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Original Research Article

Analysis of isoquinoline alkaloids from *Mahonia leschenaultia* and *Mahonia napaulensis* roots using UHPLC-Orbitrap-MS^n and UHPLC-QqQ$_{LIT}$-MS/MS

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ABSTRACT

Mahonia leschenaultia (*ML*) and *Mahonia napaulensis* (*MN*) are less known and unexplored medicinal plants of the family Berberidaceae. They are used by the Todas of Nilgiris in their religious and medical practices but chemically less identified. Hence, we decided to do extensive phytochemical analysis to explore the potential of these plant extracts. An ultrahigh performance electrospray tandem mass spectrometry (UHPLC–ESI–MS/MS) method was successfully developed for qualitative analysis of the bioactive components in Mahonia species using Orbitrap Velos Pro mass spectrometer. Sixteen compounds were identified by comparison of their retention times and mass spectra (MS) with authentic standards and reported literature. Multi-stage mass spectra (MS^{2-8}) for the identification of protoberberine and aporphine alkaloids showed the sequential expulsion of all the substituents attached with their basic skeleton followed by CO loss. Eight of the identified compounds (berberine, jatrorrhizine, palmatine, magnoflorine, isocorydine, glaucine, tetrahydropalmatine and tetrahydroberberine) were simultaneously determined by another UHPLC–ESI–MS/MS method under the multiple reactions monitoring (MRM) mode quantitatively using triple quadrupole linear ion trap mass spectrometer. The analytical method was validated for 8 bioactive compounds with overall recovery in the range 98.5%–103.6% (RSD≤2.2%), precise (RSD≤2.07%) and linear (r≥0.9995) over the concentration range of 0.5–1000 ng/mL and successfully applied in *ML* and *MN* roots, which suggests the suitability of the proposed approach for the routine analysis of Mahonia species and their quality control.

1. Introduction

Mahonia leschenaultia (Wight & Arn.) Takeda ex Gamble (*ML*) and *Mahonia napaulensis* DC (*MN*) belong to the family Berberidaceae and are the less known and unexplored medicinal plants of the genus Mahonia. Most of the *Mahonia* species are evergreen or semi-evergreen shrubs or small trees commonly distributed in the Himalayan region [1,2]. The principal chemical constituents reported for the genus Mahonia consist of isoquinoline alkaloids, mainly protoberberine (PBAs), benzylisoquinoline, and aporphine groups [3,4]. Usually, the need for phytochemical investigation of the plants is always a prerequisite for the search of new sources of medicinal plants [5–7]. The following isoquinoline alkaloids, namely berberine, jatrorrhizine, palmatine, magnoflorine, isocorydine and oxyacanthine, were previously isolated from different Mahonia species (Fig. 1) [3,4]. These constituents have been reported to possess an array of biological activities such as anti-oxidant, anti-hyperglycaemic, anti-inflammatory, hepato-protective and hypotensive properties [8–12]. The root and stem of *ML* and *MN* are a good source of berberine having antitumor activity and are also used as antimutagenic, stomachic, diaphoretic, astringent, gentle aperient, curative of piles and periodic neuralgia [11]. These plants are also used for antifungal textile dyeing based on their antifungal and anti-bacterial activity [8].

Mass spectrometry has become an indispensable tool in the investigation of the structures of molecules in complex mixtures of natural product extracts. The combination of a linear ion trap with a Fourier transform ion cyclotron resonance mass spectrometer (FTICR–MS) has become a popular choice for the characterization of chemical constituents [13–15]. Orbitrap Velos mass spectrometry is a high performance MS and MS^n technique which combines the rapid ion trap

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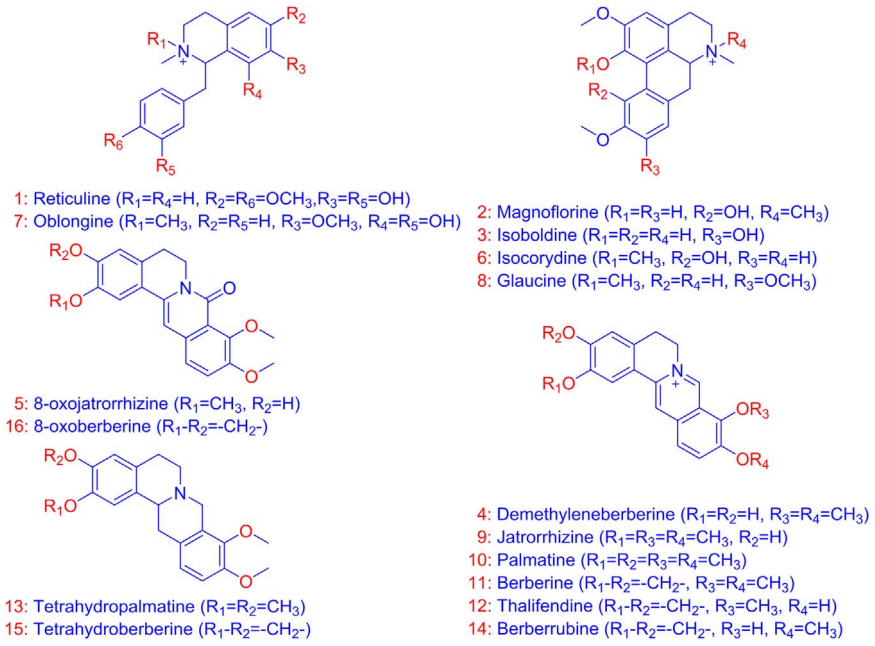

Fig. 1. Chemical structure of isoquinoline alkaloids.

Table 1
The optimized compound dependent MRM parameters and transitions for each analyte in the UPLC–ESI–MS/MS analysis.

Peak No.	RT (min)	Analyte	Precursor ion (*m/z*)	DP (V)	EP (V)	CE (eV)	Quantifier[a]	Qualifier[a]
1	1.49	Magnoflorine	342.1 $[M]^+$	50	10	27	342.1→296.7 (20)	342.1→282.0 (12)
2	1.71	Isocorydine	342.1 $[M+H]^+$	73	4.5	27	342.1→279.2 (12)	342.1→311.1 (8)
3	1.90	Glaucine	356.3 $[M+H]^+$	101	8	20	356.3→325.3 (16)	356.3→294.1 (15)
4	2.00	Jatrorrhizine	338.0 $[M]^+$	50	10	55	338.0→307.3 (15)	338.0→322.1 (11)
5	2.21	Tetrahydropalmatine	356.2 $[M+H]^+$	86	7	35	356.2→192.1 (7)	356.2→165.1 (5)
6	2.34	Tetrahydroberberine	340.0 $[M+H]^+$	55	10	35	340.0→176.0 (9)	340.0→149.1 (5)
7	2.50	Palmatine	352.2 $[M]^+$	32	10	40	352.2→336.0 (15)	352.2→308.1 (16)
8	3.11	Berberine	336.0 $[M]^+$	40	10	45	336.0→320.0 (5)	336.0→292.2 (8)

RT: Retention time; DP: Declustering potential; EP: Entrance potential; CE: Collision energy.

[a] Cell exit potential (CXP in V) is given in brackets

(IT) data acquisition with the high mass accuracy [16]. Consequently, it can perform two types of data acquisition mode, FT–MS and IT–MS. In addition, it can perform multiple gas-phase fragmentation techniques such as collision induced dissociation (CID) and higher-energy C-trap dissociation (HCD), both of which offer versatility and facilitated structural characterization [17,18]. These alternative fragmentation capabilities are essential for proposing fragmentation pathways of compounds. Similarly, the combination of triple quadrupole/linear ion trap (QqQ_{LIT}) analyzers provides rapid quantification of multiple components in a complex mixture using multiple reactions monitoring (MRM) analysis [5,19]. Therefore, the UHPLC-Orbitrap-MS^n and UHPLC-QqQ_{LIT}-MS/MS methods were developed for the investigation of isoquinoline alkaloids in crude extracts of *ML* and *MN* roots.

2. Experimental

2.1. Plant materials

Mahonia leschenaultia (*ML*) and *Mahonia napaulensis* (*MN*) roots were collected from Nilgiri region of India, and voucher herbarium specimens (Nos. 254044 and 254043 for *ML* and *MN*, respectively) were maintained and deposited in the Herbarium of National Botanical Research Institute (NBRI), Lucknow, Uttar Pradesh, India. The identity of these vouchers was matched with the available vouchers of both the plant species.

2.2. Chemicals and solvents

AR grade ethanol (Merck, Darmstadt, Germany) was used in the preparation of ethanolic extract. LC–MS grade acetonitrile, methanol and formic acid (Sigma Aldrich, St Louis, MO, USA) were used in mobile phase and sample preparation. Ultra-pure water (Type 1) was obtained from Direct-Q system (Millipore, Milford, MA, USA). The standard reference samples of berberine hydrochloride (10 mg; purity ≥96%), palmatine chloride (10 mg; purity≥97%), jatrorrhizine hydrochloride (10 mg; purity≥97%), magnoflorine iodide (10 mg; purity≥98%) and *D*-tetrahydropalmatine (THP) (10 mg; purity≥98%) were purchased from Shanghai Tauto Biotech Co., Ltd (Shanghai, China). Glaucine HBR (25 mg; lot no. 00007241-807; purity≥94.9%), tetrahydroberberine (THB) (10 mg; lot no. 00020155–02082007; purity≥98.6%), and isocorydine hydrochloride (10 mg; lot no. 00009230-213; purity≥99.9%) were purchased from ChromaDex (Irvine, California, USA).

2.3. Extraction and preparation of sample

1 g powder of shade dried plant materials of *ML* and *MN* roots (pooled five plant both) was suspended with 20 mL ethanol (100%), sonicated for 30 min at 25 °C in an ultrasonic water bath (Bandelin SONOREX, Berlin) and left for 24 h at room temperature. The extract

Table 2
HCD–MS/MS and CID–MS^n (n=2–8) data obtained in FT and IT acquisition modes of Orbitrap-MS for standard compounds by direct infusion analysis.

Compounds	HCD–MS/MS data in FT–MS mode	CID–MS^n data in IT–MS mode
Berberine *m/z* 336.1230 $[M]^+$	336.1220 (CE 50): 321.07(40),320.0905 (100), 306.0752 (40),304.0604 (28), 292.0961 (77),278.0808 (10), 275.0941 (1)	MS^2 [336 (CE 30)]: 321, 306, 292 MS^3 [336→321 (CE 30)]: 320, 318, 304, 292 MS^4 [336→321→320 (CE 38)]: 318, 290 MS^4 [336→321→ 292 (CE 42)]: 277, 264, 262, 249, 234 MS^4 [336→321→304 (CE 45)]: 289, 274, 248 MS^5 [336→321→320→318 (CE 40)]: 290, 274, 262 MS^5 [336→321→ 292 →277 (CE 35)]: 249, 219 MS^5 [336→321→ 292 →264 (CE 40)]: 249, 234 MS^5 [336→321→304→289 (CE 35)]: 260 MS^5 [336→321→320→290 (CE 38)]: 245, 262 MS^6 [336→321→320→318→290 (CE 40)]: 275, 262 MS^6 [336→321→ 292 →277→249 (CE 35)]: 248, 218 MS^7 [336→321→320→318→290→262 (CE 30)]: 261, 232, 204, 192 MS^7 [336→321→ 292 →277→249→248 (CE 35)]: 218
Jatrorrhizine *m/z* 338.1387 $[M]^+$	338.1377 (CE 50): 323.1131 (50),322.1061 (100), 308.0909 (60),306.0756 (28), 294.1128 (70),280.0959(11), 279.090 (10),265.0740 (1)	MS^2 [338 (CE 32)]: 323, 322, 294 MS^3 [338→323 (CE 30)]: 322, 294 MS^4 [338→323→322 (CE 33)]: 320, 307 MS^4 [338→323→294 (CE 33)]: 279 MS^5 [338→323→322→307 (CE 33)]: 306, 305, 279 MS^5 [338→323→ 294 →279 (CE 33)]: 251, 250 MS^6 [338→323→322→307→279 (CE 32)]: 278, 276, 262, 251 MS^6 [338→323→ 294 →279→251 (CE 35)]: 250, 234, 222 MS^7 [338→323→ 294 →279→251→250 (CE 33)]: 233, 232, 222
Palmatine *m/z* 352.1543 $[M]^+$	352.1532 (CE 50): 337.1315 (15),336.1216 (100), 322.1060 (42),320.0917 (15), 308.1270 (50),294.1113 (8), 292.0960 (5)	MS^2 [352 (CE 33)]: 337, 336, 308 MS^3 [352→337 (CE 33)]: 336, 320, 308 MS^4 [352→337→336 (CE 33)]: 334, 321, 320, 292 MS^4 [352→337→320 (CE 33)]: 318, 304 MS^4 [352→337→ 308 (CE 35)]: 293, 292, 264 MS^5 [352→337→336→321 (CE 33)]: 320, 318 MS^5 [352→337→ 336 →334 (CE 35)]: 290 MS^5 [352→337→320→318 (CE 38)]: 316, 290, 274 MS^5 [352→337→ 308 →293 (CE 35)]:292, 265, 264 MS^6 [352→337→336→321→320 (CE 32)]: 318 MS^6 [352→337→ 336 →334→290 (CE 35)]: 262 MS^6 [352→337→320→318→290 (CE 38)]: 288, 262 MS^6 [352→337→ 308 →293→292 (CE 38)]: 277, 262, 246 MS^6 [352→337→ 308 →293→265 (CE 38)]: 264 MS^7 [352→337→336→321→320→318 (CE 40)]: 290 MS^7 [352→337→ 308 →293→265→264 (CE 35)]: 249, 236, 208 MS^8 [352→337→336→321→320→318→290 (CE 35)]: 262
Tetrahydroberberine *m/z* 340.1542 $[M+H]^+$	340.1534 (CE 45): 324.1224 (4),176.0704 (100), 174.0537 (4),149.0594 (9)	MS^2 [340 (CE 28)]: 176, 149, 119 MS^3 [340→176 (CE 33)]: 161, 159, 149, 146 MS^3 [340→149 (CE 25)]: 119, 91, 77 MS^4 [340→176→149 (CE 25)]: 119, 91, 77
Tetrahydropalmatine *m/z* 356.1855 $[M+H]^+$	356.1853 (CE 42): 340.1535 (4),192.1018 (100), 190.0868 (3),165.0910 (25), 150.0675 (6)	MS^2 [356 (CE 32)]: 339, 192, 190, 165, 150 MS^3 [356→192 (CE 32)]: 177, 176, 148 MS^3 [356→165 (CE 32)]: 150, 135, 133, 119 MS^4 [356→192→177 (CE 30)]: 176, 174, 162, 160, 159, 148
Magnoflorine *m/z* 342.1704 $[M]^+$	342.1691 (CE 40): 297.1114 (100),282.0876 (14), 265.0856 (78),237.0905 (8), 219.0801 (2),58.0664 (80)	MS^2 [342 (CE 30)]: 297, 265 MS^3 [342→297 (CE 28)]: 282, 265 MS^4 [342→297→282 (CE 30)]: 267, 264 MS^4 [342→297→265 (CE 30)]: 250, 247, 237 MS^5 [342→297→282→267 (CE 30)]: 249, 239 MS^5 [342→297→265→250 (CE 30)]: 222 MS^5 [342→297→265→247 (CE 30)]: 219, 203 MS^5 [342→297→265→237 (CE 30)]: 222, 219, 209, 191 MS^6 [342→297→282→267→239 (CE 30)]: 221, 211, 193 MS^6 [342→297→265→250→222 (CE 30)]: 194 MS^6 [342→297→265→247→219 (CE 30)]: 191 MS^6 [342→297→265→237→209 (CE 30)]: 194, 191, 181 MS^7 [342→297→265→250→222→194 (CE 30)]: 193, 166, 165 MS^7 [342→297→265→247→219→191 (CE 33)]: 189, 165
Isocorydine *m/z* 342.1705 $[M+H]^+$	342.1694 (CE 42): 311.1260 (18),296.1016 (15), 279.0989 (100),264.0757 (24), 248.0809 (23), 236.0807 (13), 219.0793 (4)	MS^2 [342 (CE 25)]: 311, 297, 279 MS^3 [342→311 (CE 25)]: 296, 279 MS^4 [342→311→296 (CE 25)]: 296, 281 MS^4 [342→311→279 (CE 28)]: 264, 248 MS^5 [342→311→296→281 (CE 25)]: 263, 253, 204 MS^5 [342→311→279→264 (CE 32)]: 236 MS^6 [342→311→296→281→263 (CE 25)]: 235 MS^6 [342→311→279→264→236 (CE 32)]: 208, 206, 178 MS^7 [342→311→296→281→263→235 (CE 30)]: 207, 179
Glaucine *m/z* 356.1856 $[M+H]^+$	356.1840 (CE 25): 325.1422 (100),310.1186 (25), 294.1238 (40)	MS^2 [356 (CE 32)]: 325 MS^3 [356→325 (CE 25)]: 310, 294 MS^4 [356→325→310 (CE 28)]: 295

Table 2 *(continued)*

Compounds	HCD–MS/MS data in FT–MS mode	CID–MS^n data in IT–MS mode
		MS^4 [356→325→294 (CE 30)]: 279 MS^5 [356→325→310→295 (CE 30)]: 277, 267, 235 MS^5 [356→325→294→279 (CE 30)]: 251 MS^6 [356→325→310→295→277 (CE 30)]: 262, 249, 234 MS^6 [356→325→310→295→267 (CE 30)]: 252, 239, 224, 208 MS^6 [356→325→294→279→251 (CE 30)]: 236, 220 MS^7 [356→325→310→295→277→262 (CE 30)]: 234 MS^7 [356→325→310→295→277→249 (CE 30)]: 234, 206 MS^7 [356→325→310→295→267→239 (CE 33)]: 224, 208 MS^7 [356→325→294→279→251→236 (CE 30)]: 221, 219, 218, 208, 207 MS^8 [356→325→310→295→267→239→224 (CE 30)]: 209

was collected and filtered through filter paper (Whatman No. 1) and the residue was re-extracted three times with fresh solvent following the same procedure. The combined filtrates of each sample were concentrated using a Buchi rotary evaporator (Flawil, Switzerland) under reduced pressure at 20–50 kPa at 40 °C yielding dark yellow-brown mass which was stored at −20 °C. A fresh solution (1 mg/mL) of each sample was prepared in methanol and filtered through a 0.22 μm polyvinylidene difluoride (PVDF) membrane (MILLEX GV filter unit, Merck Millipore, Darmstadt, Germany).

2.4. Preparation of standard solutions

Stock solutions of eight reference standards (berberine, palmatine, jatrorrhizine, tetrahydroberberine, tetrahydropalmatine, magnoflorine, isocorydine and glaucine) were prepared separately in methanol (1.0 mg/mL). Then, methanol stock solution containing the mixture of eight analytes was prepared and diluted in appropriate concentration to yield a series of concentrations from 0.5 to 1000 ng/mL. The calibration curves were constructed by plotting the value of peak areas versus the value of concentrations of each analyte. All stock solutions were stored in the refrigerator at −20 °C until use.

2.5. UHPLC-Orbitrap-MS^n conditions for qualitative analysis

Qualitative analyses were performed with an Orbitrap Velos Pro™ system, which is a hybrid ion trap-orbitrap mass spectrometer (Thermo Scientific; Bremen, Germany) equipped with an electrospray ion source which was hyphenated to an Accela UHPLC (Thermo Scientific; Bremen, Germany). Accela UHPLC system consisted of an Accela PDA, Accela open AS and Accela 1250 pump system.

Chromatographic separation was carried out on a Thermo Scientific Hypersil GOLD column (100 mm×2.1 mm, 1.9 μm) operated at 20 °C. The mobile phase, which consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B), was delivered at a flow rate of 0.4 mL/min under a gradient program: 5%–15% (B) from 0 min to 3 min, 15%–60% (B) from 3 min to 8 min, 60%–90% (B) from 8 min to 10 min, and return to its initial condition over 4 min. The sample injection volume was 2 μL. The UV spectra were obtained by scanning the samples in the range of 200–600 nm.

Conditions for the ESI positive ion mode were as follows: capillary temperature, 320 °C; sheath gas flow rate, 10 arb; auxiliary gas flow rate, 5 arb; source voltage, 4 kV; source current, 100 μA; S-lens RF level, 67.50%; lens 0 voltage, −7.13 V; lens 1 voltage, −12.13 V; gate lens offset, −90 V; and front lens voltage, −13.67 V. Nitrogen was used as the sheath and auxiliary gas, and helium as the collision gas. The MS detector was programmed to perform a full scan and a data-dependent scan. For the full scan MS analysis, the spectra were recorded in the range of *m/z* 50–1000 and the FT resolution at 60000 (FWHM). The data-dependent MS^n analysis was carried out in the automatic mode on the most abundant fragment ion in $MS^{(n-1)}$. The isolation window was maintained at *m/z* 2.0 and the normalized collision energy (NCE) ranging from 20% to 60% in CID and HCD modes was applied. Data acquisition and analysis were performed using XCalibur software version 2.0.7 (Thermo Scientific).

2.6. UHPLC-QqQ_{LIT}-MS conditions for quantitative analysis

Quantitative analysis was performed on a 4000 QTRAP™ MS/MS system, which is a hybrid triple quadrupole-linear ion trap mass spectrometer (Applied Biosystem; Concord, ON, Canada), hyphenated with a Waters ACQUITY UPLC™ system (Waters; Milford, MA, USA) via an electrospray ion source (Turbo V™ source with TurboIonSpray™ probe and APCI probe) interface. Waters ACQUITY UPLC™ system was equipped with binary solvent manager, sample manager, column compartment and photodiode array detector (PAD).

Chromatographic separation of compounds was obtained with an ACQUITY UPLC BEH™ C_{18} column (100 mm×2.1 mm, 1.7 μm) operated at 25 °C. The mobile phase, which consisted of 0.1% formic acid aqueous solution (A) and acetonitrile (B), was delivered at a flow rate of 0.3 mL/min under a gradient program: 5% (B) 0 to 1.0 min, 5%–20% (B) from 1.0 to 2.0 min, 20%–30% (B) from 2.0 to 3.0 min, 30%–90% (B) from 3.0 to 4.0 min, maintained at 90% (B) from 4.0 to 5.0 min and back to initial condition from 5.0 to 5.5 min. The sample injection volume used was 2 μL.

ESI–MS (positive ion mode) was used for sample introduction and ionization process and low-energy collision dissociation tandem mass spectrometry (CID–MS/MS) was operated in the MRM mode. A Turboionspray® probe was vertically positioned 11 mm from the orifice and charged with 5500 V. Each selected analyte (10 ng/mL) was directly injected into the ESI source of QqQ_{LIT}-MS by continuous infusion to optimize compound-dependent MRM parameters such as declustering potential (DP), entrance potential (EP), collision energy (CE) and cell exit potential (CXP). Analytes tetrahydroberberine, tetrahydropalmatine, isocorydine and glaucine showed $[M+H]^+$ ion while analytes berberine, palmatine, jatrorrhizine and magnoflorine showed $[M]^+$ ion in Q1 MS scan. DP and EP were optimized to obtain the maximum sensitivity of $[M+H]^+$ and $[M]^+$ ions in Q1 multiple ion scan (Q1 MI). Identification of the fragment ions and selection of CE for each analyte were carried out in the product ion scan. All the recorded MS/MS spectra are shown in Fig. S1. Furthermore, CE and CXP were optimized to acquire the maximum sensitivity of precursor ion → product ion transition (MRM pair) in the MRM scan. Table 1 shows the optimized parameters for all the analytes.

Source dependent parameters such as temperature (TEM), GS1, GS2 and curtain (CUR) gas were set at 550 °C, 50 psi, 50 psi and 20 psi, respectively, in the flow injection analysis (FIA) by operating UHPLC with QqQ_{LIT}-MS. The collision-activated dissociation (CAD)

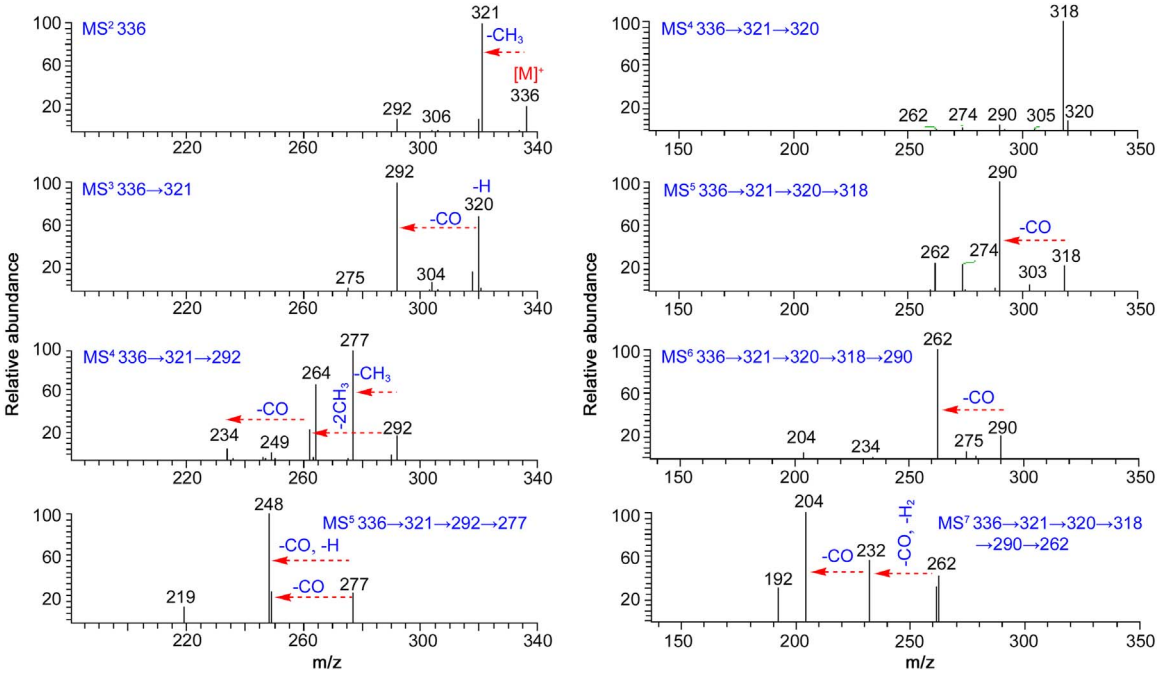

Fig. 2. ESI–MS^n spectra of the $[M]^+$ ion of berberine.

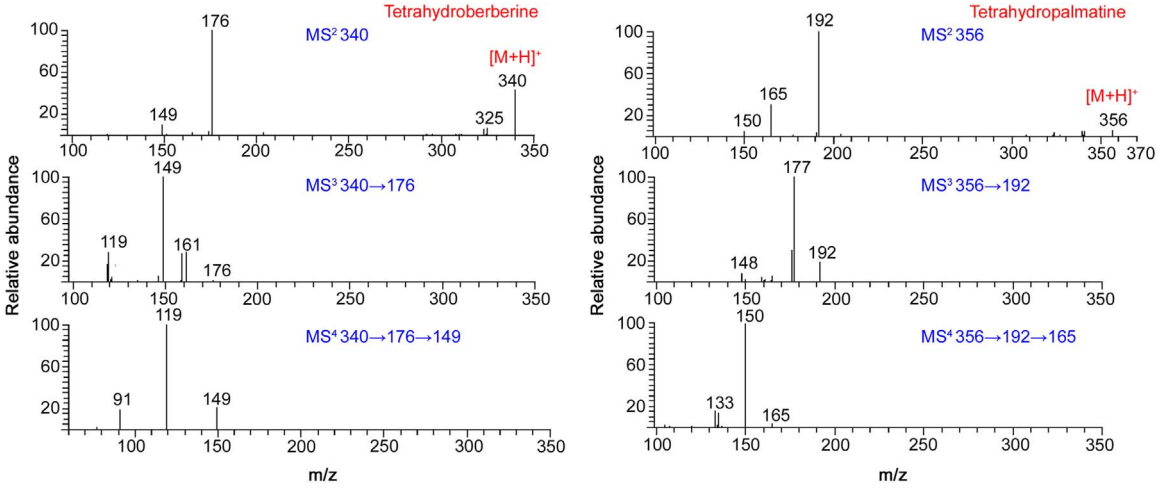

Fig. 3. ESI–MS^n spectra of the $[M+H]^+$ ion of tetrahydroberberine and tetrahydropalmatine.

gas was set as medium and the interface heater was on. High-purity nitrogen was used for all the processes. Quadrupole 1 and quadrupole 2 were maintained at unit resolution. AB Sciex Analyst software version 1.5.1 was used to control the LC–MS/MS system and for data acquisition and processing.

2.7. Validation of quantitative method

The proposed MRM method was validated for linearity, lower limits of detection (LODs), limits of quantification (LOQs), interday and intraday precisions, stability and recovery according to the International Conference on Harmonization (ICH, Q2R1) guidelines, 2005, using UHPLC-QqQ$_{LIT}$-MS [20].

This method was employed to analyze two MRM transitions in the sample matrix for each analyte but only one transition was monitored in quantitative analysis of samples due to the lack of sensitivity of the other observed product ions. The most prominent MRM transition was selected as a quantifier and the other as a qualifier (Table 1 and Figs. S1 and S2). All the peaks of the reference compounds in *ML* and *MN* roots were unambiguously identified by comparison of retention time, quantifier and qualifier transitions with MRM chromatogram of standards (Table 1). The linearity calibration curves were made from at least five experiments of each analyte and evaluated by the linear correlation coefficient (r) of the calibration curves. The LODs and LOQs were defined as a signal-to-noise ratio (SNR) equal to 3.3 and 10, respectively. The intra- and inter-day precisions were determined by analyzing known concentrations of the eight analytes in the three replicates during a single day and by triplicating the experiments on three consecutive days. The stability of sample solution stored at room temperature was investigated by replicate injection of the sample solution at 0, 1, 2, 4, 6, 8, 10, 14 and 24 h. Recovery test was carried out to investigate accuracy of this method by adding the mixed

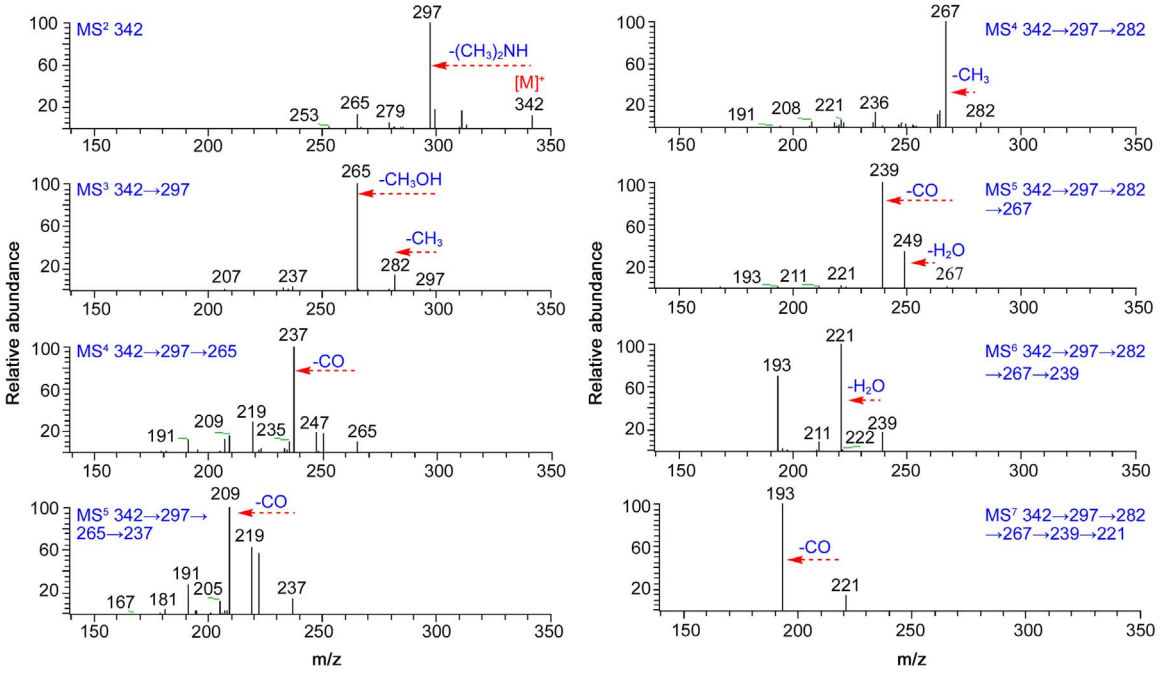

Fig. 4. ESI–MS^n spectra of the $[M]^+$ ion of magnoflorine.

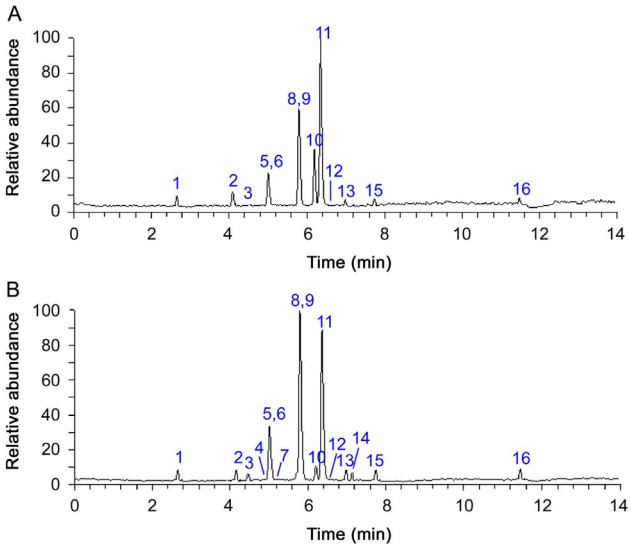

Fig. 5. Total ion chromatogram (TIC) of (A) *M. leschenaultia (ML)* and (B) *M. napaulensis (MN)* roots.

standard solutions with three different spike levels (low, middle and high) into the sample.

3. Results and discussion

3.1. Fragmentation analysis of reference standards using ESI-Orbitrap-MS^n

10 ng/mL (in MeOH) reference standards were injected into the ESI source by continuous infusion. (+)-ESI–MS was found to be adequate for measuring sensitivity to all isoquinoline alkaloids. As expected, the quaternary protoberberines (berberine, jatrorrhizine and palmatine) and one aporphine (magnoflorine) formed their respective molecular ions $[M]^+$, whereas the tetrahydroprotoberberines (THB and THP) and other aporphines (isocorydine and glaucine) afforded the protonated molecules $[M+H]^+$(Fig. 1). The precursor $[M]^+$ and $[M+H]^+$ ions were selected for HCD and CID fragmentation in FT mode to produce high resolution tandem mass (HRMS/MS) spectra. Furthermore, the MS^n spectra (n=2 to 8) were generated in IT mode to elucidate sequential fragmentation pathways (Table 2). The compounds were classified into three groups: quaternary protoberberines, tetrahydroprotoberberines and aporphines according to their chemical structures and fragmentation patterns.

3.1.1. Fragmentation of quaternary protoberberines

The $[M]^+$ ions of berberine, jatrorrhizine and palmatine were observed at m/z 336.1230, 338.1387 and 352.1543, respectively in FT mode. When CID was performed in IT mode on the berberine, the $[M]^+$ ion produced the prominent product at m/z 321, which was formed by the loss of methyl radical (Fig. 2). This product ion was further subjected to MS^3 analysis which produced ions at m/z 320 (‘1a’) and 292 (‘1b’) corresponding to sequential losses of hydrogen radical and carbon monoxide (Scheme S1). Ion ‘1a’ at m/z 320 was further subjected to MS^4 analysis which afforded the product ion at m/z 318 corresponding to loss of H_2 molecule. In the MS^5 fragmentation analysis, m/z 318 afforded the product ions at m/z 290 and 262 corresponding to sequential loss of CO molecules which was also supported by MS^6 fragmentation of ion at m/z 290. Loss of CO and H_2 molecules was observed in the MS^7 fragmentation of ion at m/z 262 which showed the expulsion of all oxygen atoms attached as substituents in protoberberine moiety. Similarly, product ion ‘1b’ at m/z 292 was subjected to MS^4 analysis which afforded the fragment ions at m/z 277, 264, 262 and 234 corresponding to loss of CH_3 radical, CO molecule, $2CH_3$ radical and $2CH_3$+CO, respectively. When CID analyses in IT mode were performed on the jatrorrhizine and palmatine, they followed the similar type of fragmentation behavior as discussed for berberine (Figs. S3 and S4). Furthermore, the $[M]^+$ ions were selected for HCD in FT mode to obtain HRMS/MS spectra. Their exact mass information of product ions acquired in FT mode gave additional support for MS^n data which was acquired in IT mode. On the basis of CID–MS^n and HCD–MS/MS fragmentation analysis, the following

Table 3
Identification of isoquinoline alkaloids in the ethanolic extracts of *M. leschenaultia (ML)* and *M. napaulensis (MN)*.

No.	RT (min)	Ion	HR–MS			Molecular formula	Assigned identity	Class	*ML*	*MN*
			m/z (calc)	*m/z* (obs)	Error (Δppm)					
1	2.7	$[M+H]^+$	330.1700	330.1701	0.30	$C_{19}H_{23}NO_4$	Reticuline	BIQS	Y	Y
2	4.2	$[M]^+$	342.1705	342.1700	−1.46	$C_{20}H_{24}NO_4^+$	Magnoflorine#	Aporphine	Y	Y
3	4.5	$[M+H]^+$	328.1543	328.1540	−0.91	$C_{19}H_{21}NO_4$	Isoboldine	Aporphine	Y	Y
4	4.9	$[M]^+$	324.1236	324.1239	0.93	$C_{19}H_{18}NO_4^+$	Demethyleneberberine	PBA	—	Y
5	5.0	$[M+H]^+$	354.1336	354.1326	−2.82	$C_{20}H_{19}NO_5$	8-oxojatrorrhizine	PBA	Y	Y
6	5.0	$[M+H]^+$	342.1705	342.1701	−1.17	$C_{20}H_{23}NO_4$	Isocorydine#	Aporphine	Y	Y
7	5.2	$[M]^+$	314.1751	314.1749	−0.64	$C_{19}H_{24}NO_3^+$	Oblongine	BIQS	—	Y
8	5.7	$[M+H]^+$	356.1856	356.1850	−1.68	$C_{21}H_{25}NO_4$	Glaucine#	Aporphine	Y	Y
9	5.8	$[M]^+$	338.1387	338.1381	−1.77	$C_{20}H_{20}NO_4^+$	Jatrorrhizine#	PBA	Y	Y
10	6.2	$[M]^+$	352.1543	352.1542	−0.28	$C_{21}H_{22}NO_4^+$	Palmatine#	PBA	Y	Y
11	6.4	$[M]^+$	336.1230	336.1231	0.30	$C_{20}H_{18}NO_4^+$	Berberine#	PBA	Y	Y
12	6.6	$[M]^+$	322.1074	322.1077	0.93	$C_{19}H_{16}NO_4^+$	Thalifendine	PBA	Y	Y
13	7.0	$[M+H]^+$	356.1856	356.1851	−1.40	$C_{21}H_{25}NO_4$	Tetrahydropalmatine#	PBA	Y	Y
14	7.2	$[M]^+$	322.1074	322.1081	2.17	$C_{19}H_{16}NO_4^+$	Berberrubine	PBA	—	Y
15	7.7	$[M+H]^+$	340.1543	340.1540	−0.88	$C_{20}H_{21}NO_4$	Tetrahydroberberine#	PBA	Y	Y
16	11.5	$[M+H]^+$	352.1179	352.1181	0.57	$C_{20}H_{17}NO_5$	8-oxoberberine	PBA	Y	Y

Compounds matched with the authentic standards; Y: Presence; —: Absence.

characteristic features for quaternary protoberberines were observed: (i) the presence of dehydrogenated fragment ions, (ii) the successive dissociations of the substituents and (iii) the absence of fragment ions below *m/z* 200.

3.1.2. Fragmentation of tetrahydroprotoberberines

The $[M+H]^+$ ions of tetrahydroberberine (THB) and tetrahydropalmatine (THP) were observed at *m/z* 340.1542 and 356.1855, respectively in FT mode. When CID was performed on these two protonated molecules (NCE 30%) with the orbitrap in IT mode, we observed the two prominent products at *m/z* 176 and 192, which were formed as a result from the retro Diels Alder cleavage for THB and THP, respectively. Similarly, the product ions at *m/z* 149 and 165 were produced by B ring cleavages of THB and THP, respectively. They were further subjected to MS^3 analysis which produced ions corresponding to loss of CH_3 radical, NH_3, H_2 and CH_2O, as shown in Fig. 3. This identification was also supported by HRMS and HRMS/MS data observed in HCD analysis in FT mode.

3.1.3. Fragmentation of aporphines

In FT mode, magnoflorine showed the $[M]^+$ molecular ion at *m/z* 342.1704; however, isocorydine and glaucine afforded the protonated molecules $[M+H]^+$ at *m/z* 342.1705 and 356.1856, respectively. In the CID analyses, aporphine alkaloids afforded product ions which were created by the loss of CH_3NH_2 and/or $(CH_3)_2NH$ depending on the N-substitution on nitrogen and the B ring cleavage. When CID was performed on the magnoflorine (NCE 30%) in IT mode, it produced the prominent product at *m/z* 297 by loss of dimethylamine molecule $[(CH_3)_2NH]$ (Fig. 4). This product ion was further subjected to MS^3 analysis which produced ions at *m/z* 282 and 265 corresponding to loss of methyl radical and methanol, respectively (Scheme S2). In the MS^4 and MS^5 analysis, ion at *m/z* 265 afforded the fragment ion at *m/z* 237 and 209, respectively, corresponding to sequential loss of CO molecule. Similarly, MS^4 analysis of ion at *m/z* 282 afforded the fragment ion at *m/z* 267 by loss of methyl radical. When ion at *m/z* 267 was subjected to MS^5 analysis, it showed the ion at *m/z* 249 and 239 corresponding to loss of water and CO molecules. In the MS^6 and MS^7 analysis, ion at *m/z* 239 showed the fragment ion at *m/z* 221 and 193, respectively, corresponding to sequential loss of water and CO molecules. Glaucine and isocorydine followed similar fragmentation pattern with the magnoflorine (Figs. S5 and S6). HRMS and HRMS/MS data observed in HCD analysis in FT mode are given in Fig. S7.

3.2. Isoquinolines analysis of ML and MN roots using UHPLC-Orbitrap-MS^n

The total ion chromatograms (TICs) of the *ML* and *MN* roots extract are presented in Fig. 5. The exact masses of targeted $[M]^+$ or $[M+H]^+$ ions of all possible isoquinoline alkaloids were extracted from their TICs using a mass tolerance window of ±5 ppm and the respective peak retention times (RT) are reported in Table 3. The mass spectra derived from these extracted ion chromatograms (EICs) showed intense $[M]^+$ and $[M+H]^+$ ions with the mass error < 2.9 ppm. These ions were further subjected to MS^n analysis at varied collision energies under CID and HCD type fragmentation (Table 4). Identified alkaloids showed distinguishable fragment ions with high mass accuracy.

Sixteen compounds have been tentatively identified based on their mass spectrometric features in which eight peaks (2, 6, 8–11, 13 and 15) were confirmed as magnoflorine, isocorydine, glaucine, jatrorrhizine, palmatine, berberine, tetrahydropalmatine and tetrahydroberberine, respectively, with the co-chromatography of reference standards. Peaks 1 and 7 showed the $[M+H]^+$ and $[M]^+$ ions at *m/z* 330.1701 and 314.1749, respectively. They produced the characteristic fragments of benzylisoquinoline alkaloids: (i) loss of the nitrogen as CH_3NH_2 or $(CH_3)_2NH$ depending on the substitution on N followed by the removal of MeOH and (ii) isoquinoline and resonance-stabilized benzyl fragments by benzylic cleavage (cleavage beta to nitrogen) which provided information about the substitution on the A and C rings [5]. In IT mode, peak 1 produced the product ion at *m/z* 299 by loss of CH_3NH_2, *m/z* 192 and 137 corresponding to the isoquinoline and the benzylic cleavage fragment. Further, product ion at *m/z* 299 produced ions at *m/z* 267 and 175 by loss of methanol and benzene moiety, respectively, in the MS^3 analysis. Ion at *m/z* 175 yielded fragments at *m/z* 145 and 115 by sequential loss of methanol and CO, respectively in MS^{4-5} analysis which indicated presence of methoxy and hydroxy substituent in isoquinoline moiety. Hence, peak 1 was tentatively identified as reticuline (Fig. 1). Likewise, MS^2 spectrum of peak 7 afforded a characteristic fragment at *m/z* 58 $[C_3H_8N]^+$ due to $[(CH_3)_2N{=}CH_2]^+$ ion which showed the presence of two methyl groups on the nitrogen corresponding to the RDA fragment. It showed loss of $(CH_3)_2NH$ (*m/z* 269), the isoquinoline fragment at *m/z* 192 and the benzylic

Table 4
HCD–MS/MS and CID–MS^n (n=2–8) data obtained in FT and IT modes for isoquinoline alkaloids present in ethanolic extracts of *M. leschenaultia (ML)* and *M. napaulensis (MN)*.

No.	Assigned identity	HCD–MS/MS data in FT–MS mode	CID–MS^n data in IT–MS mode
1	Reticuline	299.1270 (7), 267.1012 (8),192.1020 (100), 175.0753 (20),151.0754 (9), 143.0490 (21),137.0571 (39), 115.0540 (4)	MS^2 [330]: 299, 192, 137 MS^3 [330→299]: 267, 175 MS^4 [330→299→175]: 143, 115 MS^5 [330→299→175→143]: 115
3	Isoboldine	297.1090 (60), 282.0877 (40),265.0840 (100), 253.0829 (9),237.0890 (38), 219.0966 (13),191.0838 (15)	MS^2 [328]: 297 MS^3 [328→297]: 282, 265 MS^4 [328→297→265]: 237, 233 MS^4 [328→297→282]: 250 MS^5 [328→297→265→237]: 205
4	Demethyleneberberine	309.0999 (50), 308.0891 (100),306.0715 (8), 294.0739 (32),292.0970 (25), 280.0950 (71),266.0785 (23)	MS^2 [324]: 309 MS^3 [324→309]: 308, 294 MS^4 [324→309→308]: 280 MS^4 [324→309→294]: 292, 266
5	8-oxojatrorrhizine	339.1040 (15), 338.1015 (100),324.0867 (19), 322.0950 (11),310.1093 (12), 296.0893 (20),280.1080 (10)	MS^2 [354]: 339 MS^3 [354→339]: 338, 324 MS^4 [354→339→338]: 310 MS^4 [354→339→324]: 322, 296
7	Oblongine	269.1177 (15), 237.0916 (10),209.0963 (7), 192.1015 (10),175.0766 (10), 145.0623 (15),143.0496 (20), 121.0654 (11),115.0545 (11), 107.0490 (100),58.0663 (55)	MS^2 [314]: 269, 192, 107, 58 MS^3 [314→269]: 237, 175 MS^4 [314→269→175]: 143, 115 MS^5 [314→269→175→143]: 115
12	Thalifendine	307.0840 (100), 306.0742 (5),279.0870 (25), 278.0840 (15),250.0841 (5)	MS^2 [322]: 307 MS^3 [322→307]: 306, 279 MS^4 [322→307→306]: 278
14	Berberrubine	307.0838 (100), 306.0760 (3),279.0872 (20), 278.0771 (10),250.0767 (15)	MS^2 [322]: 307 MS^3 [322→307]: 306, 279 MS^4 [322→307→306]: 278
16	8-oxoberberine	337.0925 (44), 336.1041 (30),322.0717 (100), 319.0815 (6),308.0933 (8), 294.0752 (29),279.0554 (5)	MS^2 [352]: 337 MS^3 [352→337]: 336, 322 MS^4 [352→337→336]: 308 MS^4 [352→337→322]: 320, 294

cleavage fragment at *m/z* 107. In MS^3 analysis, ion at *m/z* 269 produced fragments at *m/z* 237 and 175 by loss of methanol and benzene moiety, respectively. Ion at *m/z* 175 afforded similar fragments as appeared in the case of peak 1. Hence, peak 7 was tentatively identified as oblongine. In addition, HCD–MS/MS in FT mode provided the HRMS/MS spectrum which confirmed the identifications.

Peak 3 afforded the protonated $[M+H]^+$ ion at *m/z* 328.1540. It showed initial loss of methylamine (*m/z* 297) in MS^2 followed by loss of methanol (*m/z* 265) or methyl radical (*m/z* 282) in MS^3 analysis. In MS^4 analysis, ion at *m/z* 265 produced fragment ions at *m/z* 237 and 233 by loss of CO and methanol, respectively, which indicated presence of two vicinal methoxy and –OH group. Loss of methanol was also observed in MS^4 analysis of ion at *m/z* 282 and MS^5 analysis of ion at *m/z* 237 (Table 4). Therefore, peak 3 was tentatively identified as isoboldine.

Peaks 4, 12 and 14 afforded the molecular ions $[M]^+$ at *m/z* 324, 322, and 322, respectively. They showed fragmentation pattern of quaternary protoberberine alkaloids and tentatively identified as demethyleneberberine, thalifendine and berberrubine, respectively. Peaks 12 and 14 afforded similar fragmentation pattern, hence identified as an isomeric pair (different positions of methoxy and hydroxy group at C-9 and C-10). They showed initial loss of methyl radical in MS^2 analysis (4=*m/z* 309; 12, 14=*m/z* 307), followed by sequential loss of hydrogen radical and CO molecule in MS^{3-4} analysis. Peaks 5 and 16 showed strong $[M+H]^+$ ion at *m/z* 354.1327 and 352.1180, respectively, in FT mode. They afforded sequential loss of two CH_3 followed by CO and another parallel pathway showed sequential loss of CH_3 followed by hydrogen radical and CO in MS^{2-4} spectra as shown in Table 4. Furthermore, they showed 16 u higher mass to those of jatrorrhizine (9) and berberine (11), respectively. Hence, they were tentatively assigned as 8-oxojatrorrhizine and 8-oxoberberine, respectively. All the 16 compounds were detected in root part of *MN* while compound 4, 7 and 14 were not detected in *ML* root.

3.3. Quantitative analysis of isoquinolines in ML and MN roots using UHPLC-QqQ_{LIT}-MS/MS

3.3.1. Linearity, precision and recovery results of the validated method

The calibration curve showed good linearity with correlation coefficient (r) of ≥0.9995 over the tested concentration range. The LODs and LOQs were in the range of 0.08–0.48 ng/mL and 0.24–1.46 ng/mL, respectively. Relative standard deviation (RSD) values for precision were in the range of 0.55%–2.07% for intraday assays and 0.87%–2.05% for interday assays. The RSD values for stability were found in the range of 1.01%–3.14% and recoveries of the analytes were 98.50%–103.60% (RSD 1.10%–2.20%), evaluated by calculating the ratio of amount detected versus the amount added (Table 5).

3.3.2. Method application

The established UHPLC–ESI–MS/MS analytical approach was subsequently applied to determine contents of eight bioactive compounds, namely magnoflorine, isocorydine, glaucine, jatrorrhizine, tetrahydropalmatine, tetrahydroberberine, palmatine and berberine in the ethanolic extracts of *ML* and *MN* roots. The quantitative results are summarized in Table 6, which shows remarkable differences of their contents among the *ML* and *MN* roots. For example, the total contents of eight bioactive constituents were abundant in *MN* root (155747.60 μg/g). The eight components differed greatly in their contents and quaternary protoberberine alkaloids are the major

Table 5
Linearity, LOD, LOQ, precisions, stability and recovery results of investigated components.

No.	Linearity			LOD (ng/mL)	LOQ (ng/mL)	Precision(RSD, %)		Stability (RSD, %) (*n*=5)	Recovery	
	Regression equation	*r*	Linear range (ng/mL)			Intraday (*n*=3)	Interday (*n*=9)		Mean (*n*=3)	RSD (%)
1	y=613x−41.7	0.9999	1.0–250	0.22	0.67	1.39	1.25	2.54	102.49	2.20
2	y=6060x−76.0	0.9995	1.0–1000	0.26	0.79	1.46	1.55	1.88	101.60	1.92
3	y=3690x−49.8	0.9995	1.0–1000	0.19	0.58	0.55	1.74	1.01	98.50	1.50
4	y=1100x−7.7	0.9999	0.5–1000	0.12	0.36	1.50	0.87	2.02	99.11	1.12
5	y=11800x−48.0	0.9999	1.0–200	0.18	0.55	2.07	1.67	2.17	102.0	1.40
6	y=5200x+110.0	0.9999	1.5–200	0.48	1.46	1.18	2.05	1.39	98.70	1.10
7	y=918x+1.9	0.9996	0.5–50	0.08	0.24	1.20	0.99	3.14	100.30	1.74
8	y=130x−9.7	0.9998	0.5–100	0.14	0.42	1.61	1.90	1.52	103.60	1.10

y: peak area; x: concentration of compound (ng/mL); LOD: limit of detection, S/N =3.3; LOQ: limit of quantification, S/N =10.

Table 6
Contents of eight analytes in the ethanolic extracts of the root of *M. leschenaultia (ML)* and *M. napaulensis (MN)*.

Analytes	*ML*(μg/g)[a]	*MN*(μg/g)[a]
Magnoflorine	3552.50±0.03	7107.75±0.06
Isocorydine	235.65±0.05	232.88±0.06
Glaucine	40.79±0.05	45.95±0.03
Jatrorrhizine	26637.50±0.01	47765.00±0.02
Tetrahydropalmatine	92.44±0.10	88.76±0.06
Tetrahydroberberine	32.10±0.10	49.80±0.03
Palmatine	29127.50±0.01	13877.50±0.02
Berberine	62212.50±0.01	86580.00±0.01
Total[b]	121930.68	155747.60

[a] Content=mean ± SD (*n*=3);
[b] Total: The total contents of eight compounds in ML and MN extracts.

constituents. Among them, berberine showed the highest amount (*ML*=62212.50 μg/g and *MN*=86580.00 μg/g), followed by palmatine (*ML*=29127.50 μg/g and *MN* =13877.50 μg/g) and jatrorrhizine (*ML*=26637.50 μg/g and *MN*=47765.00 μg/g), which were disclosed as the important and main active constituents of the genus Mahonia [3,4].

4. Conclusion

The present study includes the qualitative analysis of isoquinoline alkaloids from ethanolic extract of the *ML* and *MN* roots using UHPLC-Orbitrap Velos Pro-MS in positive ion mode. Sixteen alkaloids have been identified and characterized in Mahonia species for the first time. A hierarchical key of CID–MS^n data is also proposed for the assignment of alkaloids using IT–MS mode. Results showed that the quaternary protoberberines and aporphines expelled all the substituents from their basic skeleton and also ring constricted by removal of carbon atom in the form of CO wherein the substituent is attached. Further, FT–MS mode was operated to get HRMS data for the identification of precursor as well as product ions under the HCD analysis. An UHPLC-QqQ_{LIT}-MS/MS method was also developed for quantification of eight bioactive compounds in root part of *ML* and *MN* plants under MRM mode. Quantitation of magnoflorine, isocorydine, glaucine, tetrahydropalmatine, tetrahydroberberine, jatrorrhizine, palmatine and berberine was successfully completed and quaternary protoberberines were found to be the prominent compounds in the selected Mahonia species.

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Appendix A. Supplementary material

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.jpha.2016.10.002.

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