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Rapid characterization and identification of steroidal alkaloids in *Sarcococca coriacea* using liquid chromatography coupled with electrospray ionization quadropole time-of-flight mass spectrometry

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ABSTRACT

Rapid characterization of 23 pregnane-type steroidal alkaloids was studied using a positive ion electrospray ionization quadropole time-of-flight mass spectrometry (ESI-QqTOF–MS/MS) hybrid instrument. ESI-QqTOF–MS (positive ion mode) showed the presence of the protonated molecules $[M+H]^+$ which through low-energy collision-induced dissociation tandem mass spectrometric (CID–MS/MS) analysis showed the characteristic loss of dimethylamine moiety $[M+H-45]^+$ followed by the sequential lossess of attached substituents. Steroidal alkaloids having tigloyl or senecioyl group at C-3 produced diagnostic fragment ions at *m*/*z* 100 and 83. Our study also demonstrates the influence of unsaturation, and number and nature of substitutents on product ion abundance and fragment ions. Moreover, the generalization of the fragmentation pattern was linked with the structural features in steroidal skeleton. This strategy was successfully applied in LC–ESI-QqTOF–MS/MS analysis of *Sarcococca coriacea* extract to investigate and characterize pregnane-type steroidal alkaloids in complex mixture.

1. Introduction

Steroidal alkaloids are found in only handful of families and genera of the plant kingdom. Family Buxaceae one of the 20 most important alkaloid containing plant families, is comprises of four genera including *Buxus*, *Sarcococca*, *Pashysandra* and *Styloceras* [1]. The plants of genus *Sarcococca* are widely used in folk medicines in South Asia and China. Extracts of some *Sarcococca* species has been extensively used for the treatments of pain, rheumatism, malaria and other parasitic and skin diseases [2]. Most of the biological activities have been attributed to the steroidal alkaloids. Steroidal alkaloids of genus *Sarcococca* have been shown to have antileishmanial [3,4], antifungal [5], antiplasmodial [6] cholinesterase inhibitory [7] and antibacterial [8] activities.

Steroidal alkaloids of genera *Sarcococca* are pregnane derivatives. Development of a high-throughput dereplication approach is important for the analysis of trace amounts of known and unknown steroidal alkaloids in complex mixtures. Electrospray ionization mass spectrometry (ESI-MS) with collision induced dissociation (CID) has been developed as a powerful tool for the identification and characterization of molecules in mixtures [9]. This technique gives the exact mass of the molecular ion and is ideally suited for non-volatile, thermally labile and polar compounds. It is the most commonly used ionization technique for the online coupling of liquid phase separation techniques, such as liquid chromatography with mass spectrometry [10]. However, in order to characterize a compound from its MS/MS data, previous knowledge of the fragmentation pathways of homologous compounds exhibiting a conserved structural core is required [11]. These MS^n experiments, supported by ESI-QTOF–MS/MS data, identify characteristic fragments which provide useful information about the structures of compounds. An HPLC–MS method for characterizing steroidal alkaloids (including cevanine-type, veratramine-type, jervine-type and secosolanidine-type alkaloids) in *Fritillaria* spp. and related mixtures has been developed [12]. Similarly, electrospray ionization multi-stage mass spectrometry (ESI-MS) of protoverine-, germine- and zygadenin-type alkaloids from the Chinese herb *Veratrum nigrum* L. has also been conducted [13]. To the best of our knowledge, there is no published data available regarding the MS/MS analysis of *Sarcococca* steroidal alkaloids so far.

In continuation of our studies on electrospray ionization mass spectrometry and high throughput dereplication strategy development for natural products analysis [14], this paper describes the

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application of ESI tandem mass spectrometry (LC–ESI-QqTOF-MS/MS analysis) to identify fragmentation pattern and diagnostic fragments of steroidal alkaloids of *Sarcococca* species.. The results obtained can be used for characterize these types of steroidal alkaloids in crude materials like plant extracts and herbal formulations.

2. Experimental

2.1. Chemicals and reagents

Chemicals and solvents were of analytical grade or HPLC grades and were purchased from Aldrich–Sigma. Deionized water (Milli-Q) was used throughout the study. All fully characterized steroidal alkaloids were obtained from the Compound Bank facility at the Dr. Panjwani Center for Molecular Medicine and Drug Research (International Center for Chemical and Biological Sciences) University of Karachi. The steroidal alkaloids utilized in this study were isolated from *Sarcococca coriacea* and *Sarcococca hookeriana*. The isolation procedure and characterization details of the analyzed steroidal alkaloids have been reported previously [4,15].

2.2. Collection of plant material and extraction of steroidal alkaloids

The roots of *S. coriacea* (Hook, f.) were collected from Champadevi area of Kathmandu district, Nepal, during June, 2005. The plant material was identified by Prof. Dr. Krishna Kumar Shrestha, Central Department of Botany, Tribhuvan University, Nepal. A voucher specimen (Sc-5/2005) was deposited in the same section. Air dried roots (4 g) of *S. coriacea* were extracted with 80% methanol/water (15 mL). The concentrated methanolic aqueous extract (0.375 g) was dissolved in cold distilled water and defatted with petroleum ether (8 × 3 mL). The aqueous layer was then extracted with CH_2Cl_2 (8 × 3 mL) to obtain the neutral fraction. The remaining aqueous layer was then made alkaline by adding ammonia solution (pH 9–10) and extracted with CH_2Cl_2 (8 × 3 mL) to obtain the alkaline fraction (1 mg). This basic extract was then dissolved in HPLC-grade methanol (1 mL), filtered through Millipore filter (0.45 μm), and subjected to LC/MS/MS analysis.

2.3. ESI-QqTOF-MS analysis

Steroidal alkaloids were dissolved in 50% acetonitrile – water containing 0.1% formic acid (0.2 μg/μL) and analyzed by electrospray ionization (ESI) and collision-induced dissociation (CID), positive ion mode, on ESI-QqTOF-MS/MS instrument (QSTAR XL mass spectrometer Applied Biosystem/MDS Sciex, Darmstadt, Germany). High-purity nitrogen gas was used as the curtain gas and collision gas delivered from Peak Scientific nitrogen generator. The ESI interface conditions were as follows: ion spray capillary voltage of 5500 V, curtain gas flow rate 20 L min^{-1}, nebulizer gas flow rate 30 L/min, DP1 60 V, DP2 15 V, and focusing potential of 265 V. The collision energy was swept between 20 and 50 eV for MS/MS analysis. Calibration was performed using internal calibration process. Samples were introduced into the mass spectrometer using a Harvard syringe pump (Holliston, MA, USA) at a flow rate of 5 μL/min. Computational studies were performed using DFT at the B3LYP level with 6-31G* basis set in Spartan 08 v 1.2.0 (Wavefunction, CA, USA) to investigate the most probable protonation site in hookerianamide B (**6**) by utilizing the previously established strategy [14]. Theoretical fragmentation of protonated steroidal alkaloids was evaluated by using ACD/MS Fragmenter software (ACD Labs).

2.4. LC–ESI-MS/MS analysis

Chromatographic separation was performed using a capillary HPLC system (Series 1200, Agilent Technologies, Waldbronn, Germany), and reversed-phase capillary column (ZORBAX XDBC18, 150 × 4.6 mm, 5 μm, Agilent). Injection volume was 0.2 μL. The mobile phases were as follows: eluent A, H_2O (0.1% formic acid) and eluent B, ACN. The flow rate was 10 μL/min, and a gradient elution program was maintained. The chromatographic procedure was initialized at 35% B, raised to 36% B at 8 min, followed increased up to 37% B at 14 min, 38% B at 16 min, 39% B at 18 min, 40% B at 22 min, 45% B at 25 min and then a return to initial conditions 35% B at 30 min. The data were recorded *via* information-dependent acquisition (IDA) experiments with TOF scan range of *m/z* 300–600 amu at the rate of 1 scan s^{-1} with the four most abundant peaks. Switch criteria was used for masses between 350 and 600 amu. Precursor ion scans were recorded between 50 and 600 amu on a QSTAR XL mass spectrometer (Applied Biosystem/MDS Sciex, Darmstadt, Germany), equipped with an ESI spray source and running with Analyst QS 1.1 (Applied Biosystems). The system was operated in the positive ion mode and the source and inlet parameters were optimized as: ion spray capillary voltage, 5500 V; curtain gas flow rate, 15 L min^{-1}; nebulizer gas flow rate, 35 L min^{-1}; DP1 60 V; DP2 15 V; focusing potential, 265 V; and collisional energy of 25 and 35 eV for MS/MS analysis.

3. Results and discussion

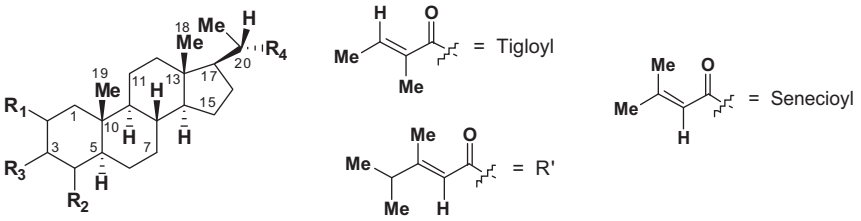
Twenty three pregnane-type steroidal alkaloids obtained from *S. coriacea* and *S. hookeriana* **1–23**, were subjected to the positive ESI-QqTOF-MS analysis and generalized results are summarized in Table 1, The product ion scan recorded from the extracted $[M+H]^+$ are presented in Supplementary material Table 1. MS/MS analysis of $[M+H]^+$ ions of various alkaloids showed largely similar fragments with minor differences due to the difference in substituents attached. Relative intensities of selected product ions of $[M+H]^+$ *versus* laboratory collision energy ranging from 20 to 50 eV (with stepping up of 5 eV each time) were plotted for sarcovagine A (**8**) taken as a representative of *sarcococca* pregnane-type steroidal alkaloids (Fig. 1). It showed that the optimum collision energy (CE) for recording product ion spectra of steroidal alkaloids is 35 eV. However, the fragmentation pattern and the peaks ion abundance were found to be significantly influenced by the variation of collision energy. Therefore, MS/MS spectra of all steroidal alkaloids were screened against laboratory collision energy ranging between 20 and 50 eV.

3.1. Fragmentation pattern in Sarcococca (pregnane-type) steroidal alkaloids

Many characteristic peaks were observed mainly by the sequential losses of substituents from the $[M+H]^+$, along with substituents were detected as ions when the steroidal skeleton was removed as a neutral molecule. Fragmentation patterns of this class is mainly influenced by the presence of substituted amine/amide groups at C-3 and C-20, the presence of hydroxy groups, unsaturation at C-16, α,β-unsaturated keto group at C-4, and acetate substituents.

A characteristic loss of $NH(Me)_2$ from C-2 that is $[M+H-45]^+$, was observed in all pregnane-type alkaloids, except in compounds **12**, **22** and **23**. Compounds **12** and **23** showed the removal of *m/z* 95 $[M+H-45-45]^+$ corresponding to the synchronous losses of $NH(Me)_2$ and NH_2CHO from C-3 and C-20, respectively, while compound **22** showed a peak at $[M+H-45-32]^+$ due to the loss of $NH(Me)_2$ and CH_3OH from C-20 and C-3, respectively. In another pathway, the protonated amine group ($C_2H_8N^+$) was cleaved from

Table 1
The structures and HR-ESI-MS data of *Sarcococca* (pregnane-type) steroidal alkaloids.



	Name	R_1	R_2	R_3	R_4	Δ	$[M+H]^+$	Exact mass	Observed mass	Error (ppm)
1	Dictyophlebine	H	H	β-NHMe	$N(Me)_2$	–	$C_{24}H_{45}N_2^+$	361.3577	361.3543	3.6
2	5,6-Dihydrosarconidine	H		β-NHMe	$N(Me)_2$	$\Delta^{16,17}$	$C_{24}H_{43}N_2^+$	359.3421	359.3408	3.5
3	Terminaline	H	α-OH	β-OH	$N(Me)_2$	–	$C_{23}H_{42}NO_2^+$	364.3210	364.3199	3.0
4	N^3-Methylepipachysamine D	H	H	β-NMeCOPh	$N(Me)_2$	–	$C_{31}H_{49}N_2O^+$	465.3839	465.3855	3.35
5	N^3-Methylpachysamine A	H	H	β-$N(Me)_2$	$N(Me)_2$	–	$C_{25}H_{47}N_2^+$	375.3733	375.3726	2.07
6	Hookerianamide B	α-OH	β-OAc	β-NH-senecioyl	$N(Me)_2$	–	$C_{30}H_{51}N_2O_4^+$	503.3843	503.3868	4.9
7	Hookerianamide C	β-OAc	H	β-NH-senecioyl	$N(Me)_2$	–	$C_{30}H_{51}N_2O_3^+$	487.3894	487.3895	0.2
8	Sarcovagine A	α-OH	β-OH	β-NH-tigloyl	$N(Me)_2$	–	$C_{28}H_{49}N_2O_3^+$	461.3737	461.3793	11.98
9	Hookerianamide N	β-OH	H	β-NH-senecioyl	$N(Me)_2$	–	$C_{28}H_{49}N_2O_2^+$	445.3788	445.3783	1.2
10	Vaganine A	H	β-OAc	β-NH-senecioyl	$N(Me)_2$	–	$C_{30}H_{51}N_2O_3^+$	487.3894	487.3890	0.9
11	Sarcovagine C	H	β-OAc	β-NH-tigloyl	$N(Me)_2$	–	$C_{30}H_{51}N_2O_3^+$	487.3894	487.3918	4.9
12	*N*-Formylchonemorphine	H	H	β-NHCHO	$N(Me)_2$	–	$C_{24}H_{43}N_2O^+$	375.3369	375.3359	2.9
13	N^3-Methyl,N^3-5-hexen-1-yl-derivative of dictyophlebine	H	H	β-NMe-C_6H_{12}	$N(Me)_2$	–	$C_{30}H_{55}N_2^+$	443.4359	443.4350	2.20
14	Saligenamide A	H	H	β-NH-R'	$N(Me)_2$	–	$C_{30}H_{53}N_2O^+$	457.4152	457.4111	9.0
15	N^3-Methyl,N^3-4-penten-1-yl-derivative of dictyophlebine	H	H	β-NMe-C_5H_{10}	$N(Me)_2$	–	$C_{29}H_{53}N_2^+$	429.4203	429.4223	4.59
16	Chonemorphine	H	H	β-NH_2	$N(Me)_2$	–	$C_{23}H_{43}N_2^+$	347.3421	347.3418	0.8
17	Isosarcodine	H	H	β-NMeCOMe	$N(Me)_2$	–	$C_{26}H_{47}N_2O^+$	403.3683	403.3705	5.5
18	Sarcorine	H	H	β-NHCOMe	$N(Me)_2$	–	$C_{25}H_{45}N_2O^+$	389.3526	389.3555	7.3
19	Epipachysamine D	H	H	β-NHCOPh	$N(Me)_2$	–	$C_{30}H_{47}N_2O^+$	451.3683	451.3696	2.9
20	Sarcovagine D	H	=O	β-NH-tigloyl	$N(Me)_2$	$\Delta^{2,3}$	$C_{28}H_{45}N_2O_2^+$	441.3475	441.3471	1.03
21	Sarcovagenine C	H	=O	β-NH-tigloyl	$N(Me)_2$	$\Delta^{2,3}$, $\Delta^{16,17}$	$C_{28}H_{43}N_2O_2^+$	439.3319	439.3202	26.64
22	Alkaloid C	H	H	α-OCH_3	$N(Me)_2$	$\Delta^{5,6}$	$C_{24}H_{42}NO^+$	360.3261	360.3255	1.6
23	Iso-*N*-formylchonemorphine	H	H	β-$N(Me)_2$	NHCHO	–	$C_{24}H_{43}N_2O^+$	375.3369	375.3303	17.82

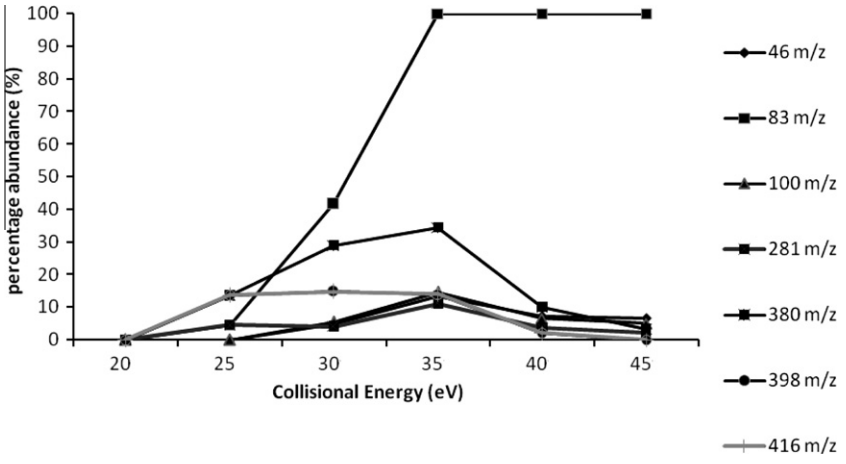


Fig. 1. Relative abundances of characteristic ions vs collisional energies of Sarcovagine A (**8**).

C-20 and detected as a characteristic ion at *m/z* 46 in all steroidal alkaloids.

Similarly compounds **4**, **14**, and **19** having an amide side chain at C-3, underwent cleavage at the C-3-N bond and found $[M+H-45-135]^+$, $[M+H-45-127]^+$, and $[M+H-45-121]^+$ product ions, respectively. Along with this, C-3 amide substituent containing compounds also showed peaks at *m/z* 136, 128 and 122 corresponding to $C_8H_{10}NO^+$, $C_7H_{14}NO^+$ and $C_7H_8NO^+$ product ions, respectively. Compounds **13** and **15** which possess alkenyl substituted amine group at C-3 likewise cleaved.

Compounds **6–11** possessing tigloyl or senecioyl moieties at C-3 showed characteristic losses of C_5H_6O (82 amu) followed by the removal of ammonia molecule (17 amu) after the sequential loss of attached substituent from the steroidal skeleton. Two characteristic peaks were also observed in steroidal alkaloids that possess tigloyl or senecioyl moiety at *m/z* 100 and 83, respectively (Fig. 2). The peak at *m/z* 100 was absent in compounds **20** and **21**, probably due to the presence of α,β-unsaturated ketone in ring A which is in conjugation with the amide group at C-3. Additionally, compounds **20** and **21** with conjugated carbonyl system in a

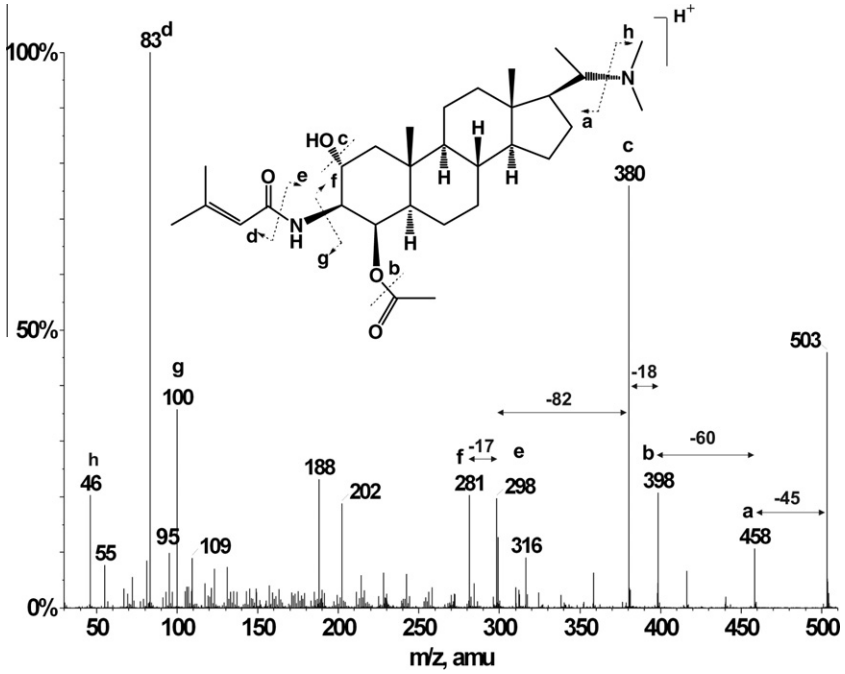


Fig. 2. MS/MS spectra of compound **6** at CE 35 eV showing neutral losses of substituents from the core skeleton and fragments at *m/z* 100 and *m/z* 83.

ring A showed the characteristic peaks at *m/z* 218 and 204. A MS/MS analysis of compounds **2** and **21** showed an intense peak of $[M+H-45]^+$ even at low collision energy (base peak at 25 eV) in comparison to the other pregnane-type steroidal alkaloids (Fig. 3).

A characteristic fragment at *m/z* 285 $[C_{21}H_{33}]^+$ probably arose by the removal of C-20 amine and/or C-3 amide moieties through the cleavage of the C-20-N and C-3-N bonds. This fragment is a diagnostic fragment for the characterization of pregnane-type steroidal alkaloids with only two substituents, i.e. at C-20 and C-3 with no unsaturation in the core skeleton. This fragment appears in the MS/MS spectra of compounds **1**, **4**, **5**, **12**, **13–19** and **23**. The same fragment were observed in compounds **2**, **3**, **7**, **9–11** and **22**, 2 amu less at *m/z* 283. This fragment ion is a characteristic of all pregnane-type steroidal alkaloids having one additional substituent or an unsaturation in the skeleton other than two *N*-substitutents at C-3 and C-20. Similarly, fragment ion at *m/z* 281 was observed in compounds **6** and **8**, which possess two more substituents attached in the skeleton, other than the two N-substituents at C-20 and C-3. Intensities of $[M+H]^+$ peaks and MS/MS ion of key fragments of compounds **1–23** are presented in Supplementary material Table 1. In the light of the above studies, a flow chart is proposed for the rapid identification and structural analysis of steroidal alkaloids with positive CID experiments (Supplementary material Scheme 1).

3.2. Elucidation of fragmentation pathway of Sarcococca (pregnane-type) steroidal alkaloids

Hookerianamide B (**6**) was selected as a representative of pregnane-type steroidal alkaloids. Three possible protonation sites were evaluated, which includes the nitrogen of amine side chain at C-20 (A), the oxygen of the amide chain at C3 (B) and the nitrogen of amide side chain at C-3 (C). It was found that the amine nitrogen (A) on protonation showed minimum energy (−1584.08844 Hartree) and therefore it was considered as the most favourable site of protonation. The oxygen of the amide chain at C-3 (B) was found to be the second most favourable site of protonation (−1584.07399 Hartree). Both forms were considered for elucidating the major fragmentation pathways of hookerianamide B (**6**) type steroidal alkaloids as the energy difference between both protonated forms was found to be 9.06 Kcal/mol. Moreover, the bond length of protonated (A–C) and nonprotonated forms of hookerianamide B (**6**) were also investigated.

Protonation at the nitrogen of amine side chain at C-20 (A) causes considerable elongation of bond c and decreases in the bond lengths of bonds a and b (Table 2), hence facilitating the cleavage of the bond between C-20 and amine nitrogen, thereby causing a loss of the secondary amine $NH(Me)_2$ (*m/z* 45 amu) to form fragment D at *m/z* 458. The secondary carbocation thus formed may rearrange to the more stable tertiary carbocation E through 1,2-H shift, followed by the sequential removal of acetate and hydroxy groups to form fragment ions E and F with *m/z* 398.3020 ($C_{26}H_{40}NO_2^+$, calc. 398.3053) and 380.2909 ($C_{26}H_{38}NO^+$, calc. 380.2947), respectively. It is proposed that the rearrangement of the amide may lead to the dissociation of the bond between the amide nitrogen at C-3 and the carbonyl carbon resulting in the loss of C_5H_6O (82 amu) to form fragment ion G at *m/z* 298 ($C_{21}H_{32}N^+$). Removal of ammonia then can occur to give fragment H, giving rise to the peak of *m/z* 281.2181 ($C_{21}H_{29}^+$, calc. 281.2263) in the MS/MS spectrum (Scheme 1). This fragment H ($C_{26}H_{40}NO_2^+$, *m/z* 281.2263) was also predicted by ACD/MS software.

Hookerianamide B (**6**) simultaneously fragment through a second pathway which is due to the protonation at the oxygen of the amide chain at C-3 (B), leading to the fragments of *m/z* 100 ($C_5H_{10}NO^+$) and 83 ($C_5H_7O^+$). The fragmentation pathway of the fragment M is proposed as described in Scheme 2. Fragments formed due to the loss of the neutral secondary amine (I), acetate (J) and water (K) molecules and can also be predicted by this pathway. Fragment K may tautomerize into L which leads to the formation of fragment M at *m/z* 83.0401 ($C_5H_7O^+$, calc. 83.0491) by the elimination of the steroidal part as a neutral moiety. This proposal is also supported by analyzing the bond lengths of protonated form C (Table 2).

This increase in the bond length of bond f was not observed in form B (i.e. on protonation at amide oxygen), suggesting that the fragment is formed through the tautomeric form L, as proposed

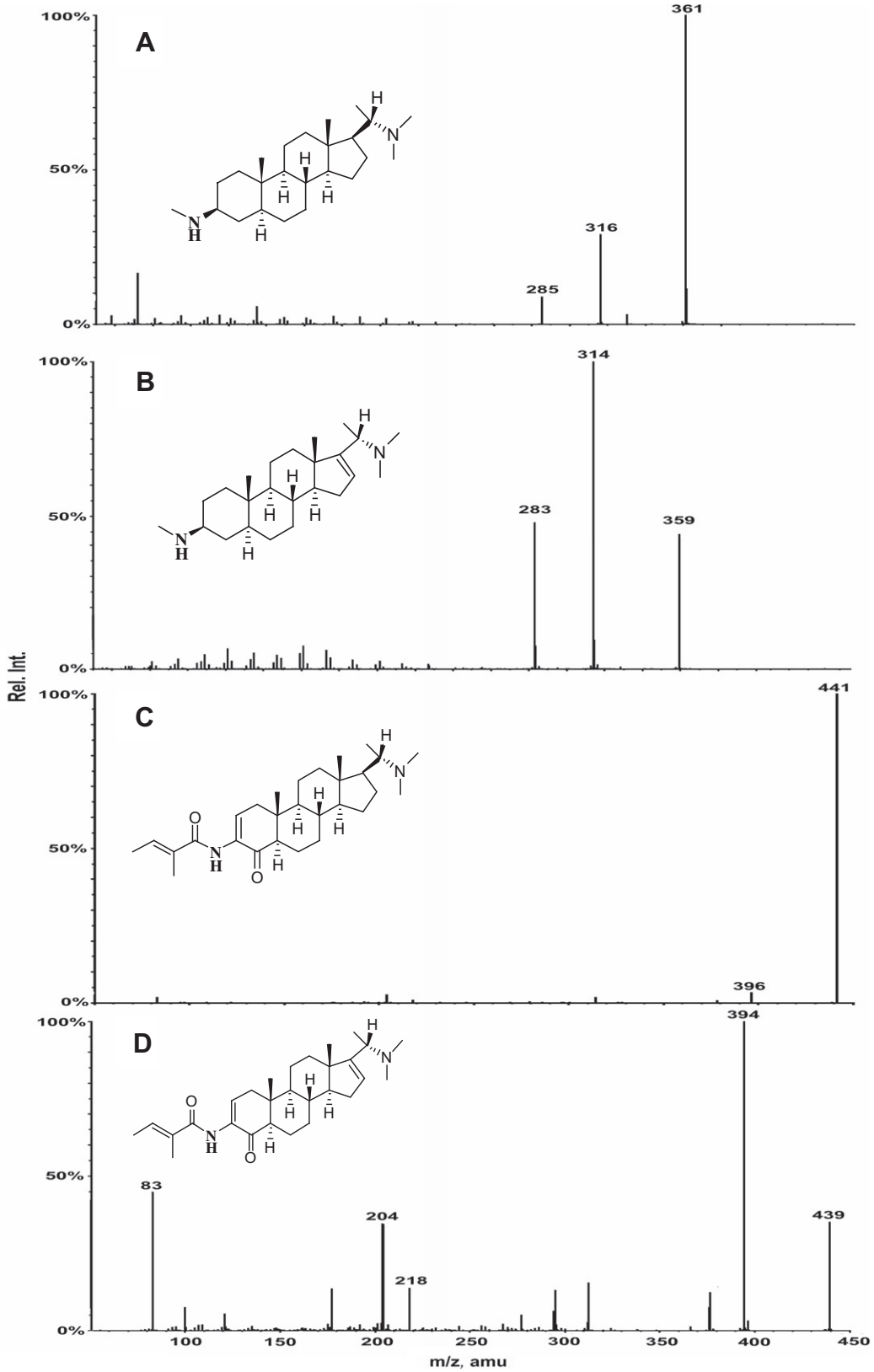
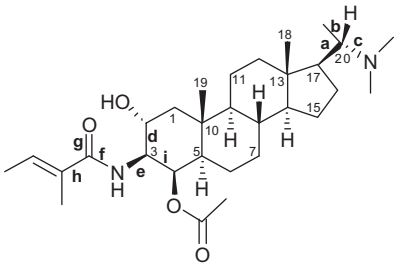


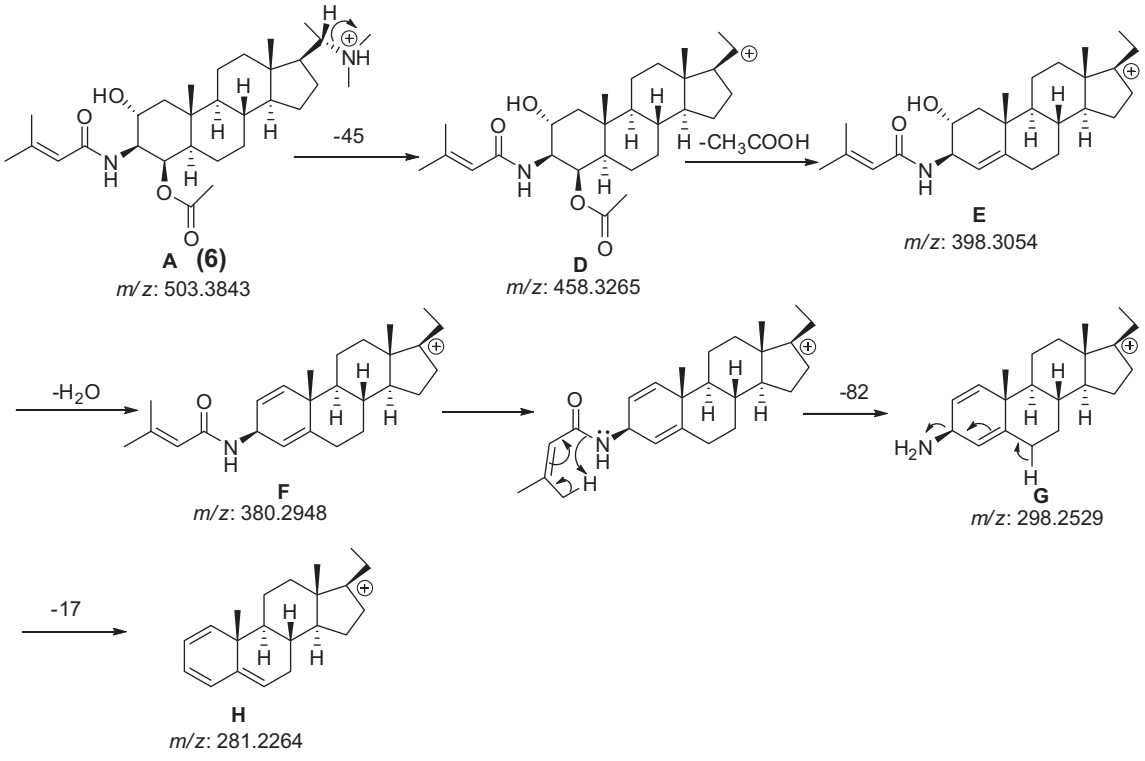
Fig. 3. Product ion spectra of compound **1** (A), **2** (B), **20** (C) and **21** (D) at collision energy of 25 eV.

in Scheme 2. The formation of fragment N at *m/z* 281 in steroidal alkaloids having two unsaturations in ring A is also proposed to occur from L through inductive bond cleavages (Scheme 2). Fragments O at *m/z* 283 (having one unsaturation in steroidal core) and P (having no unsaturation in steroidal core) at *m/z* 285, are presented in Schemes 3 and 4, respectively, and are proposed to

Table 2
Bond length data of neutral and protonated forms of hookerianamide B.



Bond	Bond length neutral (Å)	Bond length A (Å)	Δ (Å)	Bond length B (Å)	Δ (Å)	Bond length C (Å)	Δ (Å)
a	1.558	1.547	−0.011	1.555	−0.003	1.558	0.00
b	1.539	1.529	−0.01	1.538	−0.001	1.538	−0.001
c	1.487	1.570	0.083	1.487	0.00	1.487	0.00
d	1.548	1.539	−0.009	1.542	−0.006	1.553	0.005
e	1.474	1.456	−0.018	1.479	0.005	1.530	0.056
f	1.386	1.372	−0.014	1.326	−0.06	1.550	0.164
g	1.230	1.228	−0.002	1.304	0.074	1.202	−0.028
h	1.511	1.509	−0.002	1.474	−0.037	1.465	−0.046
i	1.567	1.566	−0.001	1.568	0.001	1.549	−0.018

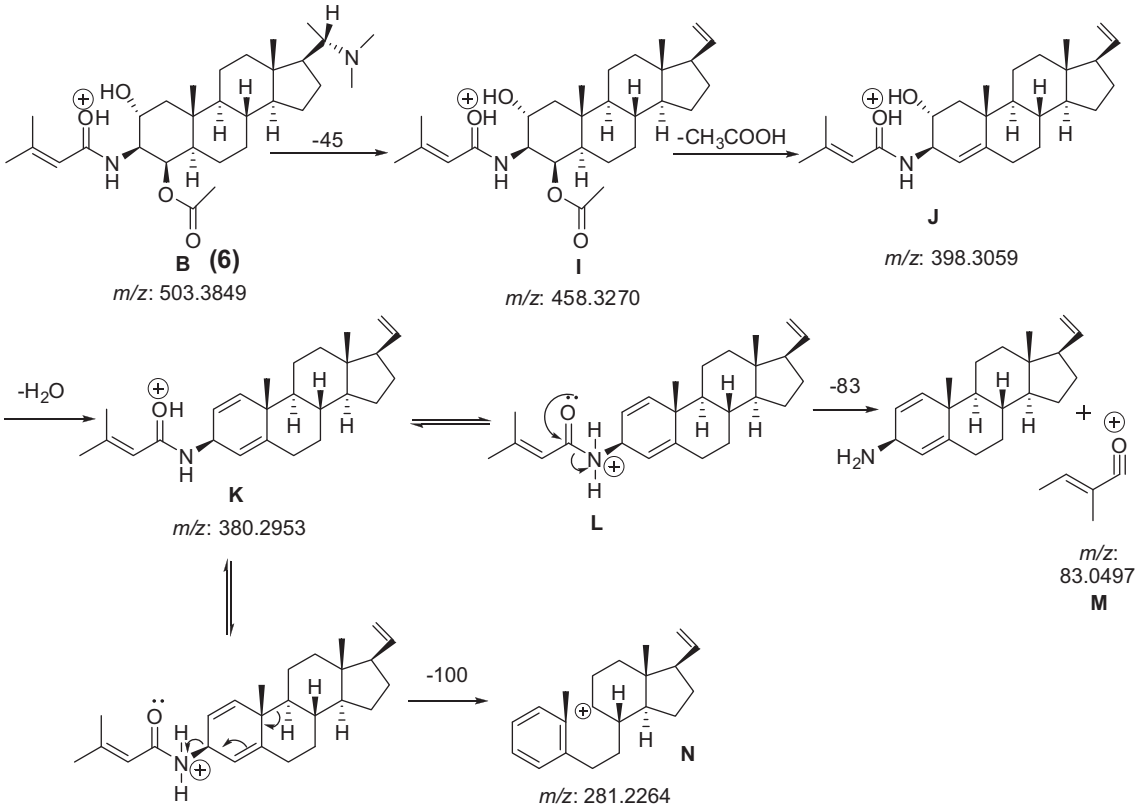


Scheme 1. Proposed fragmentation pathway for the fragments formed on protonation at nitrogen of amine side chain (A).

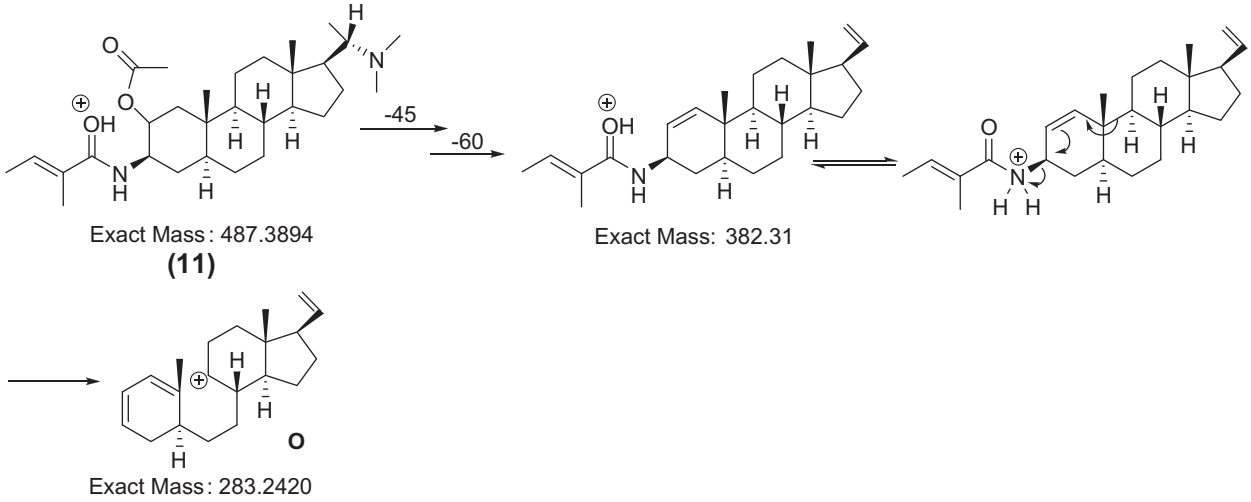
be formed through inductive cleavage. Formation of fragment Q at *m/z* 100.0652 ($C_5H_{10}NO^+$, calc. 100.0756) may occur by the abstraction of hydrogen at C-2 by the amide nitrogen (Scheme 5) in the protonated form B. The formation of this fragment through form B (protonated at carbonyl oxygen) is evident by the decrease in the lengths of bonds d and f and an increase in the length of bond e (Table 2), which facilitates the cleavage of the bond between C-3 and the amide nitrogen (bond e) with the formation of fragment Q. Fragments, M ($C_5H_7O^+$), and N ($C_5H_{10}NO^+$) were also predicted by ACD/MS software.

3.3. LC/MS/MS analysis of *S. coriacea*

The steroidal alkaloids in the roots of *S. coriacea* were investigated by LC/ESI-MS/MS. The full ESI-QqTOF-MS scan in positive mode showed the presence of 15 steroidal alkaloids as protonated $[M+H]^+$ molecular ions (Fig. 4). Fig. 5A showed the total ion chromatogram (TIC) traces in the positive ion mode. As many steroidal alkaloids are isobaric in nature and cannot be identified by only using exact mass measurement without utilizing LC, therefore separation capabilities of LC were employed. LC–MS and LC–MS/MS



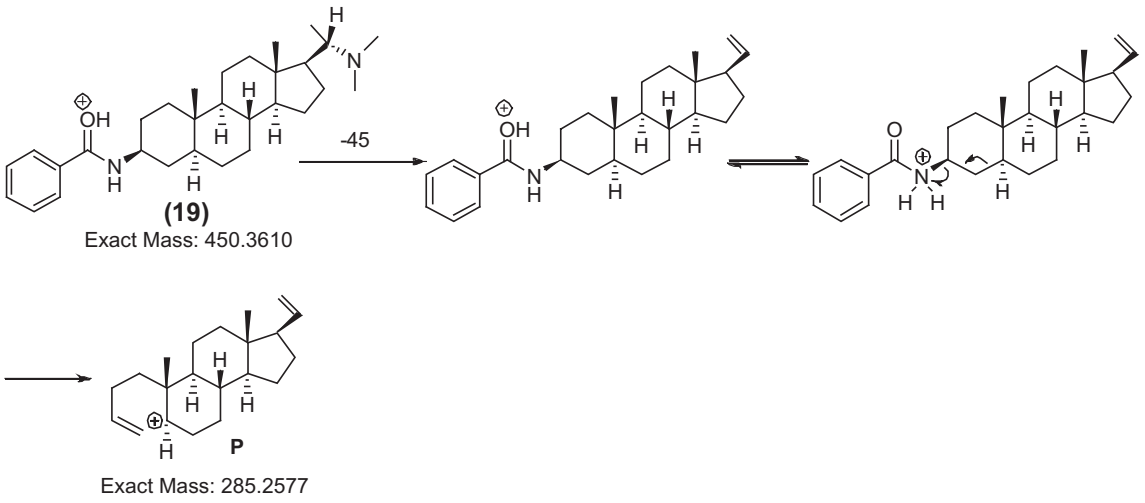
Scheme 2. Proposed pathway for fragments at *m*/*z* 281 and 83 formed on protonation at C-3 amide oxygen (B).



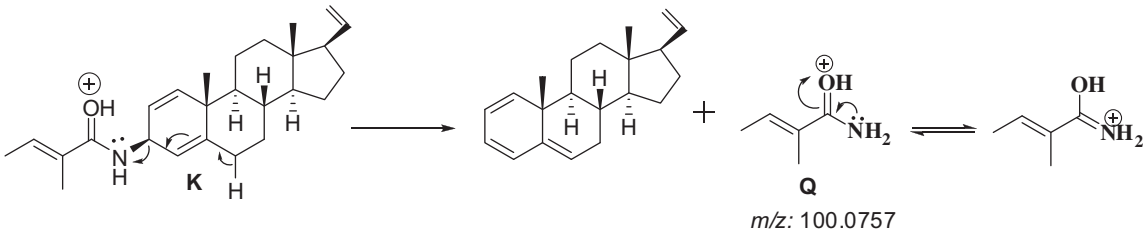
Scheme 3. Proposed pathway for fragments at *m*/*z* 283 formed on protonation at C-3 amide oxygen in compounds with one additional substituent (B).

analysis revealed the presence of 23 different steroidal alkaloids, identified from the same run of LC/MS. The retention times and MS/MS data are shown in Table 3. All compounds were searched in the updated Dictionary of Natural Products (DNP, version 19.2) on the basis of their exact deprotonated molecular masses (mass of an H^+ was subtracted before search) and respective molecular formulae, for the identification of pregnane-type steroidal alkaloids. In case of more than one matches, the search was narrowed down to genus *Sarcococca*. Finally, the compounds were identified by utilizing the proposed flow chart exploiting MS/MS data for their identification through diagnostic ions and losses (Supplementary material Scheme 1). Masses that are not reported in DNP were searched in Scifinder scholar for the most recent literature and the structures were match according to the identified fragments. Protonated ions **X, XII, XIII, XVI** and **XXI** were assigned to be the reference compounds **4**, **14**, **9**, **20** and **10**, respectively following the proposed scheme. The protonated ion **XIV** remained unidentified. The other 17 peaks were tentatively identified and are briefly discussed below.

For protonated ion **I**, *m*/*z* 545.3919 corresponding to the deprotonated molecular formula of $C_{32}H_{52}N_2O_5$ was searched in DNP (MW 544 Da). This search returned with no hits, therefore it was



Scheme 4. Proposed pathway for fragments at *m*/*z* 285 formed on protonation at C-3 amide oxygen in compounds with no additional substituent (B).



Scheme 5. Proposed fragmentation pathway for the fragment *m*/*z* 100 formed on protonation at C-3 amide oxygen (B).

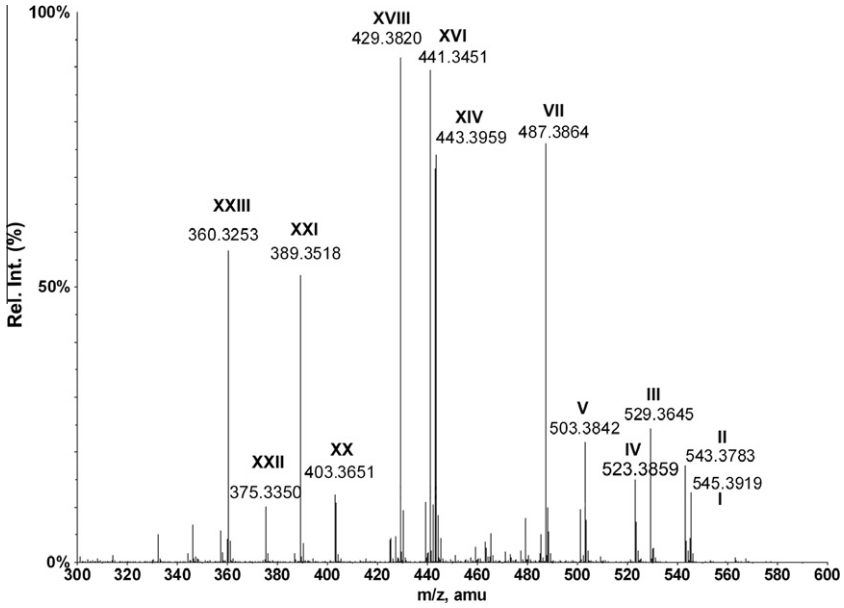


Fig. 4. Full-scan (+)ESI-QqTOF mass spectrum of the steroidal alkaloids of *Sarcococca coriacea*.

further searched by Scifinder which showed only one reported structure which is an acetyl derivative of hookerianamide B [3,16]. The exact mass and MS/MS data of this protonated ion **I** fits the spectra which showed neutral losses of dimethylamine (−45 Da), tigloyl or senecioyl group (−82 and −17 Da) and two sequential losses of acetate groups (−60 Da). Characteristic peaks at *m*/*z* 100 and 83 also showed the presence of tigloyl or senecioyl group. The characteristic peak at *m*/*z* 281 clearly showed that the compound possesses a core skeleton with two more substituents other than the substituents at C-20 and C-3. Therefore, the compound was identified as an acetyl derivative of hookerianamide B (Table 3). All the other compounds reported in Table 3 were identified by following the same strategy. Molecular ions **IV**, **III** and **XIX** were identified as hookerianamide G, nepapakistamine A and

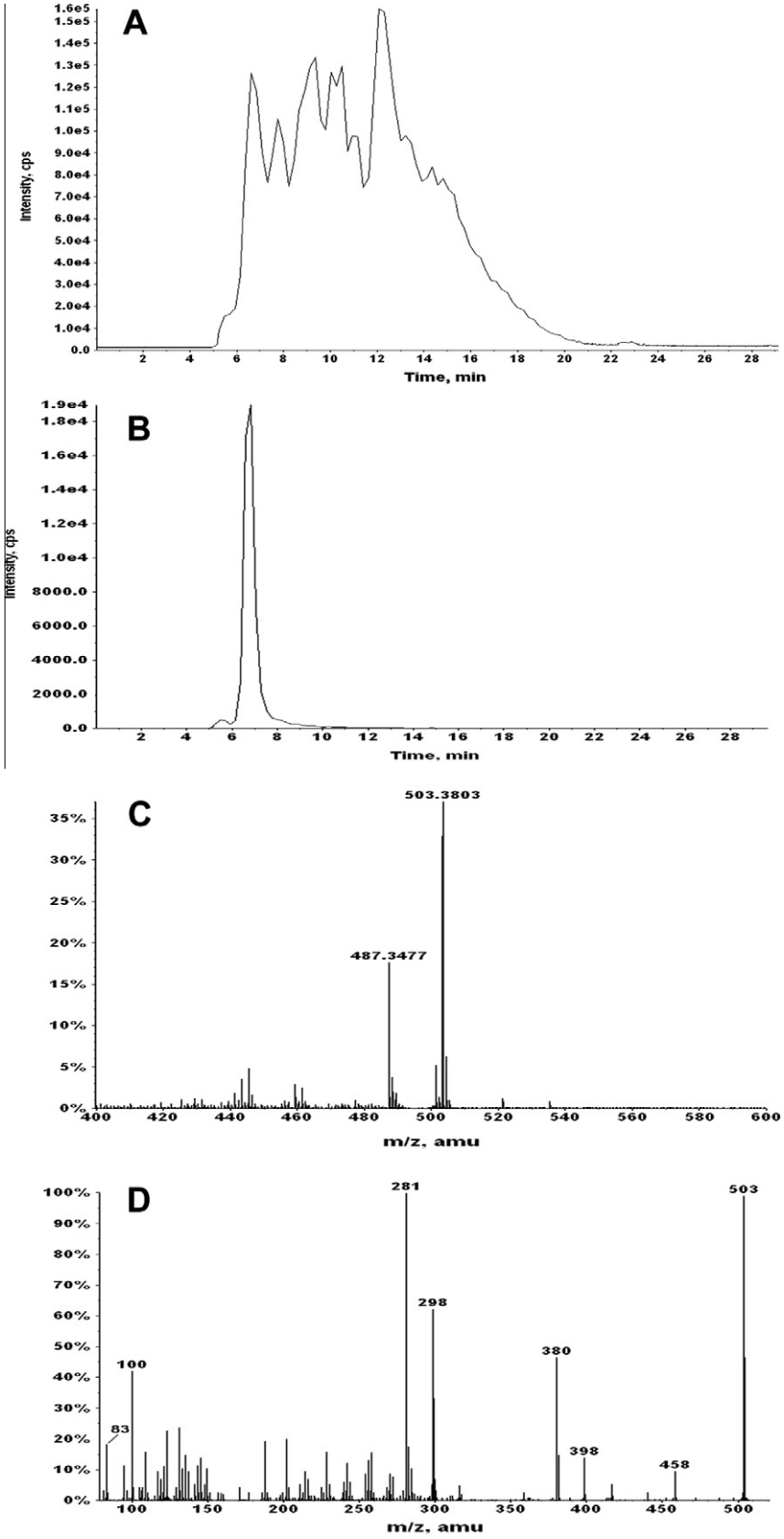


Fig. 5. LC/MS/MS spectra of *Sarcococca coriacea*. (A) Total ion chromatogram (TIC), (B) extracted ion chromatogram (XIC) of *m*/*z* 503 (**V**) (C) TOF-MS at retention time 6.846 min, and (D) MS/MS spectra of *m*/*z* 503 (**V**).

hookerianamide F, respectively. Molecular formula of $C_{32}H_{51}N_2O_5^+$ is assigned to protonated ion **II** on the basis of accurate mass measurements. No data corresponding to the molecular formula $C_{32}H_{51}N_2O_5$ has been reported for pregnane-type steroidal alkaloids in DNP and Scifinder. Therefore on the basis of fragmentation pattern, the structure of **II** was proposed to be similar to **I** but with

Table 3
Compounds identified by LC/ESI-QqTOF-MS/MS analysis of *Sarcococca coriacea*.

No.	Molecular formula	Exact mass	Observed mass	Error (ppm)	Ret. time (intensity counts)	Product ions	Neutral losses	Identified compound
I	$C_{32}H_{53}N_2O_5$	545.3948	545.3919	5.5001	9.355 (1428)	500, 440, 380, 298, 281, 100, 83	45, 60, 60, 82, 17	Acetyl derivative of hookerianamide B
II	$C_{32}H_{51}N_2O_5$	543.3792	543.3783	1.7477	9.582 (1467)	543, 498, 438.378, 296, 279, 100, 83	45, 60, 60, 82, 17	N^3-tigloyl/senecioyl, N^{20}-dimethyl, di-OAc, pregnane type steroidal alkaloid with unsaturated core structure
III	$C_{31}H_{49}N_2O_5$	529.3635	529.3645	1.7009	8.900 (2900)	498, 438, 378,100, 83	31, 60, 60, 82, 17	Nepapakistamine A
IV	$C_{33}H_{51}N_2O_3$	523.3894	523.3859	6.7261	11.180 (1685)	418, 283, 136,	105(45 + 60), 135	Hookerianamide G
V	$C_{30}H_{51}N_2O_4$	503.3843	503.3842	0.2682	6.846 (2424)	458, 398, 380, 298, 281, 100, 83	45,60,18,82,17	Hookerianamide B (**6**)/Sarcovagine B
VI	$C_{30}H_{49}N_2O_4$	501.3686	501.3678	1.765	6.618 (868)	456, 396, 378, 296, 279, 100, 83	45,60,18,82,17	Sarcovagenine B
VII	$C_{30}H_{51}N_2O_3$	487.3894	487.3864	6.1971	10.270 (5369)	442,382, 300, 283, 100, 83	45, 60, 82, 17	Hookerianamide C (**7**)/Vaganine A (**10**)/Sarcovagine C (**11**)
VIII	$C_{29}H_{47}N_2O_4$	487.3530	487.3477	10.9467	6.389 (138)	456, 396, 378, 296, 279, 100, 83	31, 60, 18, 82, 17	N^3-tigloyl/senecioyl, N^{20}-methyl, OAc,-OH pregnane type steroidal alkaloid with unsaturated core structure
IX	$C_{30}H_{49}N_2O_3$	485.3737	485.3697	8.386	9.355 (360)	440, 380, 298, 281, 100, 83	45, 60, 82,17	Hookerianamide E/vaganine D
X	$C_{31}H_{49}N_2O$	465.3839	465.3831	1.8071	17.550 (94)	420, 285, 136	45, 135	N^3-Methyl epipachysamine D (**4**)
XI	$C_{28}H_{47}N_2O_3$	459.3581	459.3679	21.2895	6.162 (23)	414, 396, 378, 279, 100, 83	45, 18, 18, 82, 17	Hookerianamide A/Salonine A/ Sarcovagenine A
XII	$C_{30}H_{53}N_2O$	457.4152	457.4085	14.7377	28.805 (14)	412, 285, 128	45, 127	Salignenamide A (**14**)
XIII	$C_{28}H_{49}N_2O_2$	445.3788	445.3751	8.4326	7.075 (179)	400, 382, 300, 283, 100, 83	45, 18, 82, 17	Hookerianamide N (**9**)
XIV	–	–	443.3959	–	15.048 (228)	398, 285, 114	45, 113	Unidentified
XV	$C_{28}H_{47}N_2O_2$	443.3632	443.3646	3.145	7.305 (640)	443, 398, 380, 298, 281,100, 83	45, 18, 82,17	N^3-tigloyl/senecioyl, N^{20}-dimethyl, hydroxy, pregnane type steroidal alkaloid with unsaturated core structure
XVI	$C_{28}H_{45}N_2O_2$	441.3475	441.3451	5.5637	13.455 (5287)	396, 378, 314, 296, 279, 218, 204, 83	45, 82,18, 17	Sarcovagine D (**20**)
XVII	$C_{28}H_{43}N_2O_2$	439.3319	439.3283	8.2066	14.365 520)	394,376,312,294, 277, 218, 204, 83	45,82,18,17	Sarcovagenine C (**21**)/14,15-dehydro sarcovagine D/2,3-dehydro sarsalignone
XVIII	$C_{28}H_{49}N_2O$	429.3839	429.3820	4.5205	12.318 (5046)	384, 285	45, 99 (82 + 17)	Epipachysamine E/Pachysamine E/ Pachysamine D
XIX	$C_{27}H_{41}N_2O_2$	425.3162	425.3127	8.3593	13.00 (243)	394, 376, 312, 294, 277, 218, 204, 83	31, 82,18,17	Hookerianaamide F
XX	$C_{26}H_{47}N_2O$	403.3682	403.3651	7.9108	7.761 (1144)	285	118 (45 + 73)	Sarcodine/Isosarcodine (**17**)
XXI	$C_{25}H_{45}N_2O$	389.3526	389.3518	2.1596	7.761 (4512)	344, 285	45, 59	Sarcorine
XXII	$C_{24}H_{43}N_2$ O	375.3369	375.3350	5.304	6.389 (1036)	285	90 (45 + 45)	*N*-formylchonemorphine (**12**)/iso-*N*-formylchonemorphine (**23**)
XXIII	$C_{24}H_{42}NO$	360.3260	360.3253	2.1973	12.318 (4268)	283, 215	77 (45 + 32)	Alkaloid C (**22**)/Pachyaxinimine A

an additional unsaturation in the core skeleton. The peak **VI** corresponding to the molecular formula $C_{30}H_{48}N_2O_4$ (MW 500 Da) afforded two hits in DNP for pregnane-type *Sarcococca* steroidal alkaloids, salignenamide C and sarcovagenine B. As the product ion spectra of **VI** showed the $[M+H-45]^+$ as the base peak, that is why there is a strong possibility of having unsaturation at C-16. The protonated ion **IX** therefore most probably corresponds to Sarcovagenine B. Protonated ions **XXIII, XX, XVIII, XVII, V, IX, XI** and **XV** were searched by their molecular formulae and confirmed by exact mass measurements. These gave more than one hit of similar compounds that are either stereoisomers or position isomers and could not be distinguished on the basis of their fragmentation pattern. Two isobaric peaks **VII** and **VIII** corresponding to the MW 486 Da were assigned the molecular formulae $C_{30}H_{50}N_2O_3$ and $C_{29}H_{46}N_2O_4$, respectively on the basis of exact mass measurements. **VII**, eluted at 10.27 min with the intensity of 5369 counts, and identified as a steroidal alkaloid with an unsaturated skeleton having N^3-tigloyl or senecioyl group, N^{20}-methyl and a acetate group, but the position of acetate group could not be assigned on the basis of fragmentation data while peak **VIII** with the molecular formula $C_{29}H_{46}N_2O_4$ eluted at 6.389 min with low intensity (138 counts) was not found in DNP and Scifinder search. The structure of **VIII** was tentatively proposed on the basis of fragmentation pattern which showed the neutral losses of methylamine (−31 Da), tigloyl or senecioyl group (−82 and −17 Da), acetate group (−60 Da) and a hydroxy group (−18 Da). The characteristic peaks at *m/z* 100 and 83 also indicated the presence of tigloyl or senecioyl group. The diagnostic peak at *m/z* 279 corresponded to the compound having a core skeleton with two more substituents other than the substituents at C-20 and C-3, with one unsaturation in the core structure. The abundant fragment ion at $[M+H-31]^+$ in the MS/MS data of **XV** showed that there is a strong possibility of unsaturation at C-16. Finally the structure of **XV** was tentatively proposed as N^3-tigloyl/senecioyl, N^{20}-methyl, OAc, -OH pregnane-type steroidal alkaloid with unsaturation at C-16. The study clearly demonstrated the utility of LC–ESI-QqTOF-MS/MS analysis as a tool for the rapid identification of steroidal alkaloids in complex mixtures.

4. Conclusions

In conclusion, fragmentation patten of *Sarcococca* (pregnane-type) steroidal alkaloids have been studied by using ESI-QqTOF-MS/MS technique. Diagnostic fragments were identified and

structure-fragmentation relationships developed by studying 23 reference steroidal alkaloids. It was observed that many characteristic neutral losses and formation of key fragment ions can provide useful structural information about the basic skeleton and attached substituents. This study clearly demonstrated the importance of combining liquid chromatography to mass spectrometry for rapid identification of pregnane-type steroidal alkaloids in complex mixtures, such as plant extracts or herbal formulations.

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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.steroids.2011.11.001.

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