



Original Article

Structural characterization of monoterpene indole alkaloids in ethanolic extracts of *Rauwolfia* species by liquid chromatography with quadrupole time-of-flight mass spectrometry[☆]Sunil Kumar^a, Awantika Singh^{a,b}, Vikas Bajpai^{a,b}, Mukesh Srivastava^{b,c}, Bhim Pratap Singh^d, Brijesh Kumar^{a,b,*}^a Sophisticated Analytical Instrument Facility, CSIR-Central Drug Research Institute, Lucknow 226031, Uttar Pradesh, India^b Academy of Scientific and Innovative Research (AcSIR), New Delhi 110025, India^c Biometry and Statistics Division, CSIR-Central Drug Research Institute, Lucknow 226031, Uttar Pradesh, India^d Department of Biotechnology, Mizoram University, Tanharil, 796004 Aizawl, Mizoram, India

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ABSTRACT

Rauwolfia species (Apocynaceae) are medicinal plants well known worldwide due to its potent bioactive monoterpene indole alkaloids (MIAs) such as reserpine, ajmalicine, ajmaline, serpentine and yohimbine. Reserpine, ajmalicine and ajmaline are powerful antihypertensive, tranquilizing agents used in hypertension. Yohimbine is an aphrodisiac used in dietary supplements. As there is no report on the comparative and comprehensive phytochemical investigation of the roots of *Rauwolfia* species, we have developed an efficient and reliable liquid chromatography-tandem mass spectrometry (LC–MS/MS) method for ethanolic root extract of *Rauwolfia* species to elucidate the fragmentation pathways for dereplication of bioactive MIAs using high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (HPLC–ESI–QTOF–MS/MS) in positive ion mode. We identified and established diagnostic fragment ions and fragmentation pathways using reserpine, ajmalicine, ajmaline, serpentine and yohimbine. The MS/MS spectra of reserpine, ajmalicine, and ajmaline showed C-ring-cleavage whereas E-ring cleavage was observed in serpentine via Retro Diels Alder (RDA). A total of 47 bioactive MIAs were identified and characterized on the basis of their molecular formula, exact mass measurements and MS/MS analysis. Reserpine, ajmalicine, ajmaline, serpentine and yohimbine were unambiguously identified by comparison with their authentic standards and other 42 MIAs were tentatively identified and characterized from the roots of *Rauwolfia hookeri*, *Rauwolfia micrantha*, *Rauwolfia serpentina*, *Rauwolfia verticillata*, *Rauwolfia tetraphylla* and *Rauwolfia vomitoria*. Application of LC–MS followed by principal component analysis (PCA) has been successfully used to discriminate among six *Rauwolfia* species.

1. Introduction

Rauwolfia species, which belong to Apocynaceae family, are widely distributed in Asia, Africa and America [1,2]. Apart from the common *Rauwolfia serpentina*, other species found in India are *R. hookeri*, *R. micrantha*, *R. verticillata*, *R. tetraphylla* and *R. vomitoria* [3]. *Rauwolfia* species have been used for the treatment of hypertension, snake bites, feverish illnesses and insanity from ancient time in Indian System of Medicine (ISM), Traditional Chinese Medicine (TCM) and Western System of Medicine (WSM) [4–6]. The ethanolic extract of the roots of *Rauwolfia* species has been also used to treat cardiovascular

diseases [7,8], cancer, hypertension [9–12], various psychiatric diseases [1] and snake bites [13]. Dried root powder of *Rauwolfia serpentina* is also used for the treatment of cancer in Ayurveda [14–16]. The *Rauwolfia* species are a rich source of bioactive monoterpene indole alkaloids (MIAs) such as reserpine, ajmaline, ajmalicine, deserpidine, rescinnamine and yohimbine [17]. Reserpine is a powerful antihypertensive and tranquilizing agent used for the treatment of hypertension, schizophrenia, paranoia, breast cancer and Parkinson's disease [18–22]. Ajmaline possesses antihypertensive and antiarrhythmic activities and is also used in high blood pressure [23,24]. Ajmalicine is an antihypertensive drug used for treatment of cardio-

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* Corresponding author at: Sophisticated Analytical Instrument Facility, CSIR-Central Drug Research Institute, Lucknow 226031, Uttar Pradesh, India.

E-mail addresses: brijesh_kumar@cdri.res, gbrikum@yahoo.com (B. Kumar).

vascular diseases [25]. Serpentine possesses antihistaminase or anti-histamine activity and is used in the treatment of snake bites [26–28]. Yohimbine has potential clinical applications in erectile dysfunction and is used as an aphrodisiac in dietary supplements [29,30].

Qualitative and quantitative analyses of MIAs in *Rauwolfia* species have been carried out using high performance liquid chromatography (HPLC) [31,32], high performance thin layer chromatography (HPTLC) [3,33], gas chromatography mass spectrometry (GC–MS) [32], direct analysis in real time mass spectrometry (DART–MS) [5,34], liquid chromatography/tandem mass spectrometry (LC–MS/MS) [3,35] and Orbitrap Velos Pro mass spectrometer [36]. LC–MS/MS offers many advantages in the analysis of these types of compounds, due to its speed, sensitivity, specificity and ability to couple with high performance chromatographic techniques [37]. There are only a few reports available for the identification and characterization of MIAs by LC–MS/MS in plant extracts [35,36,38–42]. However, there is no report on the comparative and comprehensive phytochemical investigation of the roots of *Rauwolfia* species. Therefore, we have developed a simple and specific high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry (HPLC–ESI–QTOF–MS/MS) method to establish fragmentation pathways for the identification and characterization of bioactive compounds from ethanolic extract of the root of *Rauwolfia* species.

2. Experimental

2.1. Chemicals and materials

LC–MS grade solvents were purchased from Sigma-Aldrich (St. Louis, MO, USA). Ultra-pure water was produced by Milli-Q Advantage system (Millipore, USA). AR grade ethanol, purchased from Merck Millipore (Darmstadt, Germany), was used in the preparation of ethanolic extracts. Reserpine, ajmalicine, ajmaline and serpentine were purchased from Sigma-Aldrich (St. Louis, MO, USA) and yohimbine was purchased from Chem. Faces (Wuhan, Hubei, China).

2.2. Plant materials

The roots of *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata* and *R. vomitoria* were collected from the plants growing under similar conditions in Jawaharlal Nehru Tropical Botanic Garden and Research Institute (JNTBGRI) campus, Kerala, India. The plant materials were collected in September 2012 and the voucher specimens (*R. hookeri*-66449, *R. micrantha*-66450, *R. serpentina*-66451, *R. tetraphylla*-66452, *R. verticillata*-66453 and *R. vomitoria*-66454) were deposited in the Herbarium of JNTBGRI. The roots were powdered, packed in airtight containers and stored at 20 °C until analysis.

2.3. Extraction

The powdered roots (50 g each) were extracted with 250 mL of ethanol. The extractions were performed by initial sonication for 30 min at 30 °C, followed by being kept at room temperature for 24 h. The extracts were filtered through filter paper (Whatman no. 1) and the residues were re-extracted using the same method four times with fresh solvent at room temperature for 24 h. The combined filtrates were evaporated to dryness under reduced pressure at 20–50 kPa at 40 °C using a Buchi rotary evaporator. 1 mg/mL stock solutions of each species of the dried plant extracts were prepared in methanol and filtered through a 0.22 µm polyvinylidene fluoride (PVDF) membrane into the HPLC auto sampler vial prior to LC–MS analysis.

2.4. HPLC–ESI–QTOF–MS/MS conditions

Analyses were carried out using an Agilent 1200 HPLC system interfaced with Agilent 6520 hybrid quadrupole time-of-flight mass

spectrometer (Agilent Technologies, USA). The 1200 HPLC system was equipped with a quaternary pump (G1311A), online vacuum degasser (G1322A), autosampler (G1329A), thermostatted column compartment (G1316C) and diode-array detector (G1315D).

2.5. Chromatographic conditions

Chromatographic separations were performed using a Thermo BetaSil C₈ column (250 mm×4.5 mm, 5 µm) operated at 25 °C employing a gradient elution using 0.1% formic acid in water (A) and acetonitrile (B) as mobile phase at a flow rate of 0.6 mL/min. The elution consisted of a linear gradient from 25% to 27%; 0–15 min, 27%–37%; 15–18 min, 37%–42%; 18–20 min, 42%–45%; 20–22 min, 45%–48%; 22–25 min, 48–60%; 25–30 min, then returned to the initial conditions after 5 min. The sample injection volume was 1 µL.

2.6. Mass spectrometric condition

The mass spectrometer was operated in positive electrospray ionization mode and spectra were recorded by scanning the mass range m/z 50–1500 in both MS and MS/MS modes. Nitrogen was used as drying, nebulising and collision gas. The drying gas flow rate was 12 L/min. The heated capillary temperature was set to 350 °C and nebulizer pressure at 45 psi. The source parameters capillary voltage (V_{Cap}), fragmentor, skimmer and octapole voltages were set to 3500 V, 175 V, 65 V and 750 V, respectively. For the MS/MS analysis, collision energy was set at 30 eV and 35 eV, respectively, for reserpine and ajmalicine classes of compounds whereas 40 eV for ajmaline class of compounds. The accurate mass data of the molecular ions were processed through the Mass Hunter Workstation (version B 04.00) software (Agilent Technology, USA).

2.7. Statistical analysis

HPLC–ESI–QTOF–MS data obtained from three repeats of all the samples were subjected to statistical analysis. Principal component analysis (PCA) was performed on statistical software STATISTICA Version 7.0 (StatSoft, Inc., USA).

3. Results and discussion

3.1. Analysis of standards (templates)

Major bioactive MIAs such as ajmalicine (4), ajmaline (19) serpentine (32), yohimbine (28) and reserpine (44) were selected as templates to construct the diagnostic fragmentation pathways for identification and characterization of MIAs from plant extracts. Yohimbine and reserpine showed fragment ions at m/z 323.1560 and 577.2475, respectively, due to losses of CH₃OH. Both compounds also showed fragment ions at m/z 337.1912 and 397.2108 due to losses of H₂O and C₁₀H₁₂O₅, respectively. Fragment ions at m/z 224.1266, 212.1262, 158.0941 and 144.0802 were observed in yohimbine, whereas MS/MS spectrum of reserpine showed fragment ions at m/z 448.1954, 436.1950, 188.1070 and 174.0916 due to retro Diels Alder (RDA) cleavage of the C-ring. The fragment ion at m/z 448.1950 from reserpine gave product ion at m/z 236.1255 due to loss of C₁₀H₁₂O₅. Similarly, the fragment ions at m/z 224.1266 and 212.1262 gave product ions at m/z 192.1574 and 194.1157 due to losses of CH₃OH and H₂O, respectively, in yohimbine (Fig. 1A and B, Scheme 1). The MS/MS spectrum of ajmalicine showed fragmentation ion at m/z 321.1597 due to loss of CH₃OH. RDA cleavage produced the fragment ions at m/z 222.1111, 210.1130, 158.0964 and 144.0784. The product ion at m/z 190.1114 was formed by loss of CH₃OH from the ion at m/z 222.1111 (Fig. 1C and Scheme 2). Successive losses of H₂O gave rise to the ions at m/z 309.1991 and 291.1881 in ajmaline. RDA cleavage of the C-ring gave characteristic fragment ions at m/z 170.0955,

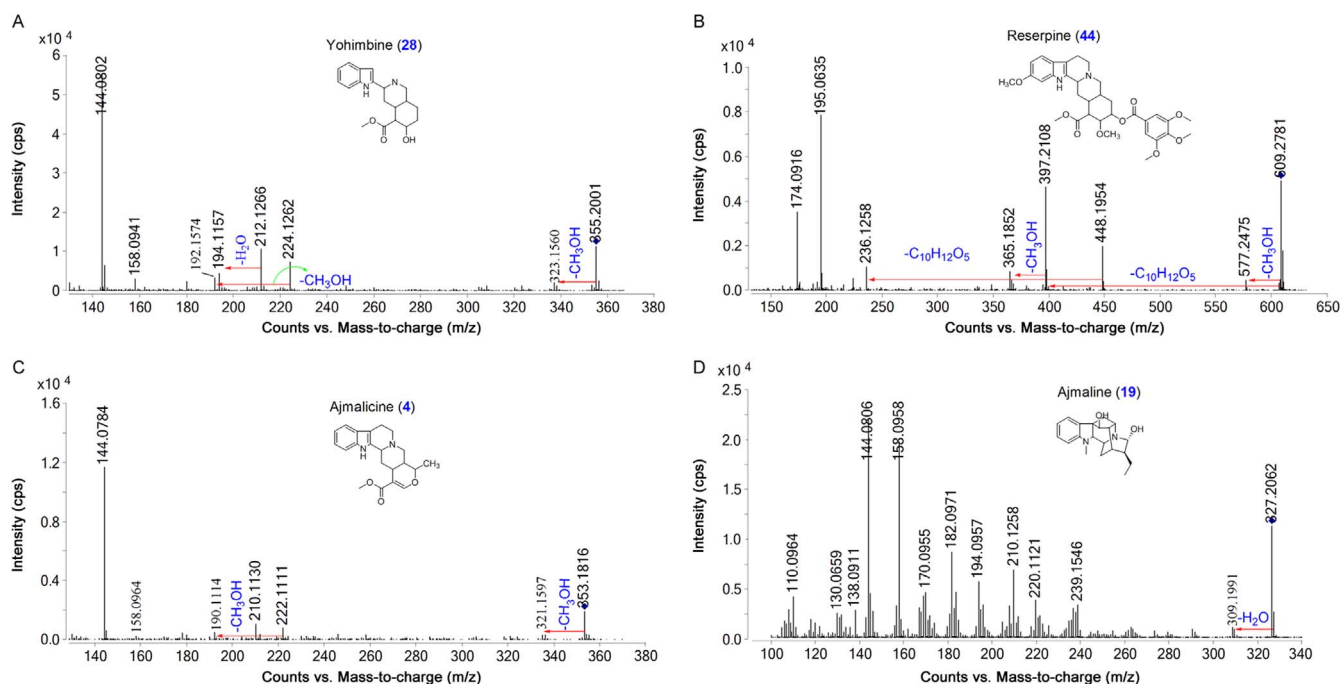


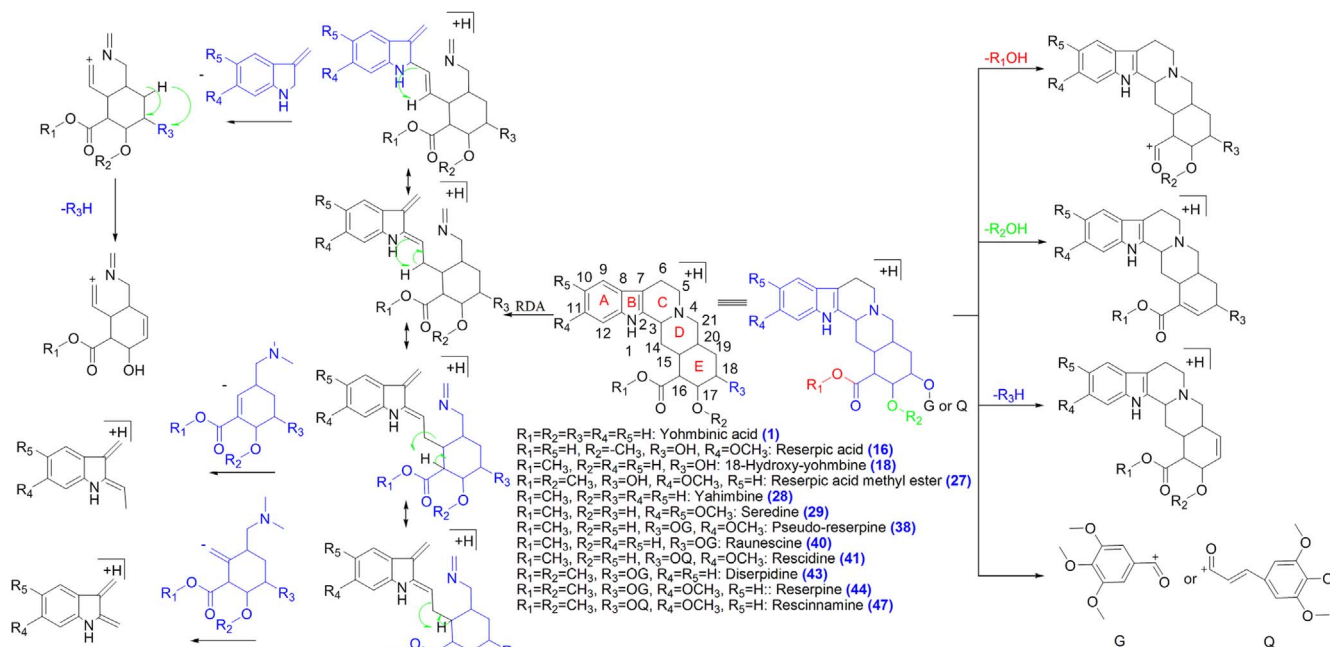
Fig. 1. MS/MS spectra of yohimbine, reserpine, ajmalicine and ajmaline.

158.0958 and 144.0806. The fragment ion at m/z 120.0795 was produced by loss of H_2O from the ion at m/z 138.0911 (Fig. 1D and Scheme 3). The MS/MS spectrum of serpentine showed fragment ions at m/z 317.1290 and 263.0789 due to losses of CH_3OH and $C_4H_7OCH_3$, respectively, whereas fragment ions at m/z 289.1314, 235.0826 and 207.0858 were formed due to successive losses of CO (Scheme 4).

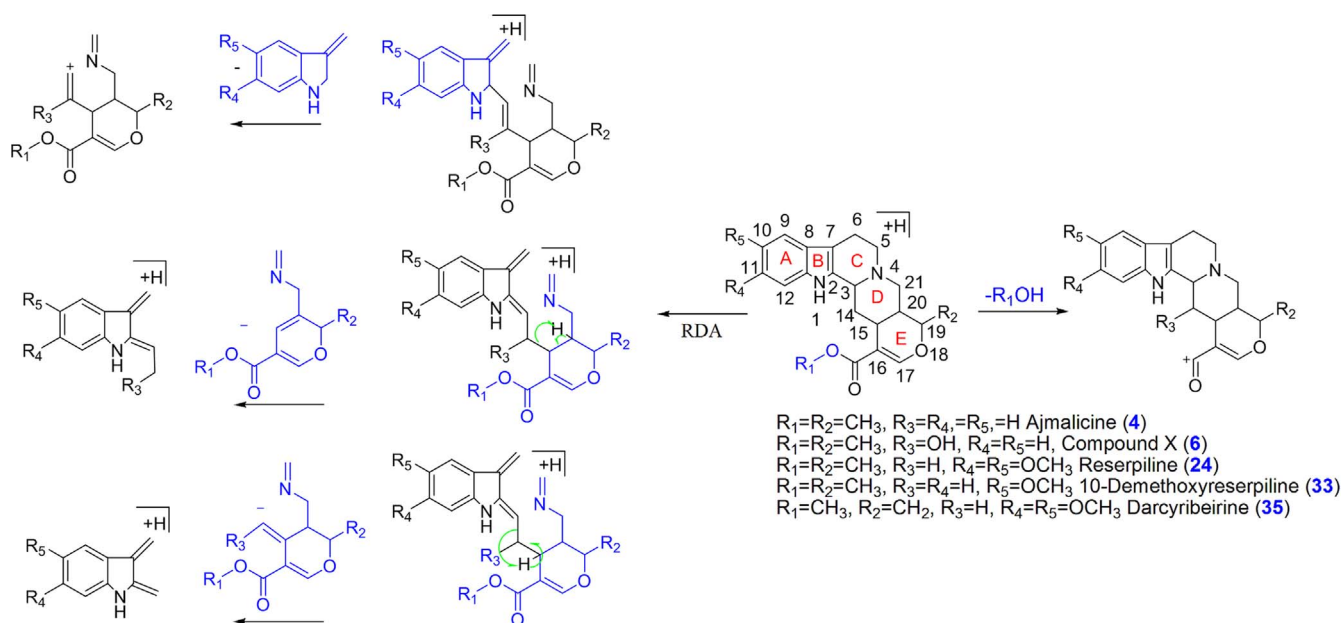
3.2. Metabolic profiling using LC–MS

All the six *Rauwolfia* species were cultivated in similar environmental conditions to study phytochemical variations. Ethanolic extract

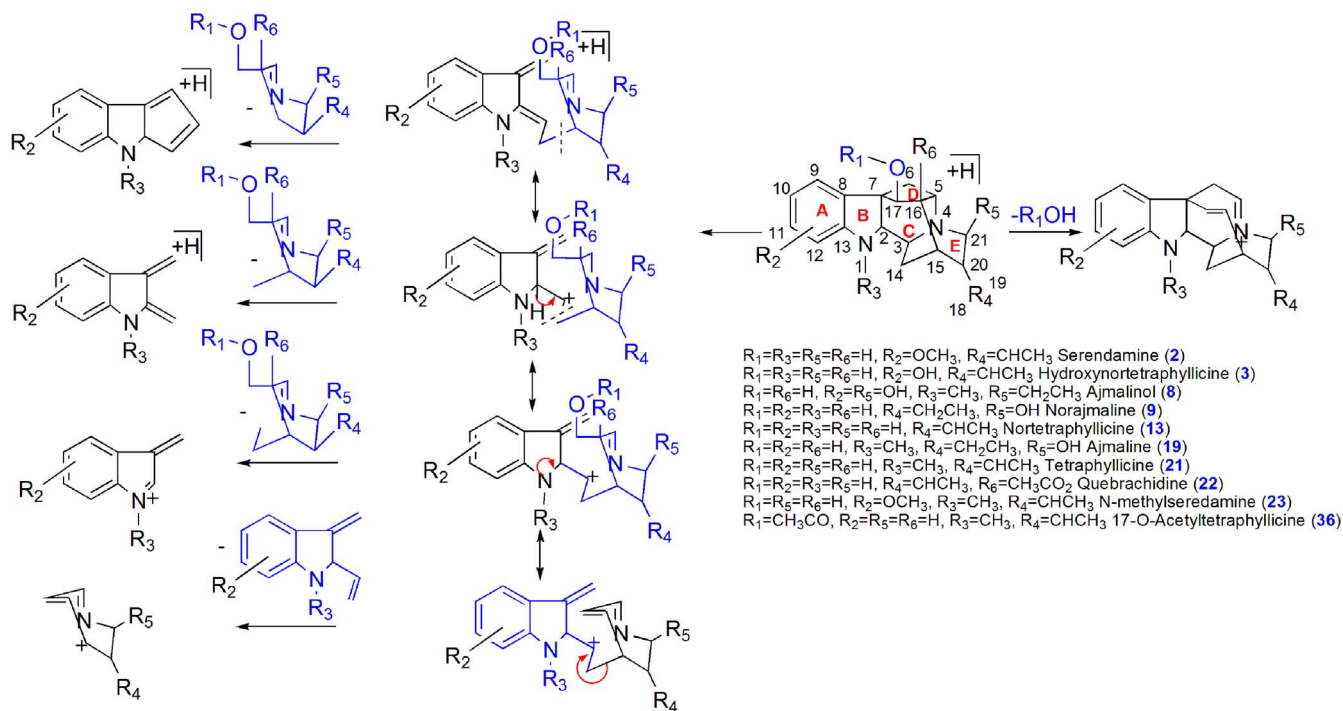
of root was analyzed using gradient mobile phase consisting of acetonitrile and 0.1% aqueous formic acid. Parameters such as column type, column temperature, mobile phase, elution conditions, flow rate and MS conditions were optimized. Base peak chromatograms (BPCs) of ethanolic extracts of *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata* and *R. vomitoria* in positive-ion mode are shown in Fig. 2. Retention time (RT), observed $[M+H]^+$, molecular formula, error (Δ ppm), major fragment ions and their relative abundance and distribution along with assignment are presented in Table 1. All these compounds were identified based on their exact mass, molecular formula, and fragmentation pattern. A total of 47 known/unknown MIAs were tentatively identified and characterized. The



Scheme 1. Proposed fragmentation pathways of reserpine class of MIAs.



Scheme 2. Proposed fragmentation pathways of ajmalicine class of MIAs (X: Methyl-14-hydroxy-19-methyl-16,17-didehydro-18-oxayohimban-16 carboxylate).



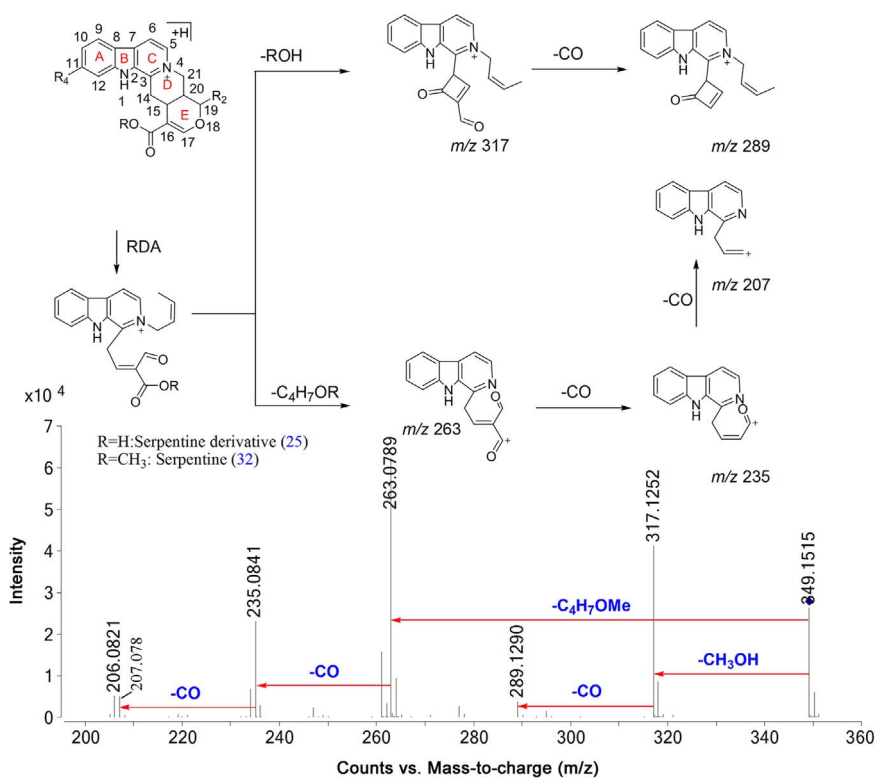
Scheme 3. Proposed fragmentation pathways of ajmaline class of MIAs.

proposed structures of the unknown compounds are shown in Fig. 3. Yohimbine, reserpine, ajmalicine, ajmaline and serpentine were unambiguously identified and characterized by comparison with their authentic standards.

3.2.1. Reserpine class of compounds

Seventeen compounds were tentatively identified and characterized on the basis of their fragmentation pathways of yohimbine and reserpine. Compounds 28 and 44 were identified and characterized as yohimbine and reserpine, respectively, by comparison with their authentic standards. Fragmentation pathways of reserpine class of compounds are shown in Scheme 1. Compounds 1 and 16 were tentatively identified as yohimbine acid and reserpine acid, respectively, which showed characteristic fragment ions at m/z 323.1754 and

383.1965 respectively, due to the losses of H₂O whereas fragment ion at m/z 369.1809 was observed due to loss of CH₃OH in compound 16. Compounds 1 (RT 3.4 min) and 11 (RT 6.9 min) gave the same fragment ions with different relative abundances in their MS/MS spectra. Both compounds 1 and 11 were tentatively identified as an isomeric pair. Compounds 16, 18, 27, 29, 38, 40, 41, 43 and 47 were identified as 18-hydroxy-yohimbine, reserpine acid methyl ester, seredine, pseudo-reserpine, raunescine, rescidine, deserpidine and rescinnamine, respectively. Compounds 18, 27, 29, 38, 40, 41, 43 and 47 showed fragment ions at m/z 339.1703, 383.1916, 383.1935, 563.2394, 533.2282, 589.2544, 547.2413 and 603.2779 due to loss of CH₃OH from [M+H]⁺ ions, respectively. Similarly, all these compounds also showed fragment ions at m/z 353.1857, 397.2122, 383.1941, 353.1805, 383.1925, 367.2009 and 397.2085, respectively



Scheme 4. Proposed fragmentation pathways of serpentine class of MIAs.

due to loss of H_2O (compounds 18 and 27), $C_{10}H_{12}O_5$ (compounds 38, 40, 43 and 47) and $C_{12}H_{14}O_5$ (compounds 41 and 47) from $[M+H]^+$ ions. Fragment ion at m/z 158.0932 was observed in compounds 1, 18, 40 and 43, whereas compounds 16, 27, 38, 40, 41 and 43 showed fragment ion at m/z 188.1045 due to loss of terpene moiety via C-ring cleavage followed by bond breaking between C14 and C15. Similarly, compound 29 gave fragment ion at m/z 218.1178. Compounds 1, 18, 40 and 43 provided RDA fragment ions at m/z 144.0794, whereas

fragment ions at m/z 174.0907 ($143\text{ Da} + OCH_3$) was observed in compounds 16, 27, 38, 41 and 47 due to loss of the terpene moiety via C-ring cleavage by RDA followed by bond breaking between C3 and C14. Similarly, the compound 29 showed fragment ion at m/z 204.1029 ($142\text{ Da} + 2OCH_3$) due to loss of terpene moiety. Compounds 38, 40 and 43 showed fragment ions at m/z 195.06 whereas fragment ion at m/z 221.0790 was observed in compounds 41 and 47 as base peak. Compounds 27 and 29 may be positional

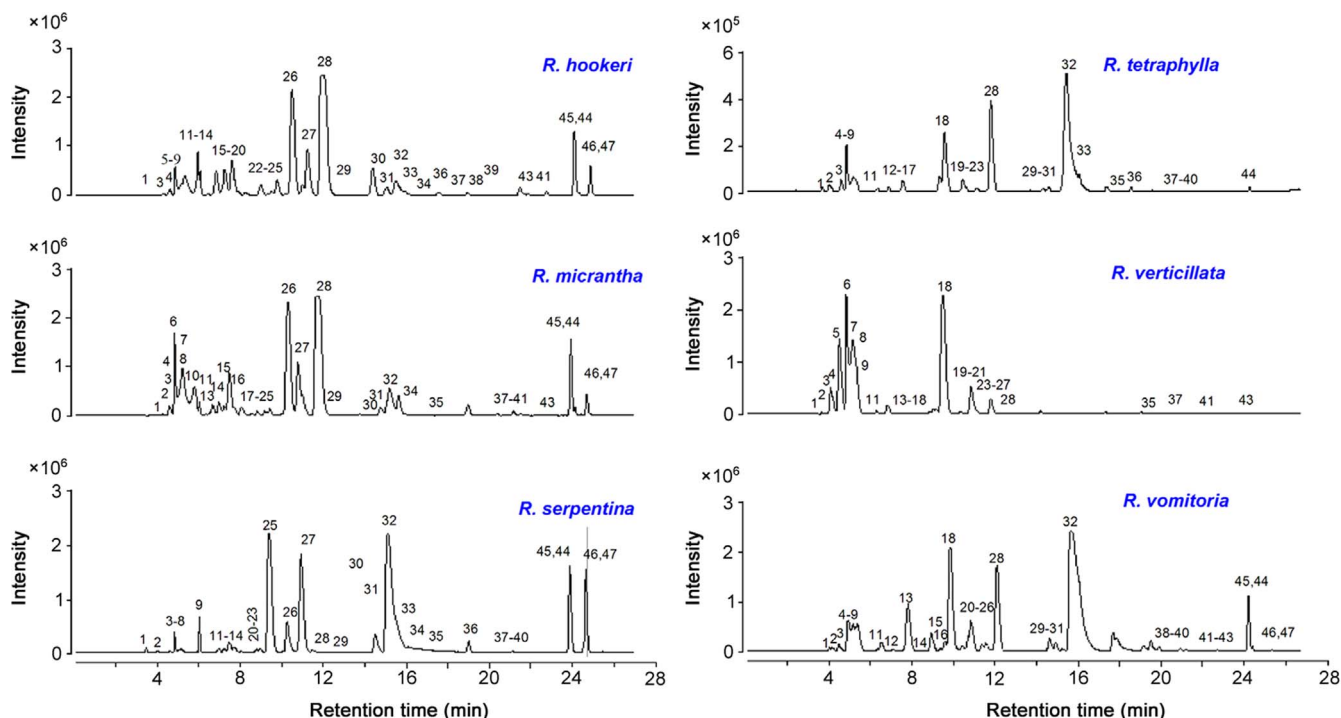


Fig. 2. Base peak chromatograms (BPCs) of ethanolic extracts of *Rauwolfia* species.

Table 1

Chromatographic and spectrometric characteristics of monoterpene indole alkaloids (MIAs) in ethanolic extracts of six *Rauwolfia* species (root) by high-performance liquid chromatography coupled with electrospray ionization quadrupole time-of-flight tandem mass spectrometry.

Compound (peak) no.	RT (min)	Error (Δppm)	Obs. <i>m/z</i>	Molecular formula	MS/MS fragment ions (Relative abundance, %)	Compounds	Distribution									
							Root									
							<i>Rh</i>	<i>Rm</i>	<i>Rs</i>	<i>Rt</i>	<i>Rv</i>	<i>Rvm</i>				
Reserpine class																
1	3.40	1.49	341.1867	C ₂₀ H ₂₄ N ₂ O ₃	323.1764 (5), 210.1119 (9), 192.1019 (4), 158.0932 (15), 144.0794 (100)	Yohimbic acid	+	+	+	+	+	+				
11	6.95	-0.21	341.1865	C ₂₀ H ₂₄ N ₂ O ₃	323.1762 (3), 210.1153 (11), 192.1018 (5), 158.0953 (15), 144.0797(100),	Yohimbic acid (isomer)	+	+	+	+	+	+				
16	7.81	0.03	401.2071	C ₂₂ H ₂₈ N ₂ O ₅	383.1965 (2), 383.1965 (2), 369.1809 (2), 321.1598 (5), 240.1200 (32), 222.1125 (1), 188.1045 (5), 174.0907 (100)	Reserpine acid	+	+	+	+	+	+				
18	8.20	-0.29	371.1966	C ₂₁ H ₂₆ N ₂ O ₄	339.1703 (3), 353.1857 (21), 240.1230 (12), 222.1125 (2), 158.0964 (10), 144.0798 (100), 228.1223 (17)	18-Hydroxy-yohimbine	+	+	+	+	+	+				
27	10.75	1.44	415.2217	C ₂₃ H ₃₀ N ₂ O ₅	383.1916 (4), 397.2122 (2), 254.1369 (51), 236.1281 (2), 222.1116 (7), 188.1059 (7), 174.0899 (100), 160.0743 (4)	Reserpine acid methyl ester	-	+	-	-	+	-				
28	10.91	-0.79	355.2019	C ₂₁ H ₂₆ N ₂ O ₃	323.1560 (10), 224.1285(19), 337.1920 (3), 212.1282 (24), 158.0959 (1), 144.0808 (100)	Yohimbine ^a	+	+	+	+	+	+				
29	11.70	1.44	415.2217	C ₂₃ H ₃₀ N ₂ O ₅	383.1935 (3), 224.1257 (9), 204.1029 (100), 218.1176 (1), 189.0771 (21), 173.0845 (45)	Seredine	+	+	+	+	+	+				
38	20.82	-0.49	595.2651	C ₃₂ H ₃₈ N ₂ O ₉	434.1748 (15), 383.1941 (30), 351.1773 (5), 222.1110 (10), 195.0631 (100), 188.10 (3), 174.0880 (43)	Pseudo-reserpine	+	+	+	+	+	+				
39	21.20	0.14	563.239	C ₃₁ H ₃₄ N ₂ O ₈	351.1711 (60), 333.1535 (42), 319.1463 (55), 222.1244 (32), 195.0672 (21), 170.0939 (34), 144.0800 (100)	Reserpine class (unknown)	+	+	+	+	-	+				
40	21.50	0.19	565.2544	C ₃₁ H ₃₆ N ₂ O ₈	533.2282 (2), 434.1714 (3), 353.1805 (57), 321.1559 (5), 222.1115 (5), 158.063 (2), 195.0622 (100), 144.0777(28),	Raunescine	-	+	+	+	+	+				
41	22.90	0.4	621.2805	C ₃₄ H ₄₀ N ₂ O ₉	589.2544 (2), 460.1930 (4), 383.1925 (11), 351.1703 (3), 221.0794 (100), 222.1115 (98), 188.1070 (5), 174.0804 (19)	Rescicine	+	+	+	-	+	+				
42	23.48	1.45	591.271	C ₃₃ H ₃₈ N ₂ O ₈	371.1896 (21), 353.1847 (28), 221.0808 (100), 144.0771 (17)	Reserpine class (unknown)	-	-	+	-	-	+				
43	23.80	0.03	579.2702	C ₃₂ H ₃₈ N ₂ O ₈	547.2413 (12), 367.2009 (79), 448.1907 (1), 335.1730 (24), 236.1262 (10), 195.0649 (100), 144.0817 (38)	Deserpidine	+	+	+	-	+	+				
44	24.31	-0.25	609.2807	C ₃₃ H ₄₀ N ₂ O ₉	577.2492 (5), 448.1930 (24), 436.1934 (5), 397.2090 (66), 365.1835 (13), 236.1261 (15), 195.0640 (100), 174.0905 (55)	Reserpine ^a	+	+	+	+	-	+				
45	24.54	1.87	607.2634	C ₃₃ H ₃₈ N ₂ O ₉	367.2017 (17), 335.1765 (11), 236.1272 (7), 221.0820 (100), 206.0577 (10), 190.0621 (14), 144.0815 (35)	Reserpine class (unknown)	+	+	+	-	-	+				
46	25.02	1.78	605.2857	C ₃₄ H ₄₀ N ₂ O ₈	369.1805 (20), 221.1805 (100), 206.0598 (11), 190.0618 (27), 174.0910 (40)	Reserpine class (unknown)	+	+	+	-	-	+				
47	25.22	0.08	635.2963	C ₃₅ H ₄₂ N ₂ O ₉	603.2779 (1), 474.2066 (5), 397.2085 (22), 236.1281 (2), 221.0791 (100), 188.1070 (2), 174.0881 (11)	Rescinnamine	+	+	+	-	-	+				
Ajmalicine class																
4	5.52	-0.36	353.1861	C ₂₁ H ₂₄ N ₂ O ₃	321.1598 (1), 222.1124 (1), 210.111 (7), 144.0808 (100), 158.0964 (2)	Ajmalicina ^a	+	+	+	+	+	+				
6	6.01	0.05	369.1809	C ₂₁ H ₂₄ N ₂ O ₄	337.1558 (19), 222.1275 (16), 351.1703 (37), 291.1496 (5), 158.0963 (30), 144.0808 (100)	Methyl-14-hydroxy-19-methyl-16,17-didehydro-18-oxayohimban-16-carboxylate	+	+	+	+	+	+				
24	10.52	1.07	413.2076	C ₂₃ H ₂₈ N ₂ O ₅	397.1763 (3), 323.1560 (3), 222.1106 (28), 218.1176 (18), 204.1005 (100)	Reserpiline	+	+	+	+	-	+				
31	14.81	0.02	413.2072	C ₂₃ H ₂₈ N ₂ O ₅	397.1766 (10), 323.1560 (2), 222.1125 (29), 204.1005 (100), 218.1176(30)	Reserpiline (isomer)	+	+	+	+	+	+				
33	15.50	0.01	383.1965	C ₂₂ H ₂₆ N ₂ O ₄	351.1677 (2), 222.1110 (10), 159.0675 (3), 188.1053 (2), 174.0895 (100)	10-Demethoxyreserpiline	+	+	+	+	-	+				
35	17.50	0.23	411.1914	C ₂₃ H ₂₆ N ₂ O ₅	379.1652 (1), 222.1110 (16), 204.1007 (100), 218.1181 (12) 173.0818 (11)	Darcyrbeirine	-	+	+	+	-	+				
Ajmaline class																
2	4.80	0.31	325.1910	C ₂₀ H ₂₄ N ₂ O ₂	307.1715 (5), 186.0896 (12), 174.0913 (57), 160.0805 (100), 146.0935 (22), 138.0954 (12)	Seredamine	+	+	+	+	+	+				

Table 1 (continued)

Compound (peak) no.	RT (min)	Error (Δ ppm)	Obs. m/z	Molecular formula	MS/MS fragment ions (Relative abundance, %)	Compounds	Distribution						
							Root						
							Rh	Rm	Rs	Rt	Rv	Rvm	
3	5.01	0.76	311.1752	C ₁₉ H ₂₂ N ₂ O ₂	293.1752 (2), 172.0763 (9), 160.0768 (21), 146.0960 (100), 136.0791 (10)	Hydroxynortetraphyllicine	+	+	+	+	+	+	
8	6.55	1.4	343.2005	C ₂₀ H ₂₇ N ₂ O ₃	325.1927 (8), 307.1830 (15), 186.0906 (54), 174.0917 (60), 160.0766 (22), 138.0912 (100)	Ajmalinol	+	+	+	+	+	+	
9	6.60	0.09	313.191	C ₁₉ H ₂₄ N ₂ O ₂	295.1806 (5), 196.1123 (21), 156.0813 (16), 144.0821 (55), 130.0664 (100)	Norajmaline	+	+	+	+	+	+	
13	7.45	1.52	503.2386	C ₂₆ H ₃₄ N ₂ O ₃	341.1898 (10), 323.1752 (62), 309.1595 (27), 291.1487 (100)	Hydoxyseredamine-O-hexoside	+	+	+	+	-	+	
14	7.52	1.26	295.1801	C ₁₉ H ₂₂ N ₂ O	277.1666 (4), 156.0799 (7), 144.0811 (27), 138.0474 (47), 130.0656 (100), 120.0791 (24)	Nortetraphyllicine	+	+	+	+	+	+	
19	8.91	0.13	327.2067	C ₂₀ H ₂₆ N ₂ O ₂	309.1991 (3), 291.1839 (3), 170.0974 (45), 158.0964 (100), 144.0812 (82)	Ajmaline ^a	+	+	+	+	+	+	
21	9.53	0.6	309.196	C ₂₀ H ₂₄ N ₂ O	291.1780 (4), 263.1536 (31), 170.0950 (19), 158.0965 (77), 144.0817 (100), 120.0822	Tetraphyllicine	-	+	+	+	-	-	
22	9.84	1.4	353.186	C ₂₁ H ₂₄ N ₂ O ₃	335.1758 (3), 275.1501 (2), 156.0808 (4), 144.0890 (100), 130.0693 (7)	Quebrachidine	+	+	+	+	+	+	
23	10.11	0.11	355.2017	C ₂₁ H ₂₆ N ₂ O ₃	309.2031 (5), 277.1667 (13), 260.1360 (12), 170.0955 (78), 158.0956 (98), 144.0805 (78)	Hydroxymethylseredamine	+	+	+	+	+	+	
36	19.31	0.28	351.2066	C ₂₂ H ₂₆ N ₂ O ₂	291.1827 (17), 182.962 (78), 170.0956 (45), 158.0962 (45), 144.0803 (100), 120.0802 (10)	17-O-Acetyltetraphyllicine	+	-	+	+	-	-	
37	20.76	0.45	489.2595	C ₂₆ H ₃₆ N ₂ O ₇	327.2073 (100), 309.1663 (80), 291.1554 (47)	Ajmaline-O-hexoside	+	+	-	+	+	+	
Quaternary alkaloids													
15	7.70	1.25	513.2232	C ₂₇ H ₃₂ N ₂ O ₈	333.1615 (14), 274.1199 (16), 246.1299 (53), 230.0986 (87), 220.1137 (67), 204.0823 (100), 195.0926 (65)	Tetradehydroyo-himbine-O-hexoside	+	+	+	+	+	+	
20	9.17	0.25	511.2373	C ₃₇ H ₃₀ N ₂ O ₈	349.1548 (100), 317.1326 (24), 289.1313 (10)	Serpentine-O-hexoside	+	+	+	+	+	+	
25	10.60	0.85	335.1389	C ₂₀ H ₁₈ N ₂ O ₃	317.1296 (100), 289.1492 (7), 263.0808 (71), 235.0863 (34), 307.0858 (5)	Serpentine derivative	-	+	+	+	+	+	
26	10.70	-1.54	351.1707	C ₂₁ H ₂₂ N ₂ O ₃	351.1674 (100), 333.1570 (4), 219.1440 (5), 271.1470 (20), 248.1024 (4), 195.0890 (6), 168.0788 (13)	Tetradehydroyo-himbine	+	+	+	+	+	+	
32	15.40	0.47	349.1547	C ₂₁ H ₂₀ N ₂ O ₃	317.1290 (66), 289.1314 (7), 263.0796 (100), 235.0826 (34), 307.0858 (6)	Serpentine ^a	+	+	+	+	-	+	
34	16.62	0.45	685.3375	C ₄₂ H ₄₅ N ₄ O ₅	653.3131 (13), 435.1947 (23), 403.1516 (3), 349.1235 (2), 375.1701 (23), 251.1558 (7)	Serpentinine	+	+	+	-	-	-	
Other compounds													
5	5.93	1.47	343.2015	C ₂₀ H ₂₆ N ₂ O ₃	326.1972 (47), 307.1794 (16), 182.0968 (57), 170.0945 (36), 158.0962 (81), 144.0795 (100), 132.0757 (11)	Rauvotetraphyllicine A	+	+	+	+	+	+	
7	6.33	1.25	313.1917	C ₁₉ H ₂₄ N ₂ O ₂	295.1845 (7), 144.0819 (70), 138.0933 (100), 122.0869 (61)	Dihydroperak-sine	+	+	+	+	+	+	
10	6.90	0.89	311.1755	C ₁₉ H ₂₂ N ₂ O ₂	293.1641 (8), 156.0808 (17), 144.0804 (52), 138.0919 (10), 130.0650 (100), 120.0805 (28)	Norajmalidine	-	+	+	+	-	-	
12	7.12	0.12	513.2231	C ₂₇ H ₃₂ N ₂ O ₈	351.1994 (100), 291.1480 (16)	Raucaffricine	+	-	+	+	+	+	
17	8.02	0.15	309.1598	C ₁₉ H ₂₀ N ₂ O ₂	291.1492, 172.0782 (24), 160.0757 (51), 146.0612 (100), 172.0782 (24), 136.0780 (38), 120.0803(15)	Normitoridine	+	+	+	+	+	+	
30	12.04	0.74	429.2018	C ₂₃ H ₂₈ N ₂ O ₆	397.1781 (87), 369.1759 (10), 339.1340 (14), 220.0972 (100), 210.1124 (35), 205.0732 (9), 189.0772 (21), 178.0845 (13), 150.0913 (21)	Carapanaubine	+	+	+	-	+	+	

^a Matched with the standards. Rh: *R. hookeri*; Rm: *R. micrantha*; Rs: *R. serpentina*; Rt: *R. tetraphylla*; Rv: *R. verticillata*; Rvm: *R. vomitoria*. RT: Retention time; Obs: observed.

isomers distinguished by RDA fragment ions. Compound 27 showed fragment ions at m/z 174.0899, 188.1059 and 254.1369 whereas fragment ions at m/z 204.1029, 218.1176 and 224.1257 were observed in compound 29. Fragments of compounds 27 and 29 showed 30 Da difference indicating -OCH₃ group on terpene and indole moiety, respectively (Fig. 4). The characteristic RDA fragment ions at m/z 210.1119 (compounds 1), 240.1200 (compounds 16), 254.1369 (compound 27), 224.1257 (compound 29), 434.1748 (compounds 38 and 40), 460.1930 (compound 41), 448.1907 (compound 43) and 474.2066 (compound 47) were observed due to losses of indole moiety via RDA

cleavage followed by bond cleavage between C2-C3. Fragment ion at m/z 222.1110 was observed due to loss of H₂O (compounds 16), C₁₀H₁₂O₅ (compounds 38 and 40) and C₁₂H₁₄O₅ (compound 41) from fragment ions at m/z 240.1200, 434.1748 and 460.1930, respectively. Similarly, compounds 27, 29, 43 and 47 showed fragmentation at m/z 236.1262 due to loss of H₂O (compound 27), C₁₀H₁₂O₅ (compound 43) and C₁₂H₁₄O₅ (compound 47) from fragment ions at m/z 254.1369, 448.1907 and 474.2066, respectively. Compounds 39, 42, 45 and 46 may be reserpine class of compounds because all these compounds followed similar fragmentation pathways with the reserpine and

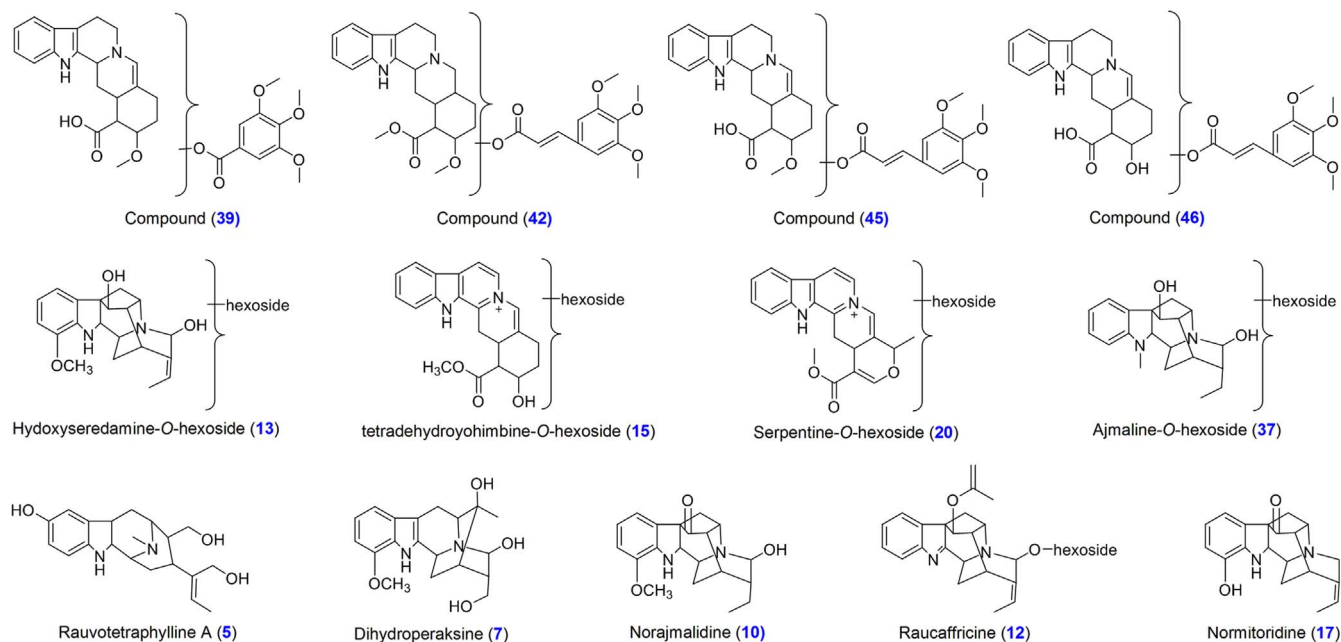


Fig. 3. Structures of unknown and other class of compounds.

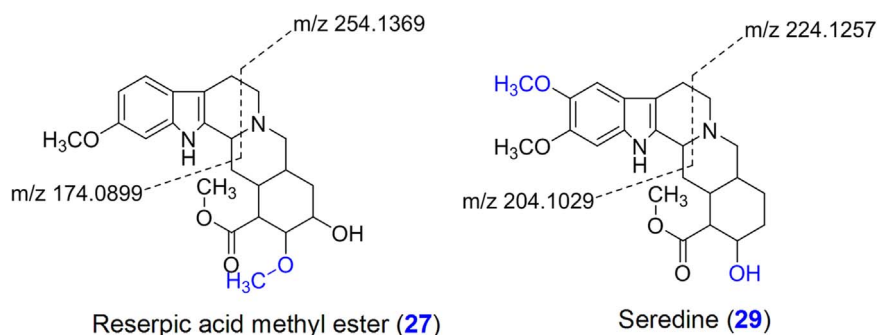


Fig. 4. Structures of isomeric compounds.

provided the characteristic fragment ions (Fig. 3).

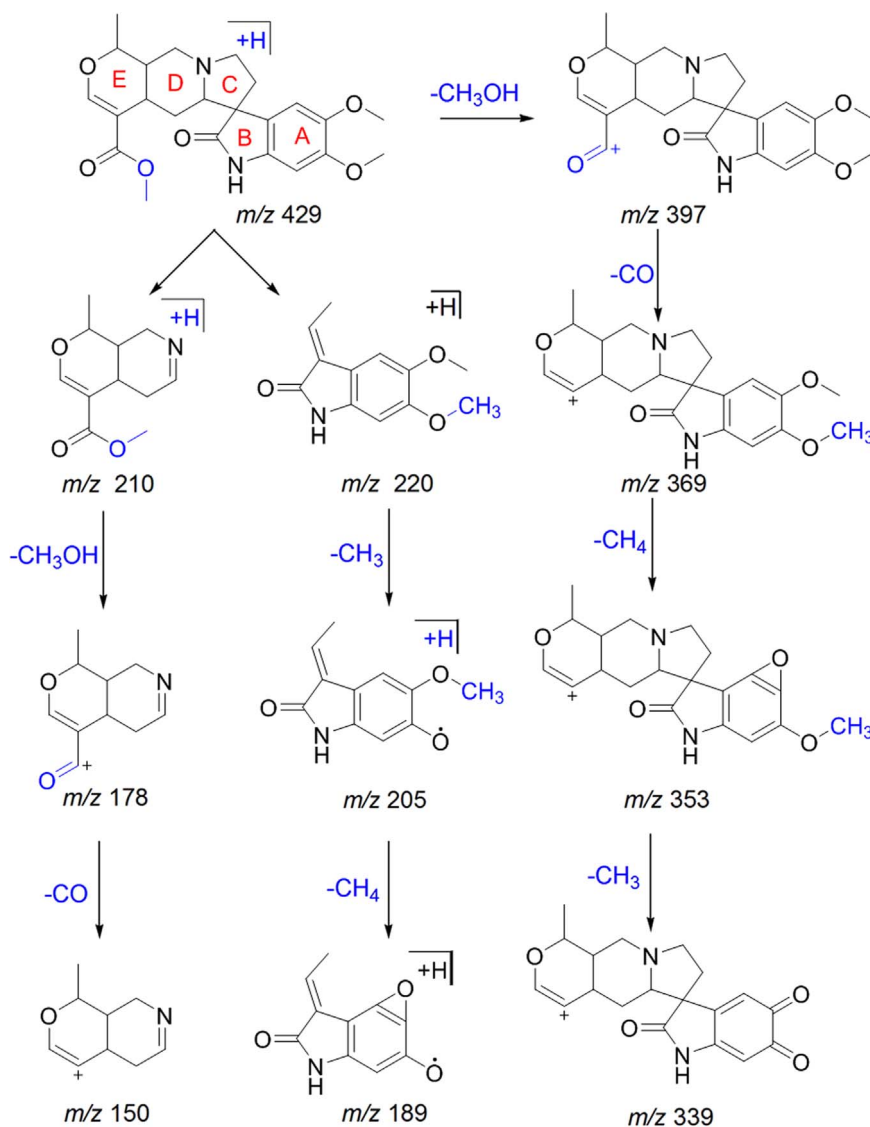
3.2.2. Ajmalicine class of compounds

Six compounds 4, 6, 24, 31, 33 and 35 were identified and characterized as ajmalicine, methyl-14-hydroxy-19-methyl-16,17-didehydro-18-oxayohimban-16 carboxylate, reserpiline, reserpiline (isomer), 10-demethoxyreserpiline and darcyribeirine, respectively (Scheme 2). Compound 4 was identified and characterized by comparison with the authentic standard of ajmalicine. Compounds 6, 24, 33 and 35 showed fragment ions at m/z 337.1558, 323.1560, 351.1677 and 379.1652 due to loss of CH_3OH , respectively. Fragment ions at m/z 158.0963 (compound 6), 218.1174 (compounds 24 and 35) and 188.1053 (compound 33) were observed due to loss of terpene moiety via RDA cleavage followed by bond cleavage between C14 and C15. Similarly, the fragment ions at m/z 144.0808 (compound 6), 174.0895 (143 Da+ OCH_3) (compound 33) and 204.1005 (142 Da+ 2OCH_3) (compounds 24 and 35) were observed as base peak due to loss of terpene moiety via RDA followed by bond cleavage between C3 and C14. Compounds 24 and 31 may be isomeric pair, showing similar fragment at retention time 10.52 min and 14.81 min with different relative abundances of fragment ions. RDA fragment ion at m/z 222.1124 was observed in all compounds due to loss of indole moiety via RDA cleavage followed by bond cleavage between C2 and C3.

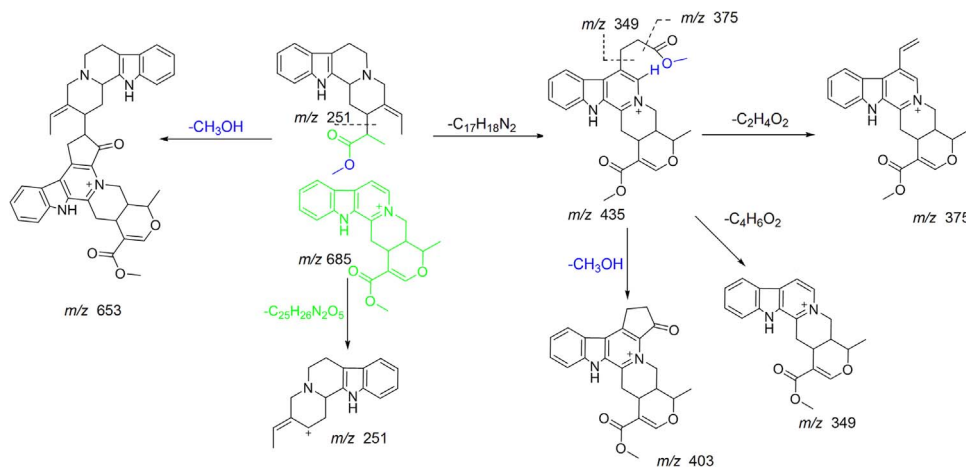
3.2.3. Ajmaline class of compounds

Twelve ajmaline class of compounds 2, 3, 8, 9, 13, 14, 19, 21, 22,

23, 36 and 37 were tentatively identified and characterized as seredamine, hydroxynortetraphylline, ajmalinol, norajmaline, hydroxyseredamine-O-hexoside, nortetraphylline, ajmaline, tetraphylline, quebrachidine, hydroxymethylseredamine, 17-O-acetyltetraphylline and ajmaline-O-hexoside, respectively (Scheme 3). Compound 19 was identified and characterized by comparison with ajmaline standard. Compounds 2, 3, 8, 9, 14, 21, 22, 23 and 36 showed fragment ions at m/z 307.1715, 293.1752, 325.1927, 295.1806, 277.1666, 291.1780, 335.1758, 309.2031 and 291.1827 due to loss of H_2O except compound 36 ($-\text{CH}_3\text{COOH}$). Fragment ions at m/z 186.0896 [155 Da+ OMe] (compound 2), 172.0763 [155 Da+ OH] (compound 3 and 8), 156.0813 (compounds 9, 14 and 22) and 170.0974 [155 Da+ CH_3] (compounds 21, 23, and 36) were observed due to loss of terpene moiety via RDA cleavage followed by bond cleavage between C14 and C15. Similarly, fragment ions at m/z 174.0913 [143 Da+ OMe] (compound 2), 160.0768 [143 Da+ OH] (compounds 3 and 8), 144.0821 (compounds 9, 14, and 22) and 158.09 [143 Da+ CH_3] (compounds 21, 23 and 36) formed due to loss of terpene moiety via RDA cleavage followed by bond cleavage between C3 and C14. All compounds showed RDA fragment ions at m/z 160.0805 [129 Da+ OMe] (compounds 2 and 8), 146.0960 (129 Da+ OH) (compound 3), 130.0656 (compounds 9, 14 and 22) and 144.0817 [129 Da+ CH_3] (compounds 21, 23 and 36) due to loss of terpene moiety via RDA cleavage followed by bond cleavage between C2 and C3. Compounds 13 and 37 were identified as hydroxyseredamine-O-hexoside and ajmaline-O-hexoside which showed a characteristic loss of 162 Da and gave fragment ions at m/z 341.1898 and 327.2073, respectively.



Scheme 5. Proposed fragmentation pathways of serpentinine.



Scheme 6. Proposed fragmentation pathways of carapanaubine.

3.2.4. Quaternary indole alkaloids

Six quaternary indole alkaloids compounds 15, 20, 25, 26, 32 and 34 were identified and characterized as tetrahydroyohimbine-*O*-hexoside, serpentine-*O*-hexoside, serpentine derivative, tetrahydroyohimbine, serpentine and serpentinine. Compound 32 was identified and characterized as serpentine which also matched with the standard. Compound 15 was identified as a hexoside of tetrahydroyohimbine and it showed fragment ion at m/z 351.1674 due to the

droyohimbine, serpentine and serpentinine. Compound 32 was identified and characterized as serpentine which also matched with the standard. Compound 15 was identified as a hexoside of tetrahydroyohimbine and it showed fragment ion at m/z 351.1674 due to the

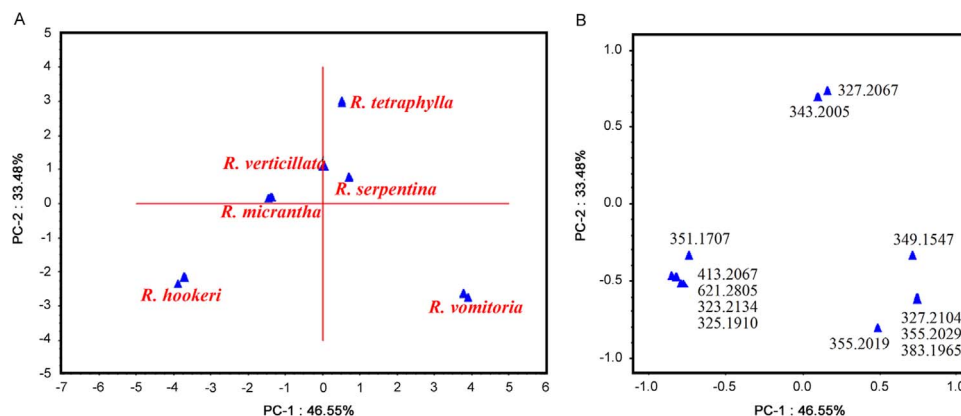


Fig. 5. (A) PCA score plot from *Rauwolfia* species showing discrimination among the *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata* and *R. vomitoria*. (B) Loading plot of the normalized LC–MS data obtained from three samples of each species showing possible ions of interest including those predominantly responsible for the discrimination of *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata* and *R. vomitoria*.

loss of $C_6H_{10}O_5$. Fragment ion at m/z 351.1674 gave product ions at m/z 333.1615 and 319.1440 due to further losses of H_2O and CH_3OH , respectively. Compound 20 showed fragment ion at m/z 349.1548 due to characteristic loss of 162 Da ($C_6H_{10}O_5$). Compound 25 was identified as serpentine derivative which showed 15 Da lower molecular weight compared to serpentine and followed similar fragmentation pattern. It showed fragment ions at m/z 317.1296 and 289.1492 due to losses of H_2O and CO , respectively. Compound 34 showed fragment ions at m/z 653.3131, 435.1956 and 251.1546 due to losses of CH_3OH , $C_{25}H_{26}N_2O_5$ and $C_{17}H_{18}N_2$, respectively. Fragment ion m/z 435.1956 showed product ions at m/z 375.1732, 349.1235, and 403.1516 due to losses of $C_2H_4O_2$, $C_4H_6O_2$ and CH_3OH , respectively (Scheme 5).

3.2.5. Other indole alkaloids

Six indole alkaloids 5, 7, 10, 12, 17 and 30 were tentatively identified and characterized as rauvotetraphylline A, dihydroperaksine, norajmalidine, raucaffricine, normitoridine, and carpanaubine, respectively. Compounds 5, 7, 10 and 17 showed fragment ions at m/z 326.1972, 295.1845, 293.1641 and 291.1492, respectively due to loss of H_2O from precursor. The RDA fragment ions at m/z 158.0962 [$143+CH_3$], 144.0795, 156.0808 and 172.0782 were observed in compounds 5, 7, 10 and 17 due to C-ring cleavage via RDA. Similarly, RDA fragment ions at m/z 130.0650 and 146.0612 [$129+OH$] were observed as base peaks in compounds 10 and 17, respectively. Compound 12 showed fragment ions at m/z 351.1994 and 291.1480 due to losses of $C_6H_{10}O_5$ and $C_2H_4O_2$, respectively. Compound 30 showed fragment ions at m/z 397.1781, 369.1759, 353.1501 and 339.1340 due to successive losses of CH_3OH , CO , CH_4 and CH_3 , respectively. Fragment ions at m/z 210.1124 and 220.0972 were observed as base peaks due to C-ring cleavage. Further fragment ions at m/z 210.1124 and 220.0972 produced fragment ions at m/z 178.0845 and 205.0732 due to losses of CH_3OH and CO , respectively. Fragment ions at m/z 150.0913 and 189.0772 formed due to losses of CO and CH_4 from m/z 178.0845 and 205.0732, respectively (Scheme 6).

3.3. Identification of markers using PCA

PCA converts a large number of data sets to a smaller number of variables. It produces overall discrimination between the closely related samples for quality control and authentication [43–45]. LC–MS data in combination with a data reduction technique such as PCA serves as an efficient and powerful tool to identify the chemical markers [34,43,44]. The LC–MS chemical fingerprints of *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata*, and *R. vomitoria* roots were analyzed by PCA to identify the chemical markers for discrimination amongst these species.

A total of 66 peaks from mass range m/z 179.0708 to 635.2984, peak area ≥ 1000 , were taken from the HPLC–ESI–QTOF–MS fingerprints ($n=3$) of roots and PCA was run. The PC1 and PC2 together were able to explain 57.96% of variance information. To obtain the best expression, peaks with lowest contribution were dropped and only twelve peaks at m/z 323.2134 (unknown), 325.1910 (seredamine), 343.2005 (ajmalinol), 327.2067 (ajmaline), 327.2104 (isomer of ajmaline), 355.2019 (yohimbine), 355.2029 (isomer of yohimbine), 349.1547 (serpentine), 383.1965 (10-demethoxyreserpiline), 351.1707 (tetrahydroyohimbine), 413.2067 (reserpiline) and 621.2805 (rescidine) were identified as marker peaks which were responsible for discrimination of six *Rauwolfia* species. Using these chemical markers PCs could explain 80.03% of the variance information as shown in the score and loading plots (Fig. 5). Peak at m/z 343.2005 (46.55%) showed a higher contribution followed by m/z 327.2104 (33.47%) and 327.2067 (12.28%), respectively. Similar report with contribution of peak area has already been reported in chromatographic fingerprinting and quantitative analysis of Xinkeshu tablet [46]. The PCA plots afforded useful qualitative information of six *Rauwolfia* species roots and revealed similarities and dissimilarities among them shown in Fig. 5A. It showed that *R. serpentina*, *R. micrantha* and *R. verticillata* were close whereas *R. hookeri*, *R. tetraphylla*, *R. vomitoria* were much apart from each other. *R. serpentina* is commonly used in herbal formulations and endangered due to over-exploitation [47]. This study showed that *R. micrantha* and *R. verticillata* might be used as substitutes for *R. serpentina* in herbal formulations. Hence, this analysis will also help to use alternate plant on the basis of phytochemical investigation which may conserve the *R. serpentina*.

4. Conclusions

A simple, sensitive, reproducible HPLC–ESI–QTOF–MS/MS method was developed in positive ion mode for the dereplication of MIAs. The diagnostic fragmentation pathways were established with the help of MS/MS spectra or diagnostic fragment ions of standard compounds. Reserpine, ajmalicine, ajmaline and yohimbine classes of MIAs showed two types of fragment ions, namely due to loss of substituents which were attached with the terpene moiety and RDA fragment ions. The established diagnostic fragmentation pathways were applied for identification and characterization of MIAs. 47 compounds were tentatively identified and characterized in ethanolic extract of roots of *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata* and *R. vomitoria*. Hydroxyseredamine-*O*-hexoside (13), ajmaline-*O*-hexoside (37), tetrahydroyohimbine-*O*-hexoside (15), serpentine-*O*-hexoside (25) and 4 reserpine class compounds 39, 42, 45 and 46 were identified as unknown/new for the first time. The isomeric compounds reserpine

acid methyl ester (27) and seredine (29) were successfully distinguished by MS/MS analysis. The marker peaks were successfully identified by PCA, which can discriminate the *R. hookeri*, *R. micrantha*, *R. serpentina*, *R. tetraphylla*, *R. verticillata* and *R. vomitoria* for quality control and authentication. Present information showed utility of accurate mass measurements which will also speed up dereplication of MIAs.

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