H_2O molecular, one and two CO_2 molecular successively. The characteristic RDA fragment ions at m/z 151.0042 ($^{1,3}A^-$) and 135.0460 ($^{0,3}A^-$) were also found, suggesting there would be dihydroxy substituted in the A-ring. According to the existing study, Peak 17 was tentatively deduced as 3, 5, 7, 2′3′, 4′, 5′-heptahydroflavonol.

Characterization of Flavonoid O-glycosides

Three flavonoid O-glycosides were identified in vine tea. They were characterized by the loss of 162 Da or146 Da from $[M-H]^-$ ions. Peak 19 displayed a $[M-H]^-$ ion at m/z 463.0857, the molecular formula provided by TOF-MS was $C_{21}H_{20}O_{12}$ and its MS^2 spectrum

showed the aglycone ion at 317.0030 as a base peak, which originated from the loss of rhamnosyl (146Da). Peak 23 exhibited an $[M-H]^-$ ion at m/z 447.0902, generated the aglycone ion at m/z 301.0347 (146 Da). Peak 19 and 23 were tentatively identified as Myricetrin and Quercetin-3-rhamnoside which were reported in vine tea[19]. Peak 21 with an $[M-H]^-$ ion at m/z 479.0802, gave the elemental composition of $C_{21}H_{20}O_{13}$; the fragment ion at m/z 317.0279, corresponding to the loss of a glucose residue (162Da). The product ion at m/z 193.0145 ($^{1,4}B^-$) and 151.0067 ($^{1,3}A^-$) originated from fission of the C-ring. By compared with the available reference[20], peak 21 was plausibly characterized as Myricetin-3-O-β-D-galactopyranoside.

Table 1: Identification of the chemical constituents of Vine Tea by UFLC-Q-TOF-MS/MS

No	T_R(min)	Formula	$[M-H]^-$(error, ppm)	Fragment ions in negative (-) ion mode	Identification
1	1.14	$C_7H_{12}O_6$	191.0550(-0.3)	173.0447$[M-H-H_2O]^-$ 171.0381 127.0295	Quinic acid
2	1.55	$C_7H_6O_5$	169.0135(2.3)	151.0040$[M-H-H_2O]^-$ 125.0245$[M-H-CO_2]^-$	Gallic acid
3	1.69	$C_{14}H_{10}O_9$	321.0235(-1.8)	303.0146$[M-H-H_2O]^-$ 277.034$[M-H-CO_2]^-$ 259.0256$[M-H-CO_2-H_2O]^-$ 193.0146;173.0248	Digallic acid
4	2.35	$C_{13}H_{16}O_{10}$	331.0663(0.9)	313.0506$[M-H-H_2O]^-$ 169.0136$[M-H-C_6H_8O_4]^-$ 125.0254$[M-H-C_6H_8O_4-CO_2]^-$	Galloy-β-D-glucose
5	3.54	$C_8H_8O_5$	183.0301(4.3)	168.0013$[M-H-CH_3]^-$	Methyl gallate
6	4.22	$C_{16}H_{14}O_6$	301.0342(-0.2)	283.0269$[M-H-H_2O]$- 257.0397$[M-H-CO_2]^-$ 191.0354 175.0050($^{0,4}B^-$) 135.0505 125.0380	Hesperetin
7	4.31	$C_9H_{10}O_4$	181.0495(-0.3)	163.0412$[M-H-H_2O]^-$ 135.0459$[M-H-H_2O-CO]^-$ 119.0514 93.0370	Veratric acid
8	4.79	$C_7H_6O_4$	153.0189(4.6)	109.0313$[M-H-CO_2]^-$ 81.0361$[M-H-CO_2-CO]^-$	Protocatechuic acid
9	5.14	$C_{15}H_{14}O_7$	305.0656(0.1)	219.0663 137.0263 125.0270	Epigallocatechin
10	5.50	$C_{15}H_{14}O_6$	289.0704(-0.8)	245.0808;205.0808 203.0697;179.0330 159.0447;109.0300	Catechin [a]
11	5.63	$C_{15}H_{14}O_6$	289.0694(-4.3)	245.0812;205.0494 203.0692;179.0330 151.0412;109.0305	Epicatechin
12	6.26	C9H8O4	179.0340(0.9)	135.0461$[M-H-CO_2]^-$ 107.0536$[M-H-C_3H_4O_2]^-$	Caffeic acid
13	6.51	$C_8H_8O_4$	167.0354(9.1)	123.0466$[M-H-CO_2]^-$ 83.0159,81.0363	Vanillic acid
14	6.73	$C_9H_6O_5$	193.0134(1.3)	165.0211$[M-H-CO]^-$ 121.0266$[M-H-CO_2-CO]^-$	3,5,7-trihydroxy
15	6.80	$C_{15}H_{12}O_8$	319.0450(0.6)	301.0361$[M-H-H2O]^-$ 193.0151(1,4B$^-$) 175.0046 151.0039($^{1,3}A^-$) 137.0253	Dihydromyricetin [a]
16	6.96	$C_9H_8O_3$	163.0396(3.8)	119.0543$[M-H-CO_2]^-$ 91.0582$[M-H-CO]^-$	p-Coumaric acid
17	7.33	$C_{15}H_{10}O_9$	333.0244(0.8)	289.0348$[M-H-H_2O]^-$ 271.0211$[M-H-H_2O-CO_2]^-$ 227.0333$[M-H-H_2O-2CO_2]^-$ 151.0042($^{1,3}A^-$) 135.0460($^{0,3}A^-$)	Unknown
18	9.29	$C_{15}H_{10}O_6$	285.0398(1.4)	241.0518$[M-H-H_2O-CO]^-$ 151.0051($^{1,3}A^-$) 133.0306($^{1,3}B^-$)	Luteolin
19	9.38	$C_{21}H_{20}O_{12}$	463.0857(-4.4)	317.0030$[M-H-Rha]^-$ 287.0197 271.0225	Myricetrin

20	9.50	$C_{15}H_{12}O_7$	303.0503(1.2)	285.0405[M-H-H_2O]$^-$ 175.0404 151.0426($^{1,3}A^-$) 125.0254	5,7,3′,4′,5′-pentahydroxyflavanone
21	9.83	$C_{21}H_{20}O_{13}$	479.0802(-3.7)	317.0279[M-H-Glc]$^-$ 193.0145($^{1,4}B^-$) 151.0067($^{1,3}A^-$)	Myricetin-3-O-β-D-galactopyranoside
22	10.44	$C_{15}H_{12}O_7$	303.0501(0.2)	285.0419[M-H-H_2O]$^-$ 151.0396($^{1,3}A^-$) 125.0627 107.0155($^{1,2}A^-$-CO-CO_2)	Taxifolin
23	10.79	$C_{21}H_{20}O_{11}$	447.0902(-4.4)	301.0347[M-H-Rha] 271.0248 255.0314[M-H-Rha-H_2O-CO]$^-$ 151.0035($^{1,3}A^-$)	Quercetin-3-rhamnoside
24	10.80	$C_{15}H_8O_9$	331.0075(-2.8)	287.0179 259.0246 203.0347	Unknown
25	11.05	$C_{15}H_{12}O_6$	287.0541(-3.2)	259.0596[M-H-CO]$^-$ 243.0692[M-H-CO_2]$^-$ 151.0049($^{1,3}A^-$) 125.0236(A)	Eriodictyol
26	11.12	$C_{15}H_{10}O_8$	317.0296(1.2)	271.0227[M-H-H_2O-CO]$^-$ 179.0013($^{0,3}B^-$) 151.0061($^{1,3}A^-$) 137.0271	Myricetin [a]
27	11.73	$C_{15}H_{10}O_7$	301.0339(-1.4)	255.0317[M-H-H_2O-CO]$^-$ 239.0408 151.0054($^{1,3}A^-$) 149.0264($^{1,3}B^-$)	Morin [a]
28	12.888	$C_{15}H_{12}O_6$	287.0544(-2.2)	269.0448[M-H-H_2O]$^-$ 243.0324[M-H-CO_2]$^-$ 151.0447 ($^{1,3}A^-$) 135.0472	Aromadendrin
29	13.53	$C_{15}H_{10}O_7$	301.0348(1.6)	273.0374[M-H-CO]$^-$ 178.9990($^{1,2}A^-$), 151.0039($^{1,2}A^-$-CO),121.0322($^{1,2}B^-$), 107.0154($^{1,2}A^-$-CO-CO_2)	Quercetin[a]
30	14.898	$C_{15}H_{12}O_5$	271.0591(-3.7)	177.0169[M-H-ring B]$^-$ 151.0038($^{1,3}A^-$), 119.0520($^{1,3}B^-$),107.0147($^{1,3}A^-$-CO_2)	Naringenin
31	15.44	$C_{15}H_{10}O_5$	269.0451(2.5)	225.0531[M-H-CO_2]$^-$ 151.0024($^{1,3}A^-$) 149.0255 117.0336($^{1,3}B^-$)	Apigenin

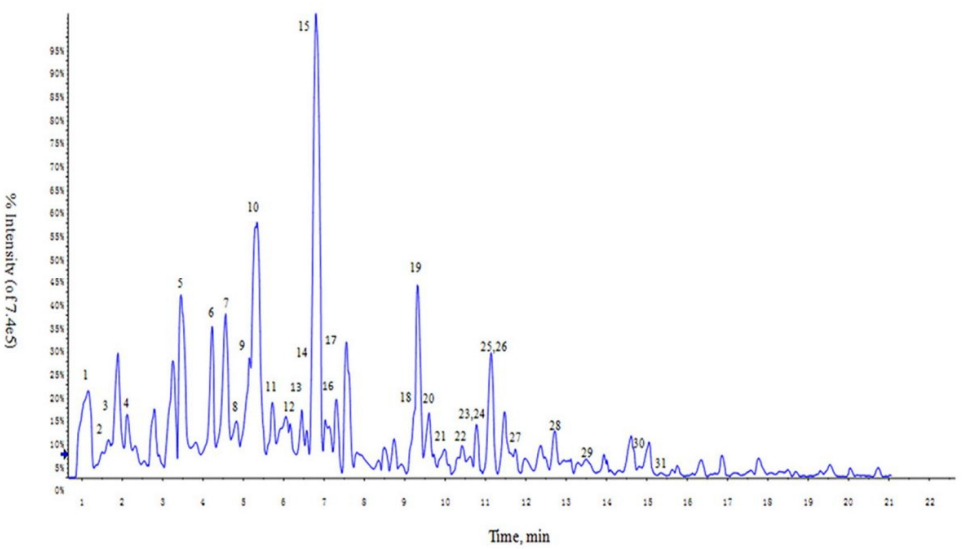

Figure 1: Total ion current profile of vine tea negative ion mode

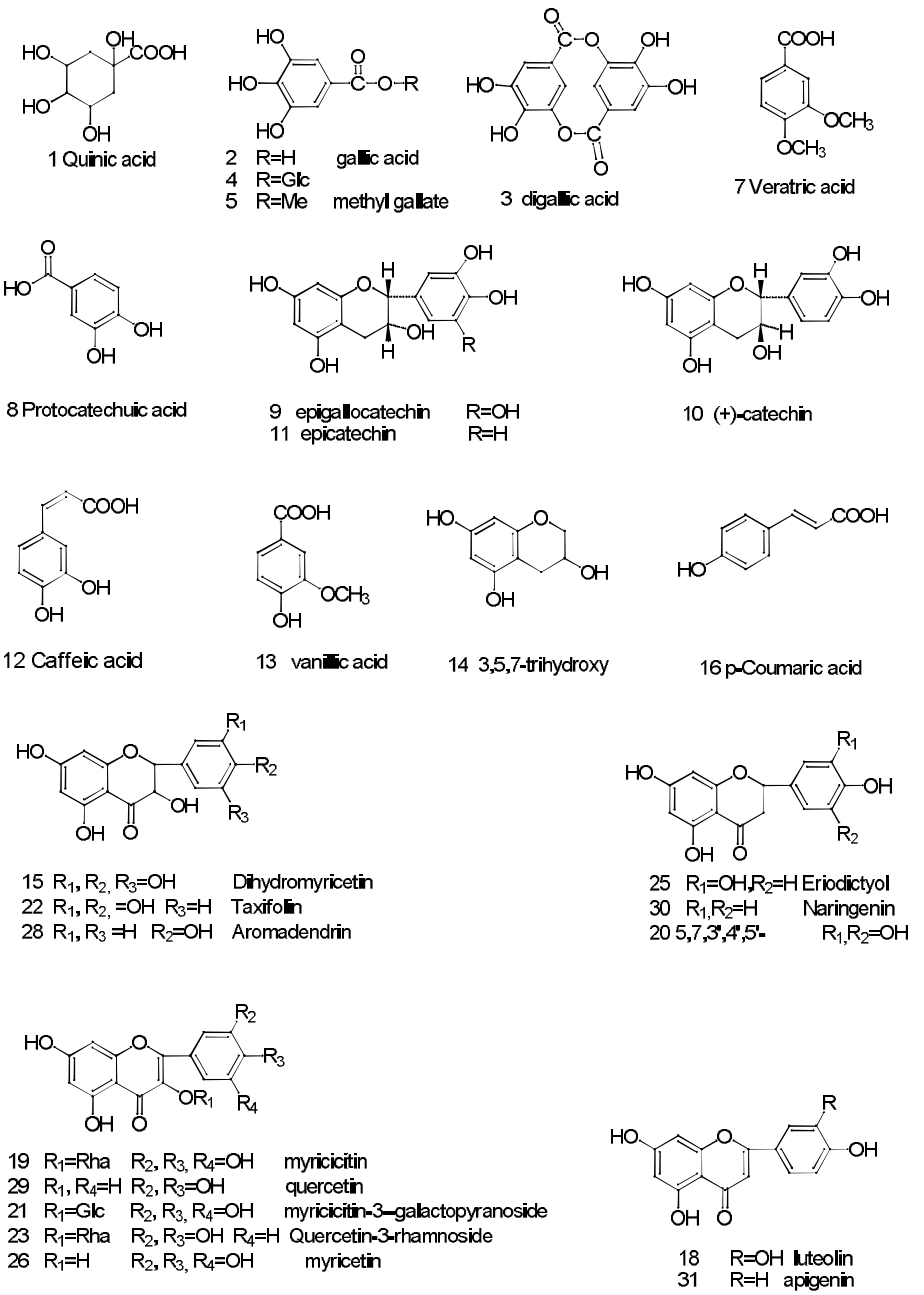

Figure 2: Chemical structures of compounds identified in VT

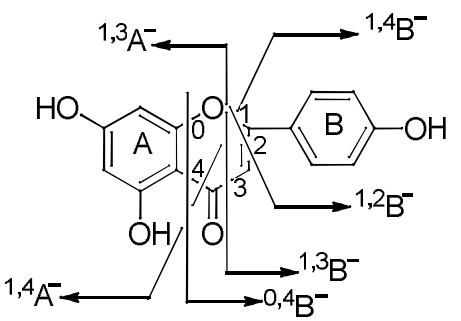

Figure 3: The different retro-cyclization cleavages of C-ring[15,16]

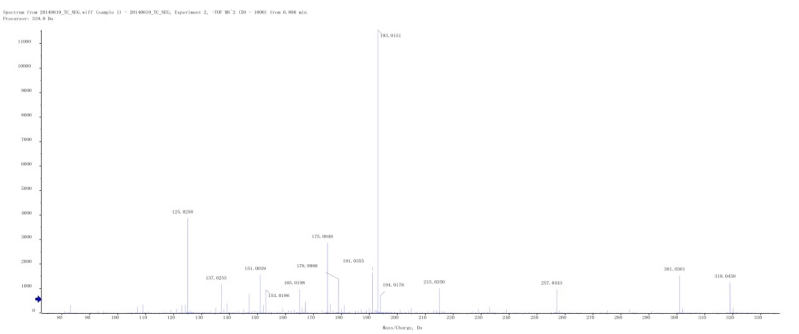

Figure 4: Spectra of ion fragments in analysis of Dihydromyricetinin negative ion mode

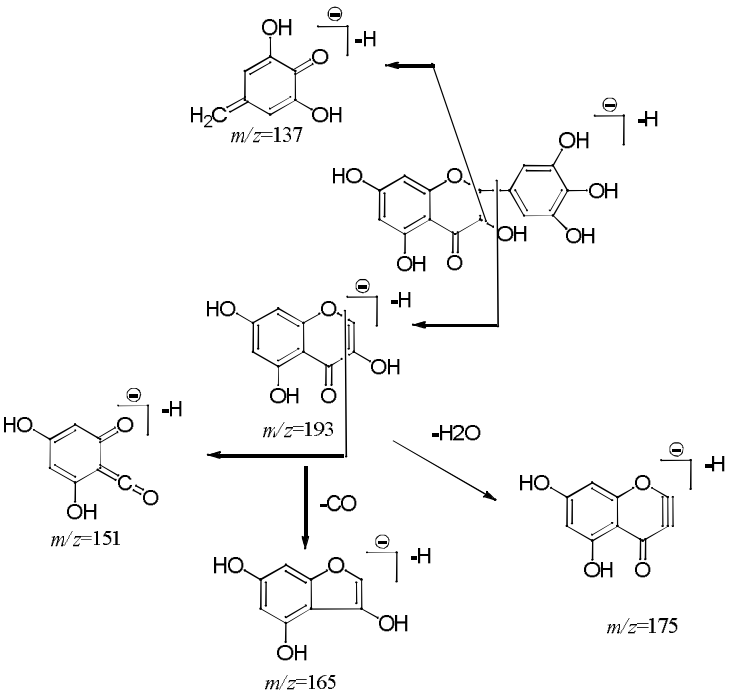

Figure 5: Proposed fragmentation pathway of Dihydromyricetin

CONCLUSION

In this study, a rapid and reliable method based on UFLC-Q-TOF-MS/MS was employed for the separation and structural identification of major constituents in Vine tea. A total of 29 ingredients were identified with reference compounds by comparing the mass spectra and retention time or tentatively characterized according to fragmentations and matching empirical molecular formula with that of the published compounds. This paper marks the first report on the chemical profiles utilizing the analytical method with UFLC-Q-TOF-MS/MS technique. As presented herein, it has been proved to be an effective tool for the systematic analysis of the constituents and improve further quality control standard for Vine tea along with other traditional Chinese medicines.

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