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Simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *Coscinium fenestratum* by liquid chromatography hybrid ion trap time-of-flight mass spectrometry

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ABSTRACT

Simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *Coscinium fenestratum* was successfully performed by liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/IT-TOF MS). A total of 32 compounds, including 2 benzylisoquinoline alkaloids, 3 aporphine alkaloids, 12 quaternary protoberberine alkaloids, 10 8-oxoprotoberberine alkaloids, 3 tetrahydroprotoberberine alkaloids, and a steroid compound were simultaneously separated and characterized by matching the empirical molecular formulae with those published in literature and the multi-stage mass spectrometry (MS^n) data obtained using structural information from IT, accurate mass measurement obtained from TOF MS, and HPLC separation. A total of 20 compounds, including 4 novel natural products were identified or tentatively identified for the first time from *Coscinium fenestratum*. In the positive-ion mode, 8-oxoprotoberberines produced $[M+H]^+$ and $[M+Na]^+$; the fragmentation of 8-oxodihydroprotoberberines produced $[M+H-{}^{\bullet}CH_3]^{+\bullet}$, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3]^+$, and $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3-CO]^+$, while 8-oxotetrahydroprotoberberines generated $[M+H-{}^{\bullet}CH_3]^{+\bullet}$, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3]^+$, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}H]^+$, and iminium ions from the cleavage of the protoberberine skeleton. The method can be applied for the analysis of 8-oxoberberine and other alkaloids in *Coptis japonica*, *Phellodendron amurense*, and other herbal medicines.

1. Introduction

It has been generally accepted that the efficacy of herbal medicines can be attributed to the synergistic activity of various major and minor components of the herbs [1,2]. The identification of the components of herbal medicines is of great importance in controlling their quality and gaining a better understanding of their pharmacological effects. Consequently, the development of rapid and reliable methods for qualitative and quantitative determination of compounds in herbal medicine has become a significant and challenging issue. The combination of high-performance liquid chromatography and multi-stage mass spectrometry (HPLC-MS^n) is being increasingly used in pharmaceutical research and for quality control in herbal medicine, because of its superior sensitivity and selectivity [2–6]. In these procedures, mass analyzers such as triple quadrupole or ion trap provide online mass information, which is useful in identifying the eluted components. However, both of these mass analyzers provide nominal mass values.

In contrast, time-of-flight mass spectrometry (TOF MS) provides higher accuracy and precision, which is remarkably efficacious in the identification of unknown compounds [7–9]. The accurately measured mass values can be used to calculate candidate empirical formulae. Mass values obtained with a mass error of less than 5 ppm can significantly reduce the number of possible structures of the separated compounds. Accordingly, the combined use of ion trap MS^n for determining structural information and TOF MS for the obtaining accurate mass measurement offers a more powerful analytical approach for identifying the constituents of herbal medicines [8,9].

Coscinium fenestratum is a medicinal plant belonging to family Menispermaceae, and it has been used as a herbal medicine in the Indochina region, India, and Sri Lanka for a long time. This plant has been used in the treatment of diarrhea, inflammation, ulcers, skin disease, and diabetes mellitus [10]. Several biological effects have been attributed to the plant extracts, including antibacterial [11], antioxidant [12], antidiabetic [13], and hypotensive [14] activities, all of which confirm its efficacy in the traditional medicinal system. A total of 15 chemical compounds, primarily quaternary and tertiary protoberberine alkaloids, have been isolated previously [15–18]. To date, several methods have been developed to

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measure the principal component of this herb, berberine; these include methods based on thin-layer chromatography (TLC) and high-performance liquid chromatography (HPLC) [18,19]. However, the pharmacological activities attributed to the herb could be due to the synergistic activity of several compounds. Therefore, it is essential to develop a method for simultaneous analysis of the compounds in *Coscinium* fenestratum in order to ensure quality control of the herb. Moreover, there are no studies on the online analysis of oxoprotoberberine alkaloids by LC/MS, which show various pharmacological effects [20–22] and occur in several important medicinal plants such as *Coptis japonica* and *Phellodendron amurense* [21,22].

In this study, we report a method for the simultaneous characterization of known and unknown compounds in *Coscinium* fenestratum by using liquid chromatography hybrid ion trap time-of-flight mass spectrometry (LC/IT-TOF MS). This method can also be applied for online analysis of both minor compounds and major compounds in other medicinal plants such as *Coptis japonica* and *P. amurense*.

2. Material and methods

2.1. Chemicals

Berberine, palmatine, jatrorrhizine, tetrahydroberberine (canadine), 20-hydroxyecdysone, methanol (LC/MS-grade) and formic acid (LC/MS-grade) were purchased from Wako Co., Ltd. (Tokyo, Japan). Ultra-pure water was produced by Milli-Q Advantage system (Millipore, USA). 8-Oxoberberine was synthesized from berberine chloride by using a previously described procedure [23]. Stock solutions of berberine (150 μg/ml), palmatine (42 μg/ml), jatrorrhizine (110 μg/ml), tetrahydroberberine (40 μg/ml), and 20-hydroxyecdysone (11.4 μg/ml) were prepared in methanol. A solution of 8-oxoberberine was prepared by dissolving 2.5 mg of the synthesized crystals in 50 ml of methanol. All the solutions were stored at 4 °C in the refrigerator. Standard solutions were prepared by diluting each stock solution in methanol.

2.2. Sample preparation and extraction

Stems of *Coscinium* fenestratum were obtained from Vientiane province, Laos in August 2006 and dried at room temperature. The stems were identified by Dr. Keooudone Rasphone (Pharmaceutical Factory No. 2, Ministry of Health, Laos). Dried *Coptis japonica* (Lot No. 230604) and *P. amurense* (Lot No. 001205003) were purchased from a herbal medicine shop (Yatsume Kampo) in Tokyo, Japan. The plants were ground in a blender (Wonder blender WB-1; Osaka Chemical, Japan), and the powder was sieved using a 60-mesh sieve to obtain a powder with particle size less than 250 μm. Extraction was performed in a Soxhlet extraction vessel by adding 0.5 g of plant powder to the extraction thimble and extracting the powder using 100 ml methanol for 11 h. The extracted liquid was filtered using a 0.45-μm membrane filter, and the filtrate was used for LC/IT-TOF MS analysis.

2.3. LC/IT-TOF MS analysis

LC/IT-TOF MS analyses were performed on an LC/IT-TOF MS system (Shimadzu, Kyoto, Japan). The LC system consisted of a Shimadzu Prominence binary pump (LC-20AD), online vacuum degasser (DGU-20As), autosampler (SIL-20AC), column oven (CTO-20AC), and a photo diode-array detector (SPD-M20A). The separation was carried out on a ZORBAX Eclipse XDB-C18 column (150 mm × 2.1 mm, 3.5 μm) (Agilent technologies, USA). The mobile phase, which consisted of a 0.1% formic acid aqueous solution (A) and methanol (B), was delivered at a flow rate of 0.2 ml/min by using the following gradient program: 20–32% (B) from 0 min to 20 min, 32–70% (B) from 20 min to 35 min, 70% (B) from 35 min to 45 min and 20% (B) from 45 min to 55 min. The sample injection volume was 2 μl. The column temperature was maintained at 40 °C. The UV spectra were obtained by scanning the samples in the range of 200–470 nm, and the peaks were simultaneously determined at 254 nm.

The IT-TOF mass spectrometer was fitted with an electrospray ionization (ESI) interface. ESI-MS^n experiments were conducted in both positive and negative modes. The following instrumental parameters were used: interface voltage, +4.5 kV (positive mode), −3.5 kV (negative mode); heat-block temperature, 200 °C; curved desolvation line (CDL) temperature, 200 °C; flow rate of nebulizing gas (N_2), 1.5 L/min; and pressure of drying gas (N_2), 100 KPa. Mass spectrometry was performed in the full-scan mode (MS^1) and automatic multiple-stage fragmentation-scan modes (MS^2–MS^4) over an m/z scan range of 100–700. In the automatic mode, all the ions were first accumulated in the octopole, and then, they were rapidly pulsed into the IT for MS^n analyses. The resulting ions were introduced into the TOF instrument for accurate mass determination. The ion-accumulation time was set at 30 ms. We chose the following parameters for collision-induced dissociation (CID): CID energy, 30%; collision-gas content, 50%. Argon was used for CID. The TOF-detector voltages for the positive and negative modes were 1.60 kV and 1.56 kV, respectively. Trifluoroacetic acid (TFA) sodium solution was used as the standard sample for calibrating the instrument. The chromatographic and mass-spectrometric analyses, including the prediction of chemical formulae were performed by using LCMS solution Ver3.41 software package (Shimadzu, Kyoto, Japan).

3. Results and discussion

3.1. Online analysis of standard compounds

The online analysis of 6 standard compounds was performed by using LC/IT-TOF MS under the optimum conditions described in the experimental section. As shown in Table 1, quaternary protoberberine alkaloids such as berberine, palmatine, and jatrorrhizine exhibited maximum UV absorption at 4 wavelength ranges: 220–230 nm, 260–280 nm, 340–350 nm, and 420–430 nm. 8-Oxoberberine exhibited maximum UV absorption at 224 nm, 341 nm, and 369 nm. In contrast, 20-hydroxyecdysone exhibited maximum absorption at a single wavelength, 249 nm. In positive mode, berberine, palmatine, and jatrorrhizine generated the molecular ion $[M]^+$ and the fragment ions $[M-{}^{\bullet}CH_3]^{+\bullet}$, $[M-{}^{\bullet}CH_3-{}^{\bullet}H]^+$, $[M-{}^{\bullet}CH_3-{}^{\bullet}H-CO]^+$ in MS^2 and $[M-{}^{\bullet}CH_3-{}^{\bullet}H-2H]^+$ in MS^3. Tetrahydroberberine generated the protonated molecule $[M+H]^+$. $[M+H]^+$ was observed to undergo the Retro-Diels-Alder (RDA) fragmentation reaction when CID was applied in MS^2. 20-Hydroxyecdysone produced $[M+H]^+$ and the sodium adduct ion $[M+Na]^+$. We also observed the cleavage of water molecules from 20-hydroxyecdysone ($M-nH_2O$, $n=1$–3). In negative mode, jatrorrhizine produced $[M-2H+HCOOH]^-$and 20-hydroxyecdysone produced $[M-H]^-$and $[M-H+HCOOH]^-$. The molecular ion and the fragment ions of berberine, palmatine, jatrorrhizine, and 20-hydroxyecdysone were consistent with those reported in previous studies [4,24,25].

$[M+H]^+$ and $[M+Na]^+$ were observed in the mass spectra of 8-oxoberberine in MS^1. Fig. 1 shows the fragmentation ion from the precursor ion at m/z 352.1169. This ion produced the fragments $[M+H-{}^{\bullet}CH_3]^{+\bullet}$, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}H-CO]^+$, $[M+H-{}^{\bullet}CH_3-H_2O]^{+\bullet}$ in MS^2, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3]^+$ in MS^3, and $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3-CO]^+$ in MS^4, which were different from the corresponding ions of berberine and tetrahydroberberine. Standard reference analysis

Table 1
Retention time (*t*R), UV(λmax) and MS data of standard compounds.

Compound	t_R (min)	UV(λmax)	*m/z* [identity] (+)ESI-MS	(−)ESI-MS	(+)ESI-MS^n fragments
Jatrorrhizine	23.05	225, 274, 343, 427	338.1390 $[M]^+$	382.1290 [M−2H+HCOOH]	323.1137, 322.1052, 320.0893, 307.0815, 294.1122
Berberine	25.45	226, 264, 346, 426	336.1236 $[M]^+$		321.0971, 320.0896, 318.0748, 292.0951
Palmatine	27.06	245, 273, 344, 425	352.1550 $[M]^+$		337.1293, 336.1228, 334.1061,320.0911, 308.1288, 292.0954
Tetrahydroberberine	21.76	285	340.1547 $[M+H]^+$		176.0712, 149.0593
20-Hydroxyecdysone	29.30	249	503.2982 [M+Na]+ 481.3164 $[M+H]^+$ 463.3059 $[M+H-H_2O]^+$ 445.2952 $[M+H-2H_2O]^+$ 427.2839 $[M+H-3H_2O]^+$	525.3061 $[M-H+HCOOH]^-$ 479.2973 $[M-H]^-$	463.3050, 445.2931, 371.2195, 303.1997
8-Oxoberberine	41.19	224, 341, 369	374.0997 $[M+Na]^+$ 352.1169 $[M+H]^+$		337.0947, 322.0696, 319.0821, 308.0927, 304.0615, 294.0757

was performed to establish the principal fragmentation pathways of quaternary protoberberines, tetrahydroprotoberberines, 8-oxoprotoberberines, and the steroid compound.

3.2. Simultaneous characterization of compounds from the stem extract of Coscinium fenestratum

The stem extract of *Coscinium fenestratum* was injected directly into LC/IT-TOF MS without any further pretreatment (Fig. 2 and Table 2). A total of 32 peaks were recorded, and the chemical structure of the compounds was characterized on the basis of the calculated accurate mass of the molecular ions, the protonated molecule, the fragment ions, the retention behavior, and the data from the UV spectra (Table 2 and Table 3). The mass error of the measured value and the predicted value was less than 5 ppm for MS^1 and less than 10 ppm for most of the fragments in MS^2–MS^4 analysis. The empirical formulae obtained from the accurate mass values were compared to those in literature to identify the known compounds. The accurate fragmentation data was used to elucidate and identify the unknown compounds. These highly accurate mass values were useful in reducing the number of candidate chemical formulae and identifying the compounds easily and rapidly. It can be concluded that most of these compounds are quaternary alkaloids, 8-oxoprotoberberine alkaloids, tetrahydroprotoberberine alkaloids, along with a single steroid compound (Table 2). Compounds 1 and 2 were characterized as benzylisoquinoline alkaloids; compounds 3, 4, and 5, as aporphine alkaloids; compounds 6, 8, and 15, as tetrahydroprotoberberine alkaloids; compounds 7, 9, 10, 11, 12, 13, 14, 16, 17, 18, 19, and 20 as quaternary protoberberine alkaloids; compound 21 as a steroid compound; and compounds 23, 24, 25, 26, 27, 28, 29, 30, 31, and 32 were characterized as 8-oxoprotoberberine alkaloids. Compound 22 was tentatively identified as a benzyl tetrahydroisoquinoline alkaloid.

3.2.1. Quaternary protoberberine alkaloids

3.2.1.1. Quaternary protoberberine alkaloids with methoxy groups at C-9 and C-10. Compounds 7, 9, 11, 16, 18, and 19 exhibited maximum absorption at 4 wavelength ranges, namely, 220–240 nm,

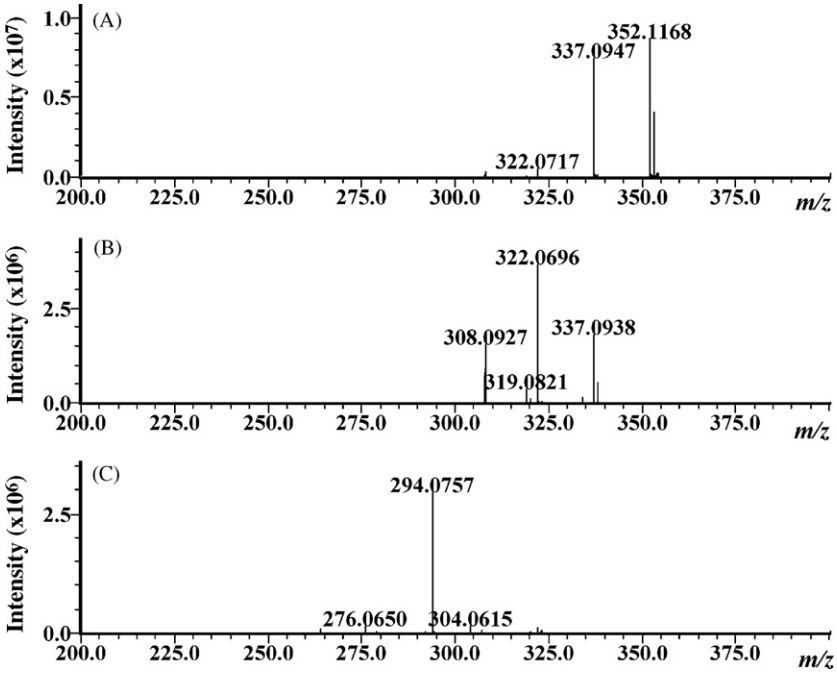


Fig. 1. MS^n mass spectra of 8-oxoberberine obtained by IT-TOF MS. (A) MS^2 spectrum of ion at *m/z* 352.1169; (B) MS^3 spectrum of ion at *m/z* 337.0947; (C) MS^4 spectrum of ion at *m/z* 322.0696.

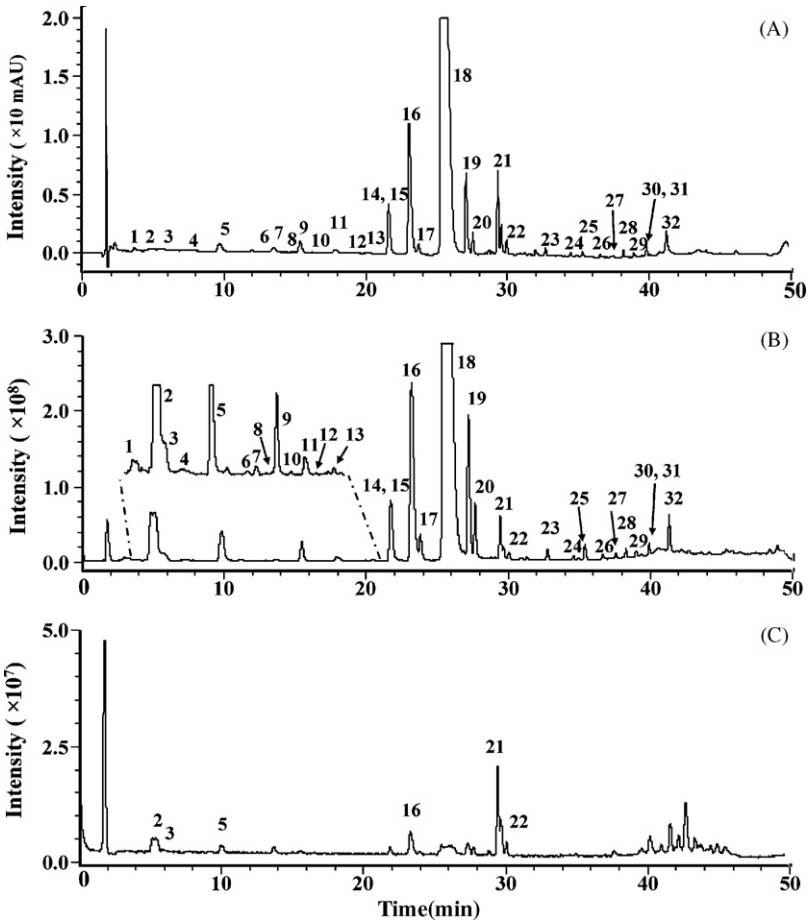


Fig. 2. HPLC-PDA and total ion chromatogram of *Coscinium fenestratum* (2 μl injected). (A) Monitored at 254 nm; (B) total ion chromatogram in positive mode; (C) total ion chromatogram in negative mode.

260–280 nm, 340–350 nm, and 410–430 nm, which suggested that they are quaternary protoberberine alkaloids [26]. Due to the low concentration of compound 12, the UV absorption of this compound could not be clearly monitored. However, it was characterized as a quaternary protoberberine compound from the fragmentation data (Table 3). The fragment ions of berberine, palmatine, jatrorrhizine (Table 2) produced $[M-{}^{\bullet}CH_3]^{+\bullet}$, $[M-{}^{\bullet}CH_3-{}^{\bullet}H]^{+}$, $[M-{}^{\bullet}CH_3-{}^{\bullet}H-CO]^{+}$ in MS^2 and $[M-{}^{\bullet}CH_3-{}^{\bullet}H-2H]^{+}$ in MS^3. The fragmentation pathways of these 3 compounds are proposed in Scheme 1 (A). These fragmentation data indicated that the 3 alkaloids lost a methyl radical in MS^2 and then produced fragment A after the loss of the H atom and the ring-closure reactions in MS^3. Since the formation of such methylenedioxy group occurs only when 2 methoxy groups are vicinal to each other, fragment A can be used as a diagnostic ion [4]. Although the exact position of methoxy groups cannot be fully elucidated by MS^n analysis, methoxy groups are rarely found at positions other than C-2, C-3, C-9, C-10, and C-11 [26]. The methoxy groups at C-9 and C-11 can be distinguished on the basis of differences in their UV-adsorption characteristics [26]. The retention times and the UV and mass spectra of compounds 16, 18, and 19 were identical to those of the standard compounds jatrorrhizine, berberine, and palmatine. Consequently, compounds 16, 18, and 19 were identified as jatrorrhizine, berberine, and palmatine, respectively. Compounds 7, 9, 11, and 12 followed the above-mentioned fragmentation pathways, which indicated that they have 2 methoxy groups at C-9 and C-10. The mass of the $[M]^+$ ions of compounds 7, 9, and 12 were 16 Da (O) higher than the mass of the $[M]^+$ ions of jatrorrhizine, berberine, and palmatine. These results suggest that these 3 compounds could be derivatives of jatrorrhizine, berberine, and palmatine with an additional hydroxy group. In addition, we also observed the loss of water molecules from these compounds in MS^4. We hypothesize that the additional hydroxy groups are at the C-13 position. This inference is based on the reasoning that the presence of the hydroxy group at C-5 would reduce the possibility of the formation of fragment A, while the presence of the hydroxy group at any other position could affect the formation of the fragment ions [4]. Thus, compounds 7, 9, and 12 were tentatively identified as 13-hydroxyjatrorrhizine, 13-hydroxyberberine, and 13-hydroxypalmatine, respectively. The lower elution times of compounds 7, 9, and 12 suggested that these compounds were more polar than jatrorrhizine, berberine, and palmatine, and supported our hypothesis. The mass of the $[M]^+$ ion of compound 11 was 14 Da (CH_2) lower than the mass of the $[M]^+$ ion of berberine; moreover, compound 11 generated fragment A, indicating the presence of 2 methoxy groups at C-9 and C-10. Therefore, compound 11 was identified as demethyleneberberine.

3.2.1.2. Quaternary protoberberine alkaloids with single hydroxy groups at C-9 or C-10. Compound 14 was characterized as quaternary protoberberine alkaloid, because it showed maximum UV absorption at 4 wavelength ranges, similar to that observed in case of berberine. However, its fragmentation pathway was not similar to the pathway of the quaternary alkaloids with 2 methoxy groups at C-9 and C-10. Compounds 10, 13, and 14 produced $[M-{}^{\bullet}CH_3]^{+\bullet}$ in MS^2, $[M-{}^{\bullet}CH_3-CO]^{+\bullet}$, $[M-{}^{\bullet}CH_3-CO-{}^{\bullet}H]^{+}$, and $[M-{}^{\bullet}CH_3-{}^{\bullet}CH_3]^{+}$ in MS^3. The generation of a single ion in MS^2 indicated that the $[M-{}^{\bullet}CH_3]^{+\bullet}$ ion was highly stable. In addition, fragment A was not observed among the fragment ions of these compounds. Thus, these

Table 2
Retention time (tR), UV(λmax) and MS data of compounds from the stem extract of *Coscinium fenestratum*.

Peak No	t_R (min)	Formula [M]+ or $[M+H]^+$	UV λmax (nm)	Measured *m/z*	Predicted *m/z*	Error (ppm)	$[M+Na]^+$	(−) ESI *m/z* [identity]	Proposed compounds
1	3.20	$[C_{19}H_{24}NO_3]^+$	223, 275	314.1762	314.1756	1.91	–	–	Tembetarine derivative
2	4.85	$[C_{20}H_{26}NO_4]^+$	281	344.1846	344.1856	−2.91	–	388.1766 $[M-2H+HCOOH]^-$, 434.1812 $[M-2H+2HCOOH]^-$	Tembetarine
3	5.73	$[C_{20}H_{24}NO_4]^+$	217, 269, 302	342.1705	342.1700	1.46	–	386.1584 $[M-2H+HCOOH]^-$	Magnoflorine
4	7.89	$[C_{20}H_{24}NO_4]^+$	222, 275	342.1686	342.1700	−4.09	–	–	*N,N* -demethyllindcarpine
5	9.71	$[C_{21}H_{26}NO_4]^+$	221, 269, 302	356.1858	356.1856	0.56	–	354.1709 $[M-2H]^-$, 400.1774 $[M-2H+HCOOH]^-$	Menisperine
6	12.98	$[C_{19}H_{19}NO_4+H]^+$	–	326.1388	326.1387	0.31	–	–	Tetrahydrothalifendine
7	13.52	$[C_{20}H_{20}NO_5]^+$	231,275,342, 412	354.1342	354.1336	1.69	–	–	13-Hydroxyjatrorrhizine
8	15.03	$[C_{21}H_{25}NO_4+H]^+$	–	356.1852	356.1862	−2.81		–	Tetrahydropalmatine
9	15.37	$[C_{20}H_{18}NO_5]^+$	231,264,342, 411	352.1178	352.1179	−0.28	–	–	13-Hydroxyberberine
10	16.60	$[C_{18}H_{16}NO_4]^+$	–	310.1074	310.1074	0.00	–	–	Demethylenethalifendine
11	17.82	$[C_{19}H_{18}NO_4]^+$	232, 275, 342, 411	324.1227	324.1230	−0.93	–	–	Demethyleneberberine
12	18.80	$[C_{21}H_{22}NO_5]^+$	–	368.1501	368.1492	2.44	–	–	13-Hydroxypalmatine
13	20.31	$[C_{19}H_{18}NO_4]^+$	–	324.1227	324.1230	−0.93	–	–	Dehydrodiscretamine
14	21.60	$[C_{19}H_{16}NO_4]^+$	228, 262, 342, 426	322.1068	322.1074	−1.86	–	–	Thalifendine
15	21.78	$[C_{20}H_{21}NO_4+H]^+$		340.1546	340.1543	0.88	–		Tetrahydroberberine
16	23.05	$[C_{20}H_{20}NO_4]^+$	225, 272, 342, 426	338.1389	338.1387	0.59	–	3 82.1285 $[M-2H+HCOOH]^-$	Jatrorrhizine
17	23.72	$[C_{20}H_{20}NO_4]^+$	240, 287, 310sh, 337	338.1384	338.1387	−0.89	–	–	Pseudojatrorrhizine
18	25.41	$[C_{20}H_{18}NO_4]^+$	228, 264, 346, 426	336.1229	336.1230	−0.30	–	–	Berberine
19	27.07	$[C_{21}H_{22}NO_4]^+$	225, 273, 342, 421	352.1544	352.1543	0.28	–	–	Palmatine
20	27.55	$[C_{21}H_{22}NO_4]^+$	239, 287, 310sh, 337	352.1547	352.1543	1.14	–	–	Pseudopalmatine
21	29.31	$[C_{27}H_{44}O_7+H]^+$	249	481.3162	481.3160	0.42	503.2979	525.3056 [M-H+HCOOH]	20-Hydroxyecdyson
22	29.93	$[C_{18}H_{19}NO_4+H]^+$	292,318	314.1374	314.1387	−4.14	336.1202	312.1239 $[M-H]^-$, 358.1295 $[M-H+HCOOH]^-$	1, 3-Dioxolo [4,5] isoquinoline-7-ol-5,6,78-tetrahydro-6-[(methoxyphenyl)methyl]
23	32.66	$[C_{19}H_{19}NO_5+H]^+$	202, 283, 369	342.1331	342.1336	−1.46	364.1153	–	8-Oxodiscretamine
24	34.47	$[C_{19}H_{18}NO_5+H]^+$	202, 222, 333, 369	340.1173	340.1179	−1.76	362.1009	–	8-Oxodehydrodiscretamine
25	35.27	$[C_{20}H_{21}NO_5+H]^+$	202, 282, 369	356.1492	356.1492	0.00	378.1306	–	8-Oxoisocorypalmine
26	36.53	$[C_{20}H_{20}NO_5+H]^+$	202, 222, 334, 370	354.1324	354.1336	−3.39	376.1163	–	8-Oxojatrorrhizine
27	37.46	$[C_{21}H_{23}NO_5+H]^+$	204, 282, 369	370.1648	370.1649	−0.27	392.1470	–	8-Oxotetrahydropalmatine
28	38.16	$[C_{19}H_{17}NO_5+H]^+$	202,291,317, 369	340.1188	340.1179	2.65	362.1011	–	8-Oxotetrahydrothalifendin
29	38.89	$[C_{21}H_{21}NO_5+H]^+$	205,221,333, 369	368.1492	368.1492	0.00	390.1299	–	8-Oxopalmatine
30	39.79	$[C_{20}H_{19}NO_5+H]^+$	-	354.1332	354.1336	−1.13	376.1161	–	8-Oxotetrahydroberberine
31	39.96	$[C_{19}H_{15}NO_5+H]^+$	206, 221, 342, 370	338.1027	338.1023	1.18	360.0894	–	8-Oxothalifendine
32	41.19	$[C_{20}H_{17}NO_5+H]^+$	203, 224, 341, 369	352.1171	352.1179	−2.27	374.0997	–	8-Oxoberberine

Table 3
MS^n data in positive mode of compounds observed in the stem extract of *Coscinium fenestratum*.

Peak No.	HPLC/ESI-MS^n *m/z* (% base peak)
1	MS^2[314.1762]: 271.1322(32), 269.1142(100), 239.1144(7), 237.0890(18), 175.0767(39), 147.0758(15), 145.0650(32), 137.0559(18), 107.0496(23) MS^3[314.1762 → 269.1144]: 175.0732(78), 145.0645(100), 137.0603(44), 107.0493(64)
2	MS^2[344.1846]: 312.1734(5), 301.1450(11), 299.1299(67), 269.1203(8), 267.1000(19), 235.0793(17), 192.0999(6), 177.0898(6), 175.0748(100), 151.0781(9), 143.0485(36), 137.0624(36) MS^3[344.1846 → 175.0748]: 160.0481(38), 143.0490 (100)
3	MS^2[342.1705]: 311.1294(26), 299.1259(19), 297.1117(100), 279.0970(17), 265.0845(53) MS^3[342.1705 → 297.1113]: 282.0889(22), 265.0848(100), 237.0951(18), 207.0779(9) MS^4[342.1705 → 297.1113 → 265.0877]: 250.0658(37), 237.0871(100), 207.0702(37), 191.0825(91)
4	MS^2[342.1686]: 297.1114 (100), 265.0842(56) MS^3[342.1686 → 297.1112]: 265.0851 (100) MS^4[342.1686 → 297.1112 → 265.0848]: 237.0900(100), 233.0613(37)
5	MS^2[356.1858]: 325.1408(14), 311.1282(100), 296.1044(12), 293.1223(6), 279.1018(16), 237.0919(16) MS^3[356.1858 → 311.1265]: 296.1037(100), 279.1029(18), 248.0860(6), 237.0929(32) MS^4[356.1858 → 311.1265 → 296.1038]: 281.0804(100), 250.1026(85), 248.0793(36), 235.0791(63)
6	MS^2[326.1388]: 176.0706(100), 149.0597(32) MS^3[326.1388 → 176.0710]: 176.0580(100), 149.0560(100)
7	MS^2[354.1342]: 339.1096(100), 338.1029(54), 310.1057(41) MS^3[354.1342 → 339.1092]: 338.1013(100), 324.084(8), 322.1064(21), 310.1071(48) MS^4[354.1342 → 339.1092 → 338.1013]: 336.0942(22), 323.0820(100), 320.0833(14), 306.0824(32)
8	MS^2[356.1852]: 192.1017(100), 177.0797(6) MS^3[356.1852 → 192.1017]: 177.0797(100) MS^4[356.1852 → 192.1017 → 177.0797]: 149.0800 (100), 148.0720(80)
9	MS^2[352.1178]: 337.0957(100), 336.0858(76), 308.0922(84) MS^3[352.1178 → 337.0957]: 336.0860(100), 334.0710(9.63), 308.0932(45) MS^4[352.1178 → 337.0957 → 336.0860]: 334.0707(100), 318.0741(47), 316.0581(35), 305.0598(5), 294.0741(7), 292.0584(6)
10	MS^2[310.1074]: 295.0818 (100) MS^3[310.1074 → 295.0839]: 267.0870 (100), 266.0790 (12) MS^4[310.1074 → 295.0839 → 267.087]: 250.0840 (28), 238.0837(100)
11	MS^2[324.1227]: 309.0997(100), 308.0918(28), 280.0984(39) MS^3[324.1227 → 309.0997]: 308.0910(100), 294.0761(42), 280.0949(55) MS^4[324.1227 → 309.0997 → 308.0901]: 306.0751(100)
12	MS^2[368.1501]: 353.1239(100), 352.1180(63), 324.1274(82) MS^3[368.1501 → 353.1254]: 352.1211(100), 324.1258(39) MS^4[368.1501 → 353.1254 → 352.1147]: 350.1015(100), 336.0865(93), 334.1066(33)
13	MS^2[324.1227]: 309.0981(100) MS^3[324.1227 → 309.0981]: 294.0738(100), 281.0997(35), 280.0872(12.27), 266.0794(19) MS^4[324.1227 → 309.0981 → 294.0738]: 266.0791(100), 238.0843(18)
14	MS^2[322.1068]: 307.0836(100) MS^3[322.1068 → 307.0836]: 292.0583(6), 279.0881(100), 278.0821(34), 251.0922(8), 250.0871(7) MS^4[322.1068 → 307.0836 → 279.0881]: 278.0813(100), 263.0615(7), 250.0871(35), 225.0758(15)
15	MS^2[340.1546]: 176.0706(100), 149.0597(5) MS^3[340.1546 → 176.0706]: 149.0614(100)
16	MS^2[338.1389]: 323.1141(100), 322.1077(56), 306.1091(6), 294.1125(69) MS^3[338.1389 → 323.1141]: 322.1074(42), 320.0903(43), 307.0824(100), 294.1133(63), 279.0919(19) MS^4[338.1389 → 323.1141 → 322.1074]: 321.1066(25), 320.0902(30), 307.0843(100)
17	MS^2[338.1384]: 338.1375(19), 323.1153(100), 322.1075(9), 294.1135(12) MS^3[338.1384 → 323.1153]: 323.1152(22), 320.0903(5.48), 308.0908(82), 307.0819(81), 295.1204(100), 294.1122(82), 279.0888(89.59), 278.0989(10), 251.0916(7), 250.0845(8) MS^4[338.1384 → 323.1153 → 295.1204]: 294.1130(100), 279.0898(50), 278.0890(17), 266.1108(6), 251.0924(11), 250.0850(33)
18	MS^2[336.1229]: 321.0987(100), 320.0918(69), 292.0969(90) MS^3[336.1229 → 321.0987]: 320.0895(100), 318.0760(50.89), 292.0960(64) MS^4[336.1229 → 321.0987 → 320.0895]: 318.0757(100)
19	MS^2[352.1544]: 352.1527(92), 337.1305(100), 336.1233(73.77), 320.1259(6), 308.1291(84) MS^3[352.1544 → 337.1305]: 337.1278(36), 334.10536(55), 321.0990(33), 320.0922(100), 308.1288(71), 292.0976(20) MS^4[352.1544 → 337.1305 → 320.0922]: 318.0755(100), 290.0819(6)
20	MS^2[352.1547]: 337.1275(30), 336.1217(100), 308.1270(31) MS^3[352.1547 → 336.1217]: 334.1068(21), 320.0922(100), 292.0977(83) MS^4[352.1547 → 336.1217 → 320.0922]: 318.0759(100), 305.0657(32), 292.0974(27), 290.0842(29), 274.0860(18), 262.0818(11)
21	MS^2[481.3162]: 445.2954(100), 371.2202(86), 303.1899(20) MS^3[481.3162 → 445.2954]: 427.2855(77), 409.2824(15), 371.2212(100), 303.1982(10), 301.1771(10)

Table 3 (*Continued* **).**

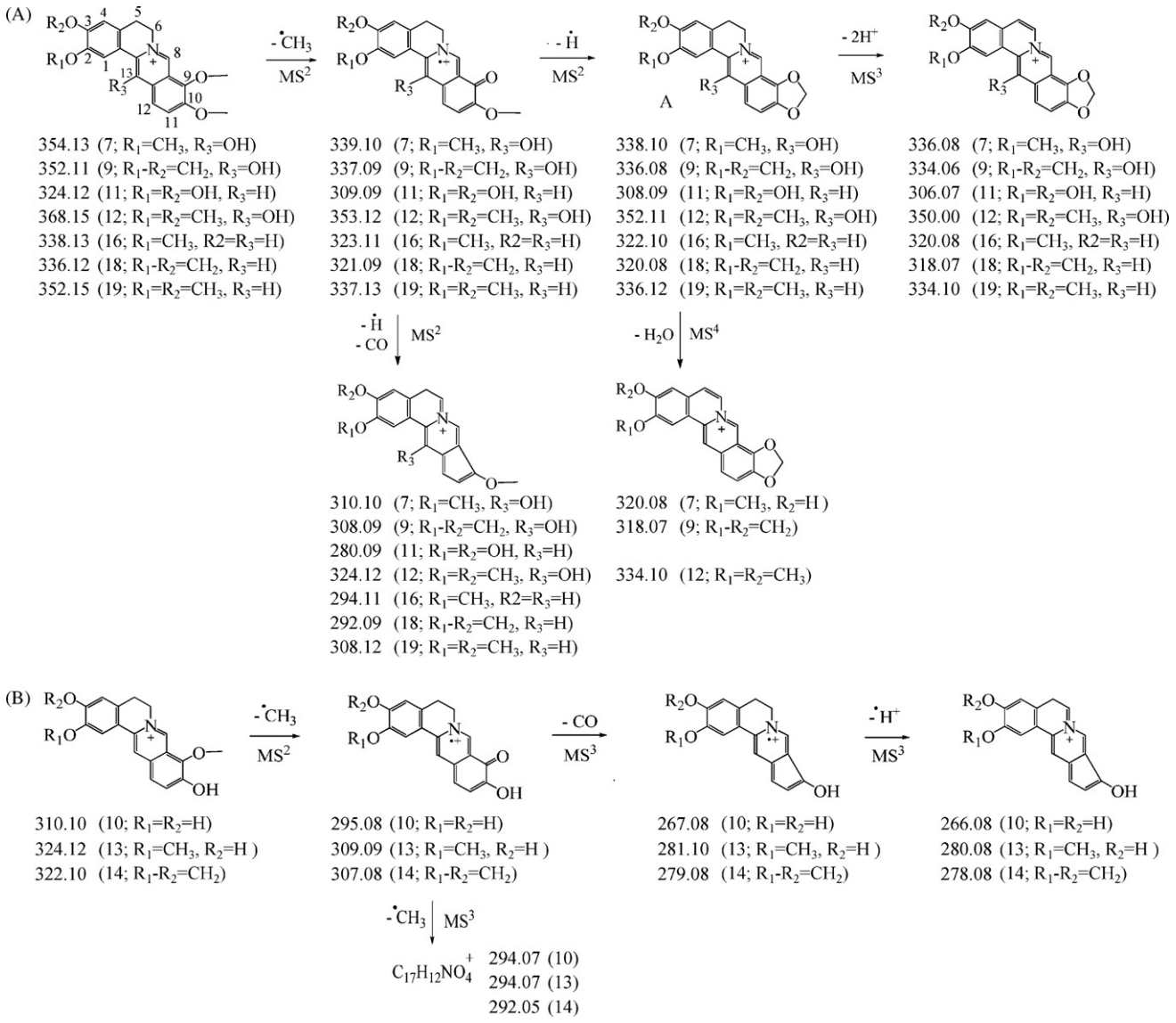
Peak No.	HPLC/ESI-MS^n m/z (% base peak)
22	MS^2[314.1374]: 177.0556(100), 145.0277(15) MS^3[314.1374 → 177.0556]: 145.0276(100), 117.0322(5) MS^4[314.1374 → 177.0556 → 145.0276]: 117.0319(100)
23	MS^2[342.1331]: 327.1092(100), 326.1032(16), 312.0862(11), 310.1058(8), 178.0874 (31) MS^3[342.1331 → 327.1092]: 326.1012(27), 312.0876 (27), 310.1046 (23), 308.0923(55), 296.0949(11), 178.0867(100), 176.0680(8) MS^4[342.1331 → 327.1092 → 178.0867]: 163.0607 (100)
24	MS^2[340.1173]: 325.0951(100) MS^3[340.1173 → 325.0951]: 310.0690(100) MS^4[340.1173 325.0951▫310.069]: 295.0475(100), 282.0743(41), 267.0524(21)
25	MS^2[356.1492]: 341.1260(61), 340.1204(10), 326.1008(20), 308.0942(5), 178.0873(28), 176.0702(100) MS^3[356.1492 → 176.0702]: 161.0467(100), 133.0534(26), MS^4[356.1492 → 176.0702 → 161.0467]: 133.0524(100)
26	MS^2[354.1324]: 339.1099(100), 324.0868(12), 310.1126(5) MS^3[354.1324 → 339.1099]: 324.0861(100), 321.0912(6), 310.1050(33) MS^4[354.1324 → 339.1099 → 324.0861]: 309.0591(17), 296.0916(100), 281.0646(18)
27	MS^2[370.1648]: 355.1396(45), 354.1353(16), 340.1187(23), 324.1191(11), 192.1005(31), 190.0868(100) MS^3[370.1648 → 190.0868]: 175.0622(96), 174.0533(79), 146.0590(100) MS^4[370.1648 → 190.0868 → 175.0622]: 174.0593(100)
28	MS^2[340.1188]: 325.0931(100), 324.0844(12), 310.0701(7), 176.0701(34), MS^3[340.1188 → 325.0931]: 324.0856(26), 310.0694(26), 306.0761(43), 295.0818(12), 176.0706(100), 175.0674(6), 174.053(6) MS^4[340.1188 → 325.0931 → 176.0706]: 174.0593(33), 149.0582(100)
29	MS^2[368.1492]: 353.1258(100), 338.1034(11) MS^3[368.1492 → 353.1258]: 338.1028(100), 324.1214(29.51) MS^4[368.1492 → 353.1258 → 338.1028]: 322.0704(6), 310.1064(100), 294.0748(9)
30	MS^2[354.1332]: 339.1086(39), 338.1059(11), 324.0854(13), 310.1092(5), 176.0718(35), 174.0559(100) MS^3[354.1332 → 176.0706]: 174.0517(100), 118.0650(26), 116.0538(10)
31	MS^2[338.1027]: 323.0784(100) MS^3[338.1027 → 323.0784]: 308.0548 (100) MS^4[338.1027 → 323.0784 → 308.0548]: 280.0599(100), 252.0628(12)
32	MS^2[352.1171]: 337.0951(100), 322.0715(7) MS^3[352.1171 → 337.0951]: 322.0710(100), 319.0841(11), 308.0908(33) MS^4[352.1171 → 337.0951 → 322.0710]: 304.0605(6), 294.0764(100), 276.0678(8)

compounds were considered to be quaternary protoberberine alkaloids with a single hydroxy group at C-9 or C-10. Compound 14 generated an $[M]^+$ ion at m/z 322.1068, suggesting that it is thalifendine or its derivative, berberrubine. To characterize the differences in the behaviors of thalifendine and berberrubine, we synthesized berberrubine by using the method described by Y. Qin et al. [27]. The synthesized dark-red powder was dissolved in methanol (50 μg/ml) and analyzed using the same procedure. The berberrubine peak was the biggest peak (86% of total peak area; 345 nm) with a retention time of 25.34 min, which was close to the berberine peak. The $[M]^+$ ion at m/z 322.1074 was observed as a base peak in the full-scan mode (MS^1) and at m/z 307.0852 in MS^2. In addition, we also observed a small peak (0.2% of total peak area) for an $[M]^+$ at m/z 322.1073 with a retention time of 21.64 min, which was identical to the retention time of compound 14. The small peak was considered to be thalifendine produced as a byproduct from the reaction. Thus, compound 14 was identified as thalifendine [15]. The $[M]^+$ ion of compound 10 had m/z value of 310.1074, which was 14 Da (CH_2) lower than that of demethyleneberberine. Compound 10 followed a primary fragment pathway that was similar to that of thalifendine. Therefore, compound 10 was identified as demethylenethalifendine or demethyleneberberrubine. Both demethylenethalifendine and demethyleneberberrubine are novel natural products that have been reported for the first time in this study. The molecular weight of compound 13 was 2 Da (2H) higher than that of thalifendine. These findings indicated that compound 13 had a methoxy and a hydroxy group at C-2 or C-3 and C-9 or C-10. Therefore, compound 13 was identified as dehydrodiscretamine or its derivative, e.g., jatrorrhizubine or stepharanine. The proposed fragmentation pathways of the quaternary protoberberines with OH groups at C-9 or C-10 are shown in Scheme 1(B).

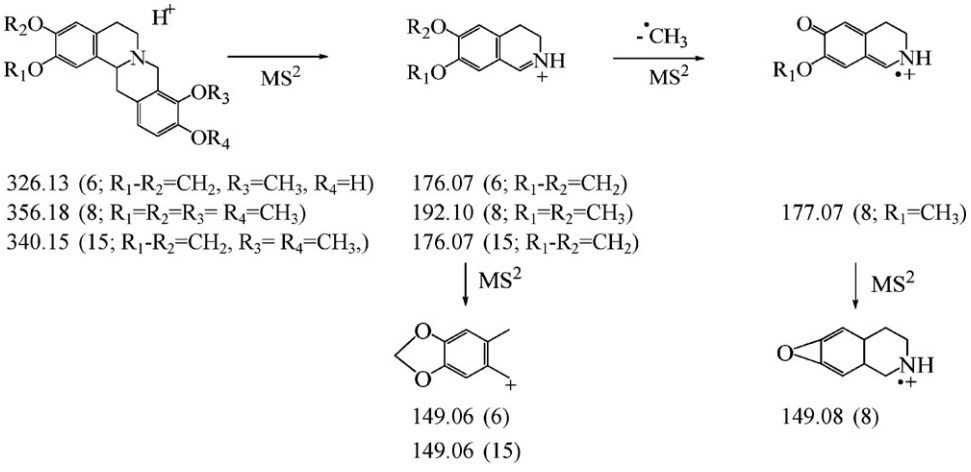
3.2.1.3. Quaternary protoberberine alkaloids with methoxy groups at C-10 and C-11. The peaks for compounds 17 and 20 appeared close to those of jatrorrhizine and palmatine, respectively. Their $[M]^+$ ions (m/z 338.1384 and 352.1547) were identical to those of jatrorrhizine and palmatine. However, their UV spectra exhibited strong maximum absorption at 287 nm and shoulder at 310 nm, which is significantly different from the spectra of quaternary protoberberines with 2 methoxy groups at C-9 and C-10. This spectrum is considered to be the characteristic of pseudoprotoberberine compounds with a methoxy group at C-11 instead of C-9 [26]. Therefore, compound 17 was proposed to be pseudojatrorrhizine and compound 20 was proposed to be pseudopalmatine. Their fragment ions are similar to those of quaternary protoberberines with 2 methoxy groups at C-9 and C-10. However, there were some differences; for example, palmatine and pseudopalmatine showed different base peaks in MS^2, and pseudojatrorrhizine generated an ion at m/z 295.1204 in MS^3, which was not observed in case of jatrorrhizine (Table 3). There are very few reports of online analysis of pseudoprotoberberine in the literature [28].

3.2.2. Tetrahydroprotoberberine alkaloids

Compounds 6, 8, and 15 generated protonated molecules ($[M+H]^+$) in the full-scan mode. The retention time, $[M+H]^+$, and the fragments of compound 15 (m/z 340.1546) were identical to



Scheme 1. Proposed fragmentation pathways of quaternary protoberberine alkaloids. (A) Compounds with 2 methoxy groups at C-9 and C-10; (B) Compounds with single hydroxy group at C-9 or C-10.



Scheme 2. Proposed fragmentation pathways of tetrahydroprotoberberine alkaloids.

those of tetrahydroberberine, which underwent the Retro-Diels-Alder (RDA) fragmentation reaction to produce an iminium ion at *m/z* 176.0706. Therefore, compound 15 was identified as tetrahydroberberine [16]. In case of compound 8, the mass of the $[M+H]^+$ ion at *m/z* 356.1852 and the fragment ion at *m/z* 192.1017 were 16 Da (CH_4) higher than the corresponding mass of tetrahydroberberine, and these mass matched with the corresponding mass of tetrahydropalmatine, which has been previously reported in *Coscinium fenestratum* [18]. Therefore, compound 8 was identified as tetrahydropalmatine. The mass of the $[M+H]^+$ ion of compound 6 was 14 Da (CH_2) lower than the mass of the $[M+H]^+$ ion of tetrahydroberberine. Compound 6 generated fragment ions at *m/z* 176.0706 and *m/z* 149.0597, which were identical to the fragment ions of tetrahydroberberine, suggesting that it contains a methylenedioxy group at C-2 and C-3. Therefore, it was tentatively identified as tetrahydrothalifendine or tetrahydroberberrubine. The fragmentation pathways of tetrahydroprotoberberine are proposed in Scheme 2.

3.2.3. 8-Oxoprotoberberine alkaloids

3.2.3.1. 8-Oxodihydroprotoberberine alkaloids. Compounds 24, 26, 29, 31, and 32 have similar UV absorption characteristics, with maximum absorption at wavelength ranges of 200–205 nm, 220–230 nm, 330–345 nm, and 365–370 nm. Compound 32 generated an $[M+H]^+$ ion at *m/z* 352.1171 (molecular formula, $C_{20}H_{17}NO_5$), and the fragments of this compound were identical to those of 8-oxoberberine. Therefore, compound 32 was identified as 8-oxoberberine [15]. The fragmentation patterns of compound 24 (*m/z*, 340.1173), 26 (*m/z*, 354.1324), 29 (*m/z*, 368.1492), and 31 (*m/z*, 338.1027) were similar to that of 8-oxoberberine. These findings indicated that the compounds 24, 26, 29, and 31 are 8-oxodihydroprotoberberine alkaloids. Compounds 26 and 29 generated $[M+H-{}^{\bullet}CH_3]^{+\bullet}$ and $[M+H-{}^{\bullet}CH_3-{}^{\bullet}H-CO]^+$ in MS^2, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3]^+$ in MS^3, and $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3-CO]^+$ in MS^4. The *m/z* values of the $[M+H]^+$ ion and the corresponding fragment ions of compound 26 and 29 were 2 Da (2H) and 16 Da (CH_4) higher than the corresponding values for 8-oxoberberine. Therefore, compound 29 was identified as 8-oxopalmatine [15] and compound 26 was tentatively characterized as 8-oxojatrorrhizine or 8-oxodihydrocolumbamine (different positions of methoxy and hydroxy group at C-2 and C-3). Compound 26 was considered to be a novel natural product. The oxodihydroprotoberberines with methoxy groups at C-9 and C-10 showed loss of water molecule in MS^2. The fragments of compounds 24 (*m/z* 340.1173) and 31 (*m/z* 338.1027) showed only $[M+H-{}^{\bullet}CH_3]^{+\bullet}$ ions at *m/z* 325.0951 and *m/z* 323.0784, respectively, in MS^2. These fragments were observed to be stable and were difficult to fragment further, indicating that they have only 1 methoxy group at C-9 or C-10. The molecular weight of compound 31 was 14 Da (CH_2) lower than that of 8-oxoberberine. Therefore, compound 31 was tentatively identified as 8-oxothalifendine. The molecular weight of compound 24 was 2 Da (2H) lower than that of compound 31. Therefore, it was tentatively identified as 8-oxodehydrodiscretamine or a derivative of 8-oxodehydrodiscretamine with a methoxy group at C-9 or C-10 and another one at C-2 or C-3. $[M+Na]^+$ was generated by all the 8-oxoprotoberberines (Table 2). The fragmentation pathways of 8-oxodihydroprotoberberine are proposed in Scheme 3 (A).

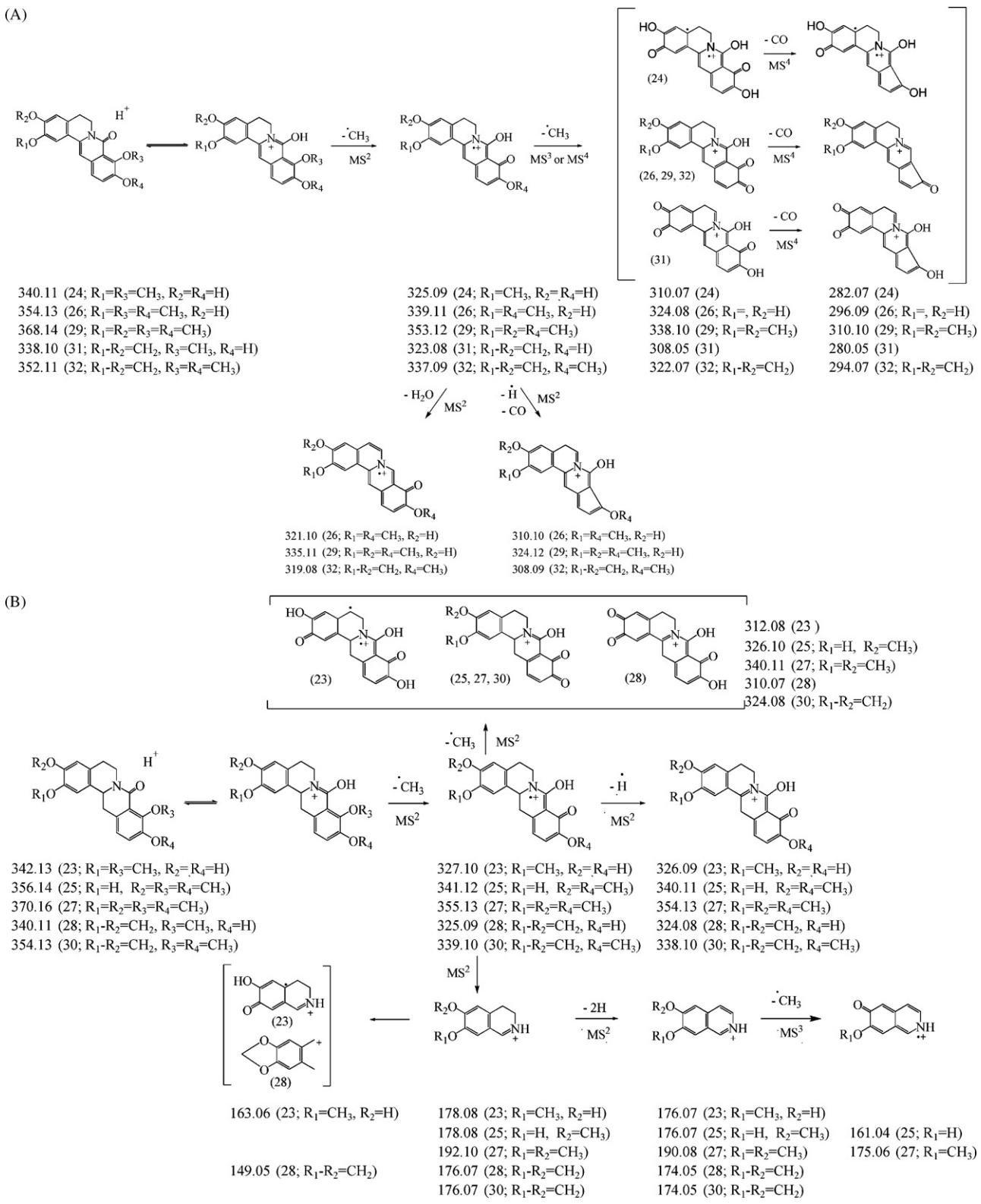
3.2.3.2. 8-Oxotetrahydroprotoberberine alkaloids. Compounds 23, 25, 27, 28, and 30 showed similar fragmentation patterns. The fragment ions at *m/z* 176.07, 178.08, and 192.10, which were iminium ions with methylenedioxy groups, methoxy and hydroxy groups, and dimethoxy groups at C-2 and C-3, suggested the possibility of ring cleavage from the protoberberine skeleton. The fragmentation patterns of these compounds were similar to those of tetrahydroprotoberberine compounds, but $[M+H-{}^{\bullet}CH_3]^{+\bullet}$, $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3]^+$, and $[M+H-{}^{\bullet}CH_3-{}^{\bullet}CH_3-H]^+$ were also observed. The formation of these additional ions could be attributed to the effect of the oxygen atom attached at C-8, which made cleavage more difficult than that in case of tetrahydroprotoberberine. Therefore, compounds 23, 25, 27, 28, and 30 were considered to be 8-oxotetrahydroprotoberberine alkaloids. The iminium ions of compounds 28 and 30 generated ions at *m/z* 176.0706 and *m/z* 176.0718, suggesting that they had methylenedioxy groups at C-2 and C-3. The $[M+H]^+$ ions of compounds 28 and 30, which were observed at *m/z* 340.1188 ($C_{19}H_{17}NO_5$) and *m/z* 354.1332 ($C_{20}H_{19}NO_5$) were identical to those of 8-oxotetrahydrothalifendine and 8-oxotetrahydroberberine (8-oxocanadine), which have been previously isolated from *Coscinium fenestratum* [15,16]. Compounds 23 and 25 may possess hydroxy group and methoxy group at C-2 or C-3 due to their iminium ions, which were observed at *m/z* 178.0874 and *m/z* 178.0873. In case of compound 25, the $[M+H]^+$ ion observed at *m/z* 356.1492 was identical to the $[M+H]^+$ ion of 8-oxoisocorypalmine, which has been reported in *Coscinium* fenestratum [15]. Compound 23 was characterized as 8-oxodiscretamine or a derivative of 8-oxodiscretamine with a methoxy group at C-9 or C-10. There are no reports of 8-oxodiscretamine alkaloids in literature. Therefore, compound 23 is considered to be a new compound. Compound 27 ($C_{21}H_{23}NO_5$) was identified as 8-oxotetrahydropalmatine. The proposed fragmentation pathways of 8-oxotetrahydroprotoberberines are summarized in Scheme 3 (B).

3.2.4. Aporphine alkaloids, benzylisoquinoline alkaloids, and a steroid compound

Aporphine alkaloids and steroid compounds have been suggested to be present in *Coscinium fenestratum*. However, only *N,N*-demethyllindcarpine (MW 341), which is an aporphine alkaloid, has been actually reported in literature [17], and there are no reports of identification of steroid compounds. As shown in Table 3, compounds 3, 4, 5 showed molecular ions at *m/z* 342.1705, *m/z* 342.1686, and *m/z* 356.1858 and generated fragment ions $[M-(CH_3)_2NH]^+$ and $[M-(CH_3)_2NH-CH_3]^+$, and $[M-(CH_3)_2NH-CH_3OH]^+$ in MS^2. These findings are considered to be a characteristic of aphorphine alkaloids. The UV absorption spectra and the mass fragments of compound 3 and 5 were similar to those of magnoflorine and menisperine [4]. Thus, compounds 3 and 5 were tentatively identified as magnoflorine and menisperine, respectively. Compound 4 generated $[M]^+$ ions and fragments similar to those of magnoflorine, indicating that it is a derivative of magnoflorine, namely, *N,N*-demethyllindcarpine. Therefore, compound 4 was identified as *N,N*-demethyllindcarpine, which has been previously reported in *Coscinium* fenestratum. The fragmentation pathways of aporphine alkaloids are proposed in Supplementary scheme (A).

Compound 2 produced $[M]^+$ ions at *m/z* 344.1846 in MS^1 and the fragment ion $[M-(CH_3)_2NH]^+$ as a major peak in MS^2. The formation of fragment ions at *m/z* 175.0748, *m/z* 151.0781, and *m/z* 143.0485 suggested a split in the skeleton structure. Compound 2 is considered to be a benzylisoquinoline alkaloid. The UV and mass spectra of this compound are similar to those of tembetarine [4]. Therefore, it was tentatively identified as tembetarine. The $[M]^+$ ion and principal fragments of compound 1 were 30 Da (CH_2O) lower than that of tembetarine. It was considered to be a derivative of compound 2. The fragmentation pathways of benzylisoquinoline alkaloids are proposed in Supplementary scheme (B).

The retention time, UV spectra, and fragmentation pattern of compound 21 were identical to those of 20-hydroxyecdysone (Table 2), which lost up to 3 water molecules in MS^1. Accordingly, compound 20 was identified as 20-hydroxyecdysone. The fragmentation pathway is proposed in Supplementary scheme (C).



Scheme 3. Proposed fragmentation pathways of 8-oxoprotoberberine alkaloids. (A) 8-Oxodihydroprotoberberines; (B) 8-Oxotetrahydroprotoberberines.

3.2.5. Structural identification of compound 22 (1,3-Dioxolo[4,5-g]isoquinolin-7-ol,5,6,7,8-tetrahydro-6-[(methoxyphenyl)methyl]-).

Fig. 3 shows the MS^n mass spectra of compound 22 in the positive and negative modes. In the full-scan mode, the spectrum showed the presence of an ion at *m*/*z* 314.1374 in the positive mode and at *m*/*z* 312.1249 in the negative mode. Using the accurate mass calculator software, we obtained the chemical formula $C_{18}H_{19}NO_4$ for the compound at *m*/*z* 314.1374. The fragments of the ion at *m*/*z* 314.1374, which was obtained in the positive mode, showed only 2 intensity peaks at *m*/*z* 177.0556 ($C_{10}H_9O_3^+$) and *m*/*z* 145.0277 ($C_9H_5O_2^+$) in MS^2, suggesting a split in the structural

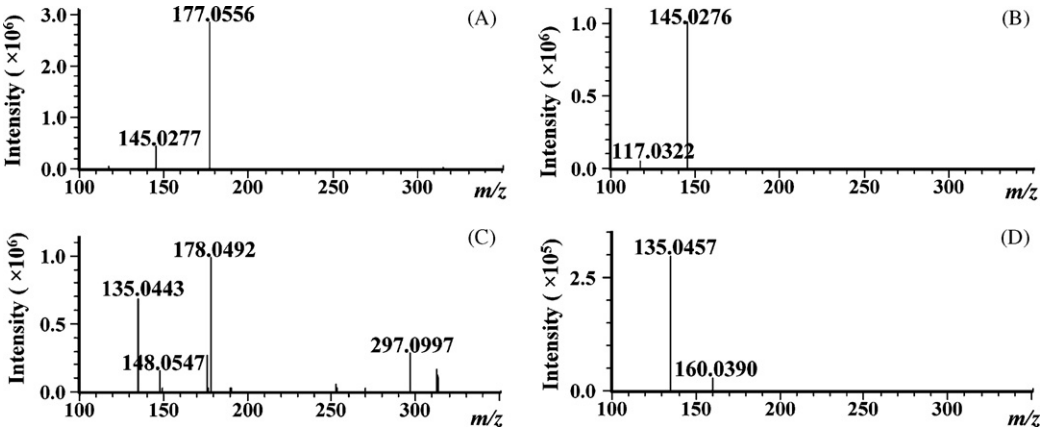
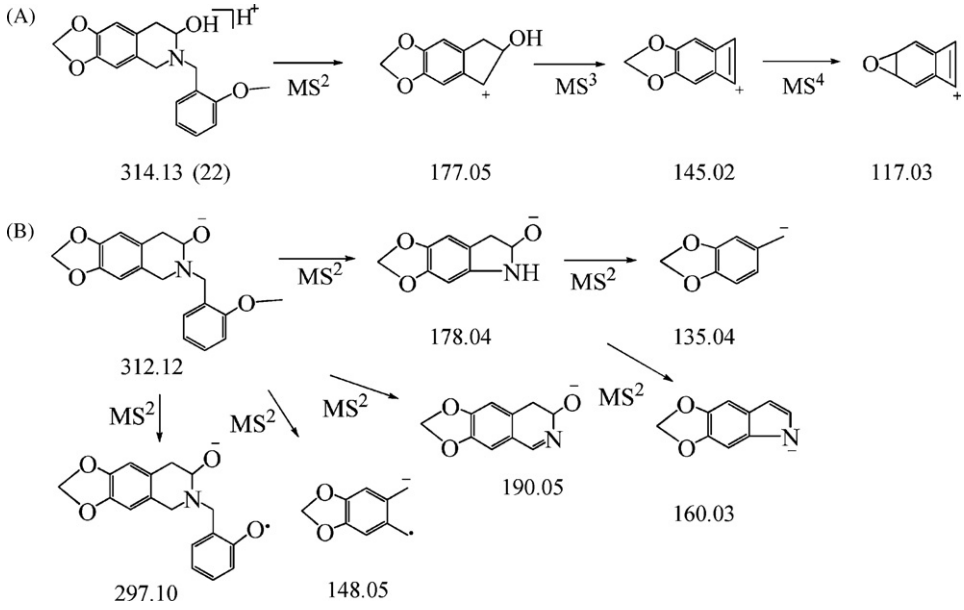


Fig. 3. MS^n data of peak No. 22. (A) MS^2 of ion at *m/z* 314.1374 ((+)ESI); (B) MS^3 of ion at 177.0556 ((+)ESI); (C) MS^2 of ion at *m/z* 312.1249 ((−)ESI); (D) MS^3 of ion at *m/z* 178.0492 ((−)ESI).

skeleton. The ion at *m/z* 177.0556 produced fragment ions at *m/z* 145.0276 and *m/z* 117.0322 ($C_8H_5O^+$). Considering the compounds found in *Coscinium fenestratum*, most of them are alkaloids that have aromatic rings. There is high possibility that the compound 22 at *m/z* 314.1374 has aromatic rings. The number of rings or unsaturations of a molecule or of a fragment can be obtained by using the double-bond equivalent values (DBE ($C_cH_hN_nO_oS_s$) = $c-(h/2)+(n/2)+1$), which is calculated from the molecular formula of the ion [29]. The odd-electron radical cations have integer DBE values, whereas even-electron ions have half integer DBE values. The fragment ion at *m/z* 145.0277 ($C_9H_5O_2^+$, DBE 7.5) indicates that its possible structure contains a benzene ring (DBE 4) and 2 other rings (DBE 2), one of the rings has a double bond (DBE 1). This ion suggests that it was formed by losing of CH_3OH molecule and generated a double bond from the ion at *m/z* 177.0556 ($C_{10}H_9O_3^+$, DBE 6.5). The DBE 6.5 of $C_{10}H_9O_3^+$ supports the hypothesis that compound 22 has a benzene ring (DBE 4) and other 2 rings (DBE 2). These fragments indicated that the compound has a benzene ring connected to a ring from a methylenedioxy group and a ring that has a hydroxy group. In contrast, fragmentation of the ion at *m/z* 312.1249 in the negative mode principally yielded ions at *m/z* 178.0492 ($C_9H_8NO_3^-$) and *m/z* 135.0443 ($C_8H_7O_2^-$). The formula $C_9H_8NO_3^-$ indicated that compound 22 was a tetrahydroisoquinoline compound. Therefore, compound 22 was tentatively identified as a tetrahydroisoquinoline compound with a hydroxy group at C-3. The hydroxy group was considered to be at the C-3 position, because the possibility of the formation of ions at *m/z* 177.0556, *m/z* 145.0277, and *m/z* 117.0322 is lower if it was at any other position. The fragmentation pathway is proposed in Scheme 4. Although we could not determine the position of the methoxy group in the phenyl ring, compound 22 was considered to be a novel natural product.

Among the 32 compounds characterized in this study, 12 compounds, namely, berberine, palmatine, jatrorrhizine, thalifendine, tetrahydroberberine, tetrahydropalmatine, *N,N*-demethylindcarpine, 8-oxoberberine, 8-oxopalmatine, 8-oxotetrahydrothalifendine, 8-oxoisocorypalmine, and 8-oxotetrahydroberberine have been isolated previously[15-18]. The other 20 compounds have been reported for the first time from *Coscinium* fenestratum in this study. To our knowledge, compounds 10, 22, 23, and 26 are the novel natural products tentatively identified in this study. The structural conformation of these compounds will be confirmed by NMR spectroscopy in future work. However, some compounds that have been reported to be present in *Coscinium* fenestratum, such as oxothaicanine (MW 385) [15] and β-sitosterol (MW 413), were



Scheme 4. Proposed fragmentation pathway of compound 22.

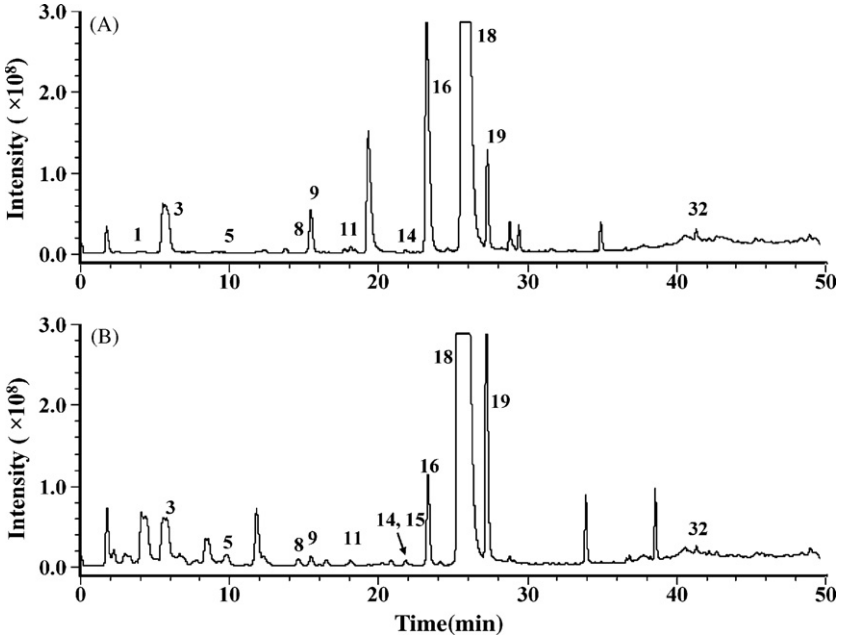


Fig. 4. (+)ESI Total ion chromatogram of *Coptis japonica (A)* and *P. amurense* (B) (2 μl injected). 0.5 g of each dried material was extracted in 100 ml methanol using Soxhlet extraction. Extract was diluted 4 times for *Coptis japonica* and 2 times for *P. amurense* in methanol before LC/IT-TOF MS analysis.

not found, although the standard sample of β-sitosterol could be detected at a retention time of 38.76 min under the experimental conditions used in this study.

3.3. Comparison of compounds in Coscinium fenestratum with those in Coptis japonica and P. amurense

Coptis japonica and *P. amurense* have been widely used in Japanese and Chinese herbal medicine. However, HPLC and LC/MS/MS analyses of these plants have been restricted to the investigation of the major quaternary protoberberine compounds such as berberine, palmatine, jatrorrhizine, coptisine, and epiberberine [25,30]. However, for the quality control of these herbs, it is essential to develop a technique for accurate simultaneous determination of both major and minor compounds. We used the method developed in this study to analyze both minor and major alkaloids in *Coptis* japonica and *P. amurense*. Fig. 4 shows the total ion chromatogram of the extract of *Coptis japonica* and *P. amurense*. There were 11 compounds in *Coptis japonica* (compounds 1, 3, 5, 8, 9, 11, 14, 16, 18, 19, and 32) and *P. amurense* (compounds 3, 5, 8, 9, 11, 14, 15, 16, 18, 19, and 32) that were identical to those in *Coscinium* fenestratum. These compounds were the tembetarine derivative, magnoflorine, menisperine, tetrahydropalmatine, 13-hydroxyberberine, demethyleneberberine, thalifendine, tetrahydroberberine, jatrorrhizine, berberine, palmatine, and 8-oxoberberine. All these compounds, except for the tembetarine derivative, demethylenberberine, 13-hydroxyberberine, have been previously isolated from *Coptis japonica* and *P. amurense*. 8-Oxoberberine was detected in both *Coptis japonica* and *P. amurense* at a retention time of 41.19 min. Thus, the technique can be applied to characterize oxoprotoberberine compounds in other medicinal plants or herbal medicines. This evidence also suggests that *Coscinium* fenestratum can be potentially used as alternative for *Coptis japonica* and *P. amurense*, although further studies on the biological activities of other minor compounds may be required.

4. Conclusions

In this study, we developed a method for simultaneous characterization of quaternary alkaloids, 8-oxoprotoberberine alkaloids, and a steroid compound in *Coscinium fenestratum* by using LC/IT-TOF MS. A total of 32 compounds were detected, of which 20 compounds, including 4 novel natural products, were identified or tentatively identified for the first time from *Coscinium fenestratum*. This information may be useful for further studies on the pharmacological activities of the herb. The proposed method is an accurate and rapid method for characterizing various compounds in *Coscinium* fenestratum. 8-oxoprotoberberines produced $[M+H]^+$ and $[M+Na]^+$ ions in MS^1 operated in the positive-ion mode. The fragmentation pathways of 8-oxoprotoberberines are different from those of quaternary protoberberines and tetrahydroprotoberberines. In addition, 8-oxotetrahydroprotoberberines generated iminium ions, which were formed by the cleavage of the protoberberine skeleton. This method can also be applied to identify 8-oxoberberine in other medicinal plants such as *Coptis* japonica and P. amurense. This study suggests that LC/IT-TOF MS has a great potential in the simultaneous analysis of secondary metabolites in medicinal plants as well as herbal medicines.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.jpba.2009.05.023.

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