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Isolation and structural elucidation of indole alkaloids from *Geissospermum vellosii* by mass spectrometry

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ABSTRACT

Alkaloids from the stem bark of *Geissospermum vellosii* possess a variety of therapeutic properties including antimalarial activities, activity as a sexual stimulant and inhibition of the proliferation of HIV and herpes viruses. Methods currently used to isolate the active components from *G. vellosii* are time-consuming, labor intensive, and result in low recovery. In addition, there is a lack of sensitive and accurate analytical methods for the structural characterization and identification of alkaloid components in minor quantities. A combination of high performance counter-current chromatography and ESI tandem mass spectrometry (MS^n) was established to isolate alkaloids from the stem bark of *G. vellosii*, and study their electrospray ionization mass spectrometry fragmentation behavior. Five indole alkaloids were successfully isolated and identified by nuclear magnetic resonance and mass spectrometry. The multi-stage tandem mass spectrometric data were used to study their fragmentation pattern and set a model for detailed structure characterization of related indole alkaloids. The presence of the even mass fragment ion suggestive of an odd number of nitrogen at m/z 144 corresponding to $C_{10}H_9N$ was characteristic to indole alkaloids. The results of the experiments demonstrated that the combination of high performance counter current chromatography and ESI-MS^n is a sensitive, selective and effective approach for rapid isolation and characterization of alkaloids from *G. vellosii*.

1. Introduction

Geissospermum vellosii is a native tree of Brazil found primarily in the Northeast, South and Southeast regions. This tree is commonly named Pao Pereira and its stem bark is traditionally used to treat malaria, constipation, liver pain and as a sexual stimulant [1–3]. *G. vellosii* has several classes of compounds like other plants of the family Apocynaceae, and its stem bark is particularly rich in indolic alkaloids [2]. Latest studies revealed that alkaloids from the stem bark of *G. vellosii* showed promising results in suppressing further proliferation of HIV and herpes viruses [4].

While alkaloids from the stem bark of *G. vellosii* showed a variety of health benefits as mentioned above, there is a need for in depth in vitro and in vivo studies to determine the biological properties of these compounds. Unfortunately, there are no cost-effective, fast, efficient and reliable methods available to obtain alkaloids in sufficient yields and purity required. Current purification methods include the use of labor intensive and time-consuming columns with a variety of stationary phases and sequential solvent extraction [5–8]. High performance countercurrent chromatography (HPCCC) is a liquid–liquid chromatographic separation technique used for the isolation of natural compounds [9–12]. Compared with traditional liquid adsorption chromatography in which a solid phase is involved, HPCCC has numerous advantages such as no irreversible adsorption and complete recovery in samples. Because of these advantages, HPCCC is gaining popularity as a purification tool for natural products [9–11,13].

Natural compound characterization is one of the most important steps in the discovery of new drug candidates. It typically involves compound purification and off-line identification by mass spectrometry, nuclear magnetic resonance (NMR), and other spectroscopic methods. However, it is usually a challenge to identify minor active components with a traditional method like NMR. High-performance liquid chromatography with tandem mass spectrometry (LC–MS^n) is a selective and proficient method for qualitative characterization of known compounds as well as the identification of unknown compounds [14–16]. Due to its high sensitivity and specificity, LC–MS^n has been proven to be a prevailing analytical tool for the identification of drug metabolites in

Abbreviations: ESI-IT-TOF-MS, electrospray ionization ion trap time of flight mass spectrometry; ESI-MS^n, electrospray ionization tandem mass spectrometry; HPCCC, high performance counter current chromatography; HPLC, high performance liquid chromatography; HRESIMS, high resolution electrospray ionization mass spectrometry; LC–MS^n, liquid chromatography–tandem mass spectrometry; NMR, nuclear magnetic resonance; TLC, thin layer chromatography.

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biological matrices [17–19]. Several investigations on alkaloids from *G. vellosii* described traditional isolation and structural identification by means of NMR [20–22], but very few studies have reported their fragmentation behavior.

In the present study, a combination of HPCCC and LC–MS^n was established to isolate alkaloids from the bark of *G. vellosii* and study their electrospray ionization mass spectrometry fragmentation behavior. We report the MS^n fragmentation of five alkaloids representing the mass spectrometry fragmentation pattern of indole alkaloids. The objective of this study is to develop a rapid method to screen and identify indole alkaloids from *G. vellosii*, especially the minor components that are difficult to disclose by traditional methods.

2. Materials and methods

2.1. Chemicals and reagents

Solvents for extraction (HPLC grade) and formic acid (ACS reagent grade) were purchased from Fisher Scientific (Waltham, MA). LCMS-grade solvents and 0.1% formic acid in water were purchased from Honeywell Burdick and Jackson (Muskegon, MI). Water was purified using a Milli-Q water purification system (Millipore, Bedford, MA). Purasil silica gel (70–230 mesh) for flash chromatography was purchased from Whatman Inc. (Piscataway, NJ).

2.2. Instrumentation

Countercurrent chromatography separation was performed on a Midi HPCCC system (Dynamic Extractions Ltd., Berkshire, United Kingdom) connected to an Armen high pressure pump (Armen Instrument, St-Ave, France). The Armen high pressure pump compartment is a fully automated system consisting of a pump, an injector, a UV/vis detector, a fraction collector, an integrated computer, and Armen Glider CPC software (Armen Instrument, St-Ave, France). The Midi HPCCC system has two preparative columns and two analytical columns of 450 mL and 18 mL each respectively, connected to a ThermoFlex 2500 chiller (Thermo Scientific, Waltham, MA). Electrospray ionization ion-trap time-of-flight mass spectrometry (Shimadzu Scientific Instruments, Columbia, MD) was used for compound analysis, formula determination and structural elucidation. NMR spectra were recorded on Bruker Avance 600 MHz spectrophotometer (Bruker BioSpin Corporation, Billerica, MA).

2.3. Plant material and preparation of crude extract

Stem bark from *G. vellosii* was purchased from Tropilab Inc. (St. Petersburg, FL) with the voucher specimen number: 5:21.20. Freeze-dried stem bark was first cut into small pieces using a knife and then ground into a fine powder using a Waring stainless steel blender (Waring Commercial, Torrington, CT). The powdered material (200 g) was extracted at room temperature three times each with 3 L methanol for 3 days as described previously [23]. The filtered extract was concentrated using a rotary evaporator (BUCHI Corporation, New Castle, DE). The dried crude extract (10 g) was kept at −20 °C until use for alkaloid isolation.

2.4. Alkaloid isolation

The methanol crude extract (2 g) was separated by counter current chromatography with 2:3:5 extended HEMWat (ethyl acetate:butanol:water) solvent system selected as described previously [23]. Elution–extrusion method was performed with the upper phase as stationary phase. After solvent system equilibration in the columns as described earlier [23], the methanol crude extract in 30 mL of 1:1 (v/v) mixture of each phase of the solvent system was injected through the sample loop. The mobile phase was pumped into the inlet of the preparative columns (900 mL) at a flow rate of 10 mL/min for 90 min and the stationary phase at the same flow rate for another 90 min. The column speed was set at 1200 rpm and the column temperature was maintained at 29 °C using a chiller. The UV detection was set at 254 nm and fractions eluted in 15-mL tubes were subjected to Dragendorff's test for alkaloids [24]. Reproducible results were obtained by three repeated HPCCC experiments. Five fractions were collected according to the positive test with Dragendorff's reagent. Collected fractions were concentrated and dried using a Buchi rotary evaporator. Some collected fractions were further purified by silica gel column chromatography. Indeed, fractions (40–120 mg) were loaded on the top of the column (20 mm × 200 mm) and were step gradient eluted with a combination of toluene–methanol with an initial composition of 95:5 (v/v) after a previous column wash with 100% toluene. The stepwise gradient elution was performed by increasing the amount of methanol by 5%. The volume of the solvent mixture used in each step was 250 mL and was collected in fractions of 50 mL. The purity of the isolated compounds was verified by HPLC and TLC. Compounds were identified by NMR and mass spectrometry methods, and comparison with available reported data.

2.5. NMR analysis of compounds

Different NMR experiments were performed to identify the five isolated compounds, including ^1H, ^{13}C, DEPT, HMQC, HMBC, COSY and NOESY NMR. Spectra were acquired in CD_3OD at 600 MHz for proton and 150 MHz for ^{13}C NMR.

2.6. Liquid chromatography/mass spectrometry analysis of compounds

Alkaloid analysis was performed by ESI-IT-TOF-MS on a Shimadzu LC–MS-IT-TOF instrument equipped with a Prominence HPLC system (SIL-20A HT autosampler, LC-20AD pump system, SDP-M20A diode array detector). On this system, purified compounds were injected into the mass spectrometer via a Shim-pack XR-ODS column (3 mm i.d. × 75 mm, 2.2 μm; Shimadzu Scientific Instruments, Columbia, MD) at 40 °C connected to a C18 guard column. The LC separation was performed using a binary solvent system comprising 0.1% formic acid in water (solvent A) and 0.1% formic acid in acetonitrile (solvent B) at a flow rate of 0.3 mL/min with the following linear gradient: 5–20% mobile phase B over 2 min, 20–50% B over 6 min, 50–95% B over 2 min, isocratic at 95% B over 2 min, and return to 5% B over 1 min. Prior to the next injection, the column was re-equilibrated for 7 min at initial conditions (5% mobile phase B).

The mass spectrometry conditions were as follows: the heat block and curved desolvation line were maintained at 200 °C. Nitrogen gas was used as nebulizer and drying gas with the flow rate set at 1.5 L/min and 10 L/min respectively. Argon gas was used as collision gas. The ESI source voltage was set at 4.5 kV and the detector was set at 1.5 V. The instrument was calibrated to <5 ppm error in mass accuracy with an external standard of sodium TFA solution. Ionization was performed using a conventional ESI source in positive ionization mode. Shimadzu's LCMS Solution software was used for data analysis. The formula predictor function of LCMS Solution was used in identification and confirmation of unknown signals. The full-scan data were acquired from 100 to 1000 Da with 300 ms and 10 ms event time and accumulation time respectively. The MS^n product ion spectra were produced by collision induced dissociation of the protonated molecule ion. Collision energy was 50% for

both MS^2 and MS^3 experiments. The MS^2 mass scan range was from 100 to 700 Da with the event time and accumulation time 573 ms and 50 ms respectively. For MS^3 experiments, the mass scan range was from 100 to 500 Da with 801 ms and 70 ms event time and accumulation time respectively.

3. Results and discussion

3.1. HPCCC separation of alkaloids

The solvent system for alkaloid separation by HPCCC was optimized as described previously [23]. The settling time of the selected solvent system HEMWat SS4 [ethyl acetate–butanol–water (2:3:5, v/v/v)] did not exceed 20 s, which is consistent with a successful HPCCC separation [25]. After the hydrodynamic equilibrium of the two phase solvent system, 70–83% stationary phase were retained in the columns which is consistent with the retention of the stationary phase's rule stating that "the higher the retention of the stationary phase, the better the peak resolution" [12].

The comprehensive HPCCC separation of the methanol crude extract is illustrated by the UV chromatogram at 254 nm as shown in Fig. 1. Collected fractions were subjected to Dragendorff's test for alkaloids by TLC, and only fractions eluted in the retention time range 18 min–72 min were positive. Some collected fractions showed a single band by TLC analysis, but the amounts collected were not enough for downstream experiments. Consequently, the HPCCC experiment efficiently separated alkaloids from a complex mixture of methanol crude extract and each alkaloid could be qualitatively isolated. Collected fractions were then merged into 5 major fractions according to the intensity on TLC with the Dragendorff's reagent and the UV absorption at 254 nm. Merged fractions were dried and the isolation efficiency was assessed by HPLC and TLC. Fractions 3 and 5 were pure and corresponded to compounds **1** (59.9 mg) and **5** (42.6 mg) with a relative quantification by peak area ratio on the chromatogram of 99.5% and 85.6% respectively (Fig. 2). The other fractions underwent further purification by flash column chromatography and compounds **2** (14.9 mg), **3** (9 mg), and **4** (3 mg) were purified from the HPCCC fractions 4, 1 and 2 respectively (Fig. 1). The relative quantification of compounds **2**, **3**, and **4** by peak area ratio on the chromatogram indicated 88%, 72% and 60% respectively (Fig. 2). These recoveries were relatively low compared to the amount of sample (2 g) injected into the HPCCC columns. This might be due to the complex mixture of the methanolic extract and the loss through further purifications.

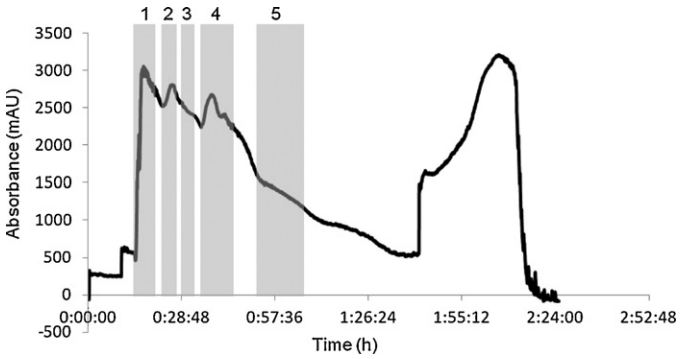

Fig. 1. HPCCC-UV chromatogram of the methanol crude extract of *G. vellosii*. Experimental conditions – solvent system: ethyl acetate/butanol/water (2:3:5, v/v/v); mobile phase: lower phase; revolution speed: 1200 rpm; flow rate: 10 mL/min; wavelength: 254 nm. Fractions 1–5 correspond to collected fractions with positive Dragendorff's test selected for further purification.

The retention time of compounds **1**, **2**, **3**, **4** and **5** were 5.63, 5.12, 4.69, 5.80 and 5.07 min respectively as determined by HPLC analysis (Fig. 2). These retention times were very close, thus explaining the difficulties of individual alkaloid isolation from *G. vellosii* by one HPCCC run. The pH-zone-refining counter-current chromatography method could be used to improve compound concentration in fractions with minimum overlap and impurities [26–29]. This technique would be particularly efficient for alkaloid separation as it is applied to ionizable compounds. Indeed, a retainer which can be an acid or base is used in the stationary phase to retain the analytes of interest while an eluter (acid or base) consistent with

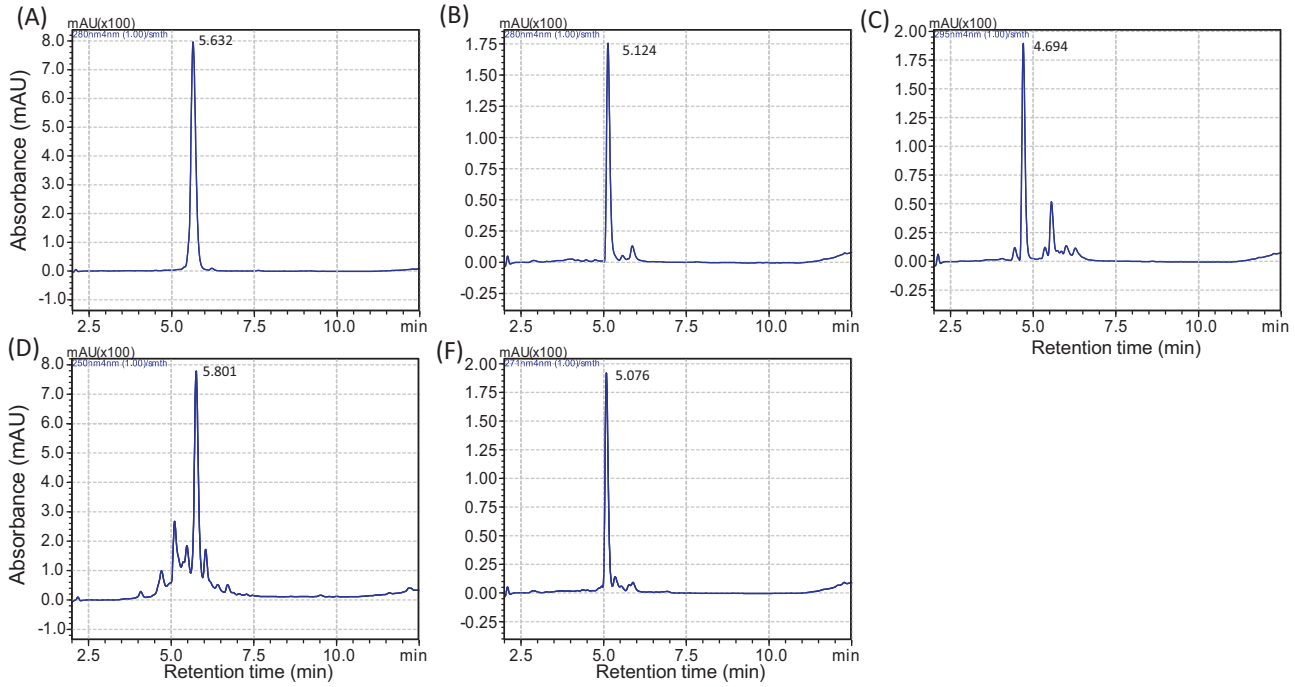

Fig. 2. HPLC chromatograms of the isolated indole alkaloids from HPCCC fractions. (A) Compound **1** from HPCCC fraction 3. (B) Compound **2** from HPCCC fraction 4. (C) Compound **3** from HPCCC fraction 1. (D) Compound **4** from HPCCC fraction 2. (E) Compound **5** from HPCCC fraction 5.

Table 1
Accurate mass measurements of the major ions observed in the ESI-MS^n spectra of compounds **1**–**5**. t_R, retention time.

Cp	t_R (min)	Measured m/z	Predicted m/z	Error (ppm)	Elemental composition	Main product ions m/z (relative abundance, %)	Identification
1	5.632	287.1825 $[M+2H]^{2+}$	287.1830	−1.74	$C_{38}H_{44}N_4O$	309 (100), 265 (15), 208 (19), 144 (17)	Geissolosimine
2	5.124	317.1931 $[M+2H]^{2+}$	317.1936	−1.70	$C_{40}H_{48}N_4O_3$	144 (100), 490 (93), 383 (12), 309 (10), 251 (10)	Geissospermine
3	4.694	299.2113 $[M+H]^+$	299.2118	−1.67	$C_{19}H_{26}N_2O$	300 (100), 194 (82), 144 (23), 182 (24), 281 (14)	Geissoschizoline
4	5.801	311.2114 $[M+H]^+$	311.2118	−1.29	$C_{20}H_{26}N_2O$	312 (100), 158 (29), 208 (30), 250 (23), 144 (16)	Geissoschizone
5	5.076	295.1795 $[M+H]^+$	295.1805	−3.39	$C_{19}H_{22}N_2O$	277 (100), 296 (29), 138 (48), 146 (43), 144 (23)	Vellosiminol

the pK_a and hydrophobicity of the analytes is used for compound elution.

3.2. Analysis and identification of alkaloids

Five alkaloids were isolated from the stem bark of *G. vellosii* using a combination of HPCCC and flash column chromatography. Compounds were then analyzed by NMR and mass spectrometry methods, and comparison to the available literature.

Mass spectrometry analysis of compound **1** revealed the molecular formula $C_{38}H_{44}N_4O$ from the positive HRESIMS data [m/z 287.1825 $(M+2H)^{2+}$, Δ −1.74 ppm] (Table 1). The molecular structure of compound **1** was deduced from the comprehensive analysis of one- and two-dimensional NMR data (see Suppl. Tables 1 and

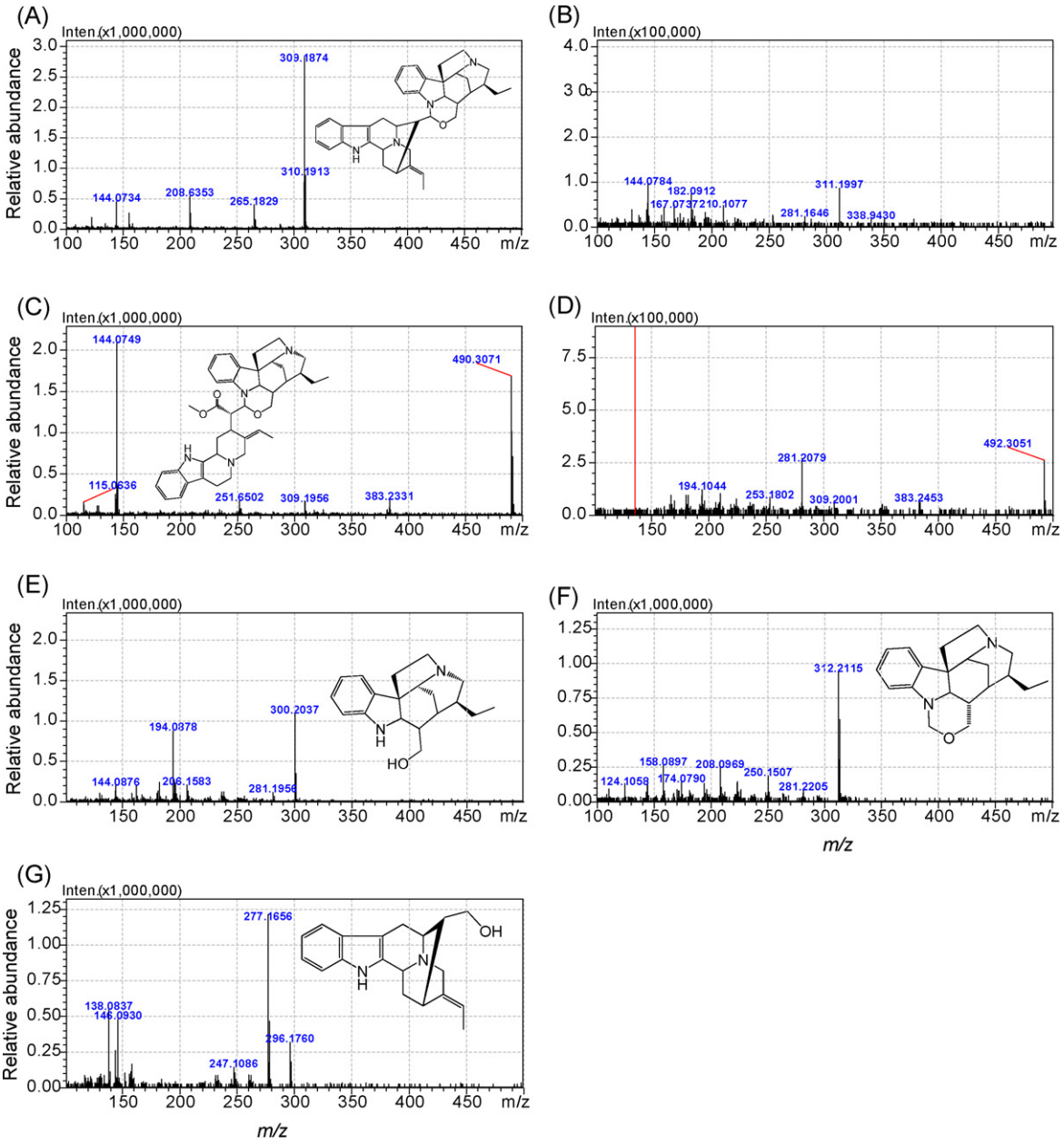

Fig. 3. ESI-MS^n spectra and structures of the isolated compounds **1**–**5**. (A) MS^2 spectrum of $[M+2H]^{2+}$ ion for compound **1**. (B) MS^3 spectrum of $[M+H-265]^+$ ion for compound **1**. (C) MS^2 spectrum of $[M+2H]^{2+}$ ion for compound **2**. (D) MS^3 spectrum of $[M+H-144]^+$ ion for compound **2**. (E) MS^2 spectrum of $[M+H]^+$ ion for compound **3**. (F) MS^2 spectrum of $[M+H]^+$ ion for compound **4**. MS^2 spectrum of $[M+H]^+$ ion for compound **5**.

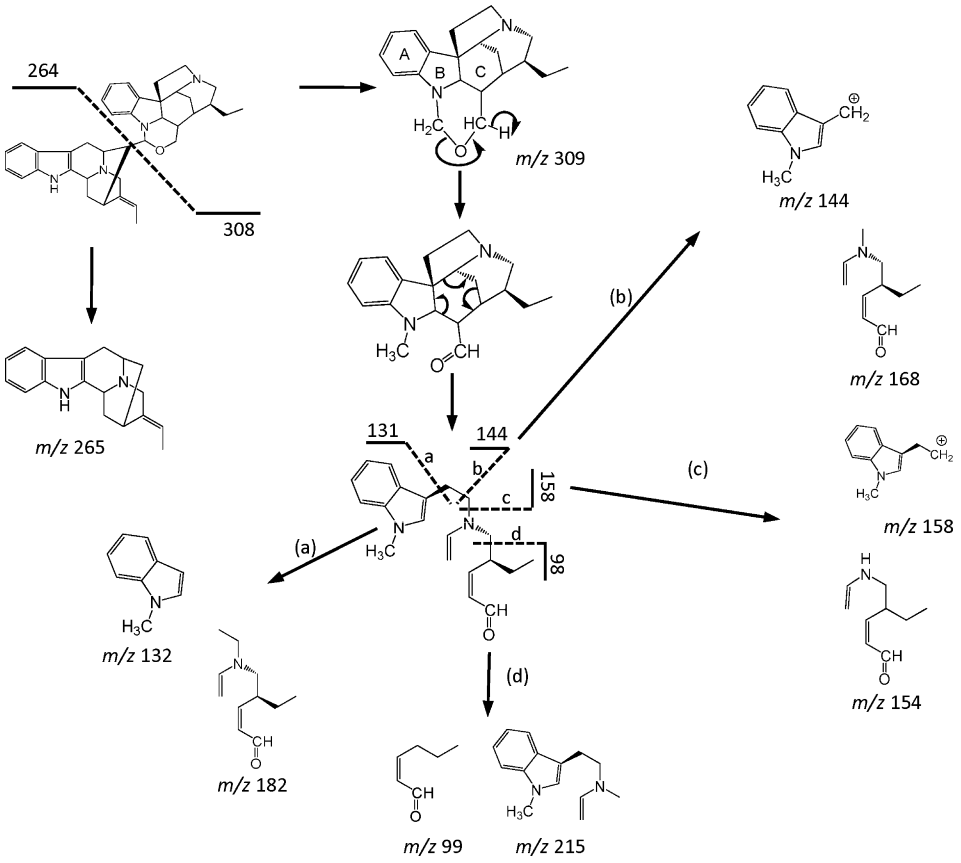

Fig. 4. Proposed fragmentation pathways of the bis-indole alkaloid geissolosimine (compound **1**) in ESI-MS^n. *a*, *b*, *c* and *d* represent labilized positions for fragmentation.

2 for ^{13}C and ^{1}H NMR) and long-range correlations obtained by HMBC as geissolosimine. Geissolosimine was previously identified in *G. vellosii* [20]. Compound **2** was shown to have the molecular formula $C_{40}H_{48}N_4O_3$ from positive HRESIMS data [*m*/*z* 317.1931 $(M+2H)^{2+}$, Δ −1.70 ppm]. The ^{1}H and ^{13}C NMR spectra data (Suppl. Tables 1 and 2) were similar to the reported data for geissospermine [21,22] and was confirmed to be the bis-indole alkaloid geissospermine. The mass spectrometry analysis of compound **3** showed the molecular formula $C_{19}H_{26}N_2O$ from positive HRESIMS data [*m*/*z* 299.2113 $(M+H)^+$, Δ −1.67 ppm]. ^{1}H and ^{13}C NMR spectra indicated close resemblance to the upper unit of compounds **1** and **2** and matched with what was previously published for geissoschizoline isolated from *G. sericeum* [30]. Compound **4** was shown to have the molecular formula $C_{20}H_{26}N_2O$ from positive HRESIMS data [*m*/*z* 311.2114 $(M+H)^+$, Δ −1.29 ppm] (Table 1). The ^{13}C NMR spectrum was very close to **3** with an extra signal at δ_c 76.3 suggesting a carbon atom located between oxygen and nitrogen, as shown in Fig. 3F [20]. Compound **4** was identified as geissoschizone [31] based on the comprehensive 2D NMR analysis (Suppl. Tables 1 and 2). Compound **5** was shown to have the molecular formula $C_{19}H_{22}N_2O$ and [*m*/*z* 295.1795 $(M+H)^+$, Δ −3.39 ppm]. ^{1}H and ^{13}C NMR of compound **5** was very close to the lower unit of compound **1**. Compound **5** was identified as vellosiminol based on the comprehensive 2D NMR analysis, and it was previously isolated from *G. vellosii* [20].

3.3. Structural elucidation of alkaloids using ESI-MS^n

Identification of alkaloids from *G. vellosii* by mass spectrometry cannot be based solely on ESI-MS. Multiple tandem mass spectrometry (MS^n) is needed to obtain considerable structural information, which has been successfully used for the structure elucidation of alkaloids from *Rhizoma coptidis* [19] and *Stemona saxorum* [16]. To validate the structure of the alkaloids by ESI-MS, and to demonstrate the power of MS^n to characterize the structure of alkaloids, isolated compounds from *G. vellosii* bark were first analyzed by NMR. ESI-MS spectra in both positive and negative modes were investigated in this study and the positive mode was found to be more sensitive. Compounds **1**–**5** showed abundant $[M+2H]^{2+}$ and $[M+H]^+$ ions in the positive ion full-scan spectra, which were selected as precursor ions for collision-induced dissociation experiments.

The MS^2 product ions for compound **1** were *m*/*z* 309, 265, 208 and 144 (Fig. 3A). The most abundant MS^2 product ion being $[M+H-265]^+$ at *m*/*z* 309 corresponding to one half of the bis-indole and the other half being $[M+H-309]^+$ at *m*/*z* 265 as illustrated on the suggested fragmentation pathway of this compound (Fig. 4). This fragmentation pattern is characteristic to bis-indole and for compound **1** (geissolosimine), it is cleaved in concentrated hydrochloric acid at room temperature to the indole, vellosimine and the indoline, geissoschizoline [32]. This facile cleavage indicates a linking of the two portions through acetal formation with the aldehyde group of vellosimine. According to the stereochemistry of vellosimine, the formation of geissolosimine may involve the indoline –NH and the $-CH_2OH$ of geissoschizoline [20]. In the MS^3 experiment, the ion $[M+H-265]^+$ at *m*/*z* 309 was chosen as the precursor ion (Fig. 3B). The most abundant fragment ions detected were *m*/*z* 311, 144, 158, and 182. The ion *m*/*z* 311 resulted from the protonation of the precursor ion and the fragment ion *m*/*z* 144 is characteristic to indole alkaloid fragmentation by mass spectrometry [33]. Note that this fragment ion (*m*/*z* 144) was detected in the product ions of all alkaloids investigated in this study. After having undergone the rearrangement as illustrated in the fragmentation pathway (Fig. 4), the ring *C* is opened in a six-membered transition state (see arrows), thus releasing the strain inherent in the fused polycyclic system

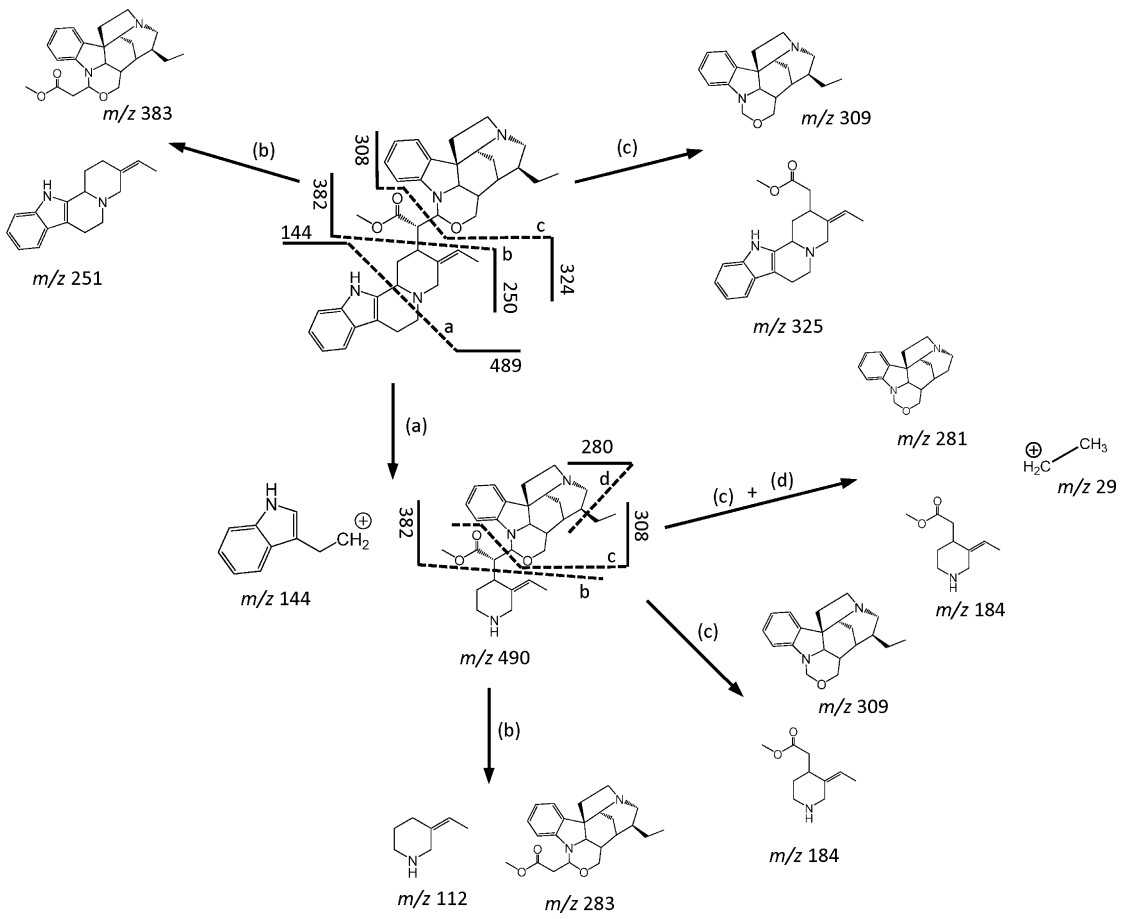

Fig. 5. Proposed fragmentation pathways of the bis-indole alkaloid geissospermine (compound **2**) in ESI-MS^n. *a*, *b*, *c* and *d* represent the labilized positions for fragmentation.

with simultaneous aromatization of the dihydroindole moiety [34]. Further cleavages then occur at the labilized positions marked *a*, *b*, *c* and *d* leading to the observed ions *m*/*z* 144, 158, 182 and 168 (Fig. 4). Product ions *m*/*z* 144 and 158 contain the indole fragment while *m*/*z* 168 and 182 contain the non-aromatic portion of the molecule.

The MS^2 fragment ions of compound **2** (geissospermine) were *m*/*z* 144, 490, 309, 383 and 251, with *m*/*z* 144 being the base peak (Fig. 3C). The fragment ion [M+H−489]$^+$ at *m*/*z* 144 resulted from the characteristic fragmentation pattern (marked position *a* in Fig. 5) of bis-indole as previously illustrated on the fragmentation of communesins, a class of insecticidal indole alkaloids [35]. Further fragmentations on this molecule ion at the labilized position marked *b* and *c* led to the observed fragment ions *m*/*z* 383 and 309 respectively (Fig. 3C). In the MS^3 experiment, fragment [M+H−144]$^+$ at *m*/*z* 490 was chosen as the precursor ion (Fig. 3D). Fragmentations on this ion occur at the labilized positions marked *b*, *c* and *d* leading to the observed ions *m*/*z* 383, 309 and 281 respectively (Fig. 5). The presence of the fragment ion *m*/*z* 309 revealed the structural similarity between compounds **1** and **2**. Fragment *m*/*z* 309 then underwent further fragmentations as described above for compound **1** (Fig. 4).

For compound **3** (geissoschizoline), the MS^2 fragment ions were *m*/*z* 144, 194, and 281 (Fig. 3E), and this fragmentation pattern is similar to the MS^3 experiment in compound **2** (Fig. 3D). The loss of −18 u was due to the elimination of water leading to the fragment ion *m*/*z* 281. This molecular ion underwent similar fragment pathway with the fragment *m*/*z* 309 in compound **1**. Fragmentations occurred at the marked labilized positions *c* and *d* leading to the fragment ions *m*/*z* 144 and 194 instead of *m*/*z* 158 and 215 respectively (Fig. 4). Geissoschizoline is also known as an acid hydrolysis product of the bis-indole alkaloid geissospermine and Steele et al. [30] demonstrated that this compound is a significant component of the original extract and not an artifact of the extraction procedure. The MS^2 fragment ions of compound **4** were *m*/*z* 144, 158, 250 and 281 (Fig. 3F). The fragmentation pattern is similar to the MS^3 experiment in compound **1** and the MS^2 of compound **3**. Compounds **3** and **4** were structurally similar to the upper half indole moiety of compound **1**. For compound **5** (vellosiminol), the MS^2 fragment ions were *m*/*z* 277, 247, 138, 144 and 146 (Fig. 3G). The loss of −18 u was due to the elimination of water leading to the base peak fragment ion *m*/*z* 277. The chemical and physical structure of this compound was similar to the lower half indole moiety of compound **1**. It is a significant component of the original extract and not an artifact of the isolation procedure since it has been previously isolated from *G. vellosii* [20].

4. Conclusions

A combination of HPCCC and ESI tandem mass spectrometry (MS^n) techniques has been developed and applied to the separation of indole alkaloids from the methanolic crude extract of *G. vellosii* stem bark and their electrospray ionization mass spectrometry fragmentation behavior was established. The presently accumulated information on the mass spectral fragmentation behavior of indole alkaloids investigated can be of considerable aid in structural elucidation of unknown indole alkaloids. The construction of a library including ESI-MS^n spectra for known indole alkaloids would greatly facilitate the identification of new indole alkaloids in

medicinal plant extracts. The mass spectra of indole alkaloids can often lead to the precise location of certain substituents on the aromatic rings and when combined with NMR analysis and the conventional UV and IR spectral measurements, these physical tools can lead with a minimum of chemical manipulation to accurate structural definitions.

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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.jchromb.2011.12.018.

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