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Characterization of alkaloids in bark extracts of *Geissospermum vellosii* by HPLC-UV-diode array-multistage high-resolution mass spectrometry

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ABSTRACT

A number of analytical studies, started in the sixties of the last century, concerning the stem bark of *Geissospermum vellosii,* have documented the presence of a number of indole alkaloids whose molecular identity was defined by NMR technique. The potential bioactivity of these compounds has inspired more recent analogous studies either devoted to structural elucidation of new alkaloid molecules or to the investigation of the role of some of them in cancer therapy. Anyway, a complete fingerprinting of the bark content is still lacking. In this paper, after a suitable extraction step, we obtain a chromatographic separation showing a number of components higher than the number of alkaloids so far described.

Considering the great number of substances present in the stem bark, their identification is practically impossible to reveal by NMR techniques. As we presume that there are other stem bark unidentified alkaloids with important bioactivity, we propose to characterize their molecular structures by UV–Vis Diode Array spectrophotometry and High-Resolution Multistage Mass Spectrometry.

The two adopted detection techniques were first tested on the already known *Geissospermum vellosii* molecules, and, after an inspection of their efficacy, were applied to the substances that have not yet been described. Herewith we propose the molecular structures of 10 substances that were never previously described, and in addition we provide experimental evidence of the presence of 6 already known substances which were never reported in the *Geissospermum* genus. A far more detailed description of the bark constituents is therefore provided.

1. Introduction

Geissospermum vellosii Allemão (familiarly known as Pao Pereira) is a tree from the Amazonian rain forest, stretching from Guyana to Brazil, that belongs to the Apocynaceae family. The same plant was denoted as *Geissospermum laeve* Baillon for some years, and, in spite of the subsequent standardization of its botanical classification, both names still survive. Its bark extracts are peculiarly rich in indole alkaloids and have been known for centuries, in Brazilian folk medicine, to have febrifuge effects [1], and curare-like activity [2]. Furthermore, several additional therapeutic properties have been proposed over the years. The dispersion of the bark in hot water has been used by the native population as a remedy for malaria, stomach disorders, constipation and liver pain [3]. More recently a number of papers have been published on the use of Pao Pereira bark extract as a suppressor of prostate cancer cells [4], an inhibitor of pancreatic cancer and expander of gemcitabine effects [5], to potentiate the effects of carboplatin against ovarian cancer and in many other important pathologies [6–12]. *Geissospermum reticulatum* and *Geissospermum sericeum* belong to the same genus and share the antimalarial properties of *Geissospermum vellosii*. *Geissospermum vellosii* (Syn. laeve) is the most extensively studied species in this genus.

The alkaloid content of the stem-bark extracts of this plant was extensively studied in the late 1950s and early 1960s. However, the structural investigation tools of those years were primarily chemical in nature, relying mainly on degradation, derivatization and the synthesis of structural subunits for molecular resolution, meaning that large amounts of starting herbal material were necessary overall in the cases where the final molecular identification was achieved by NMR analysis,

Abbreviations: Alk., G.Vell.; HPLC, HR-MS.

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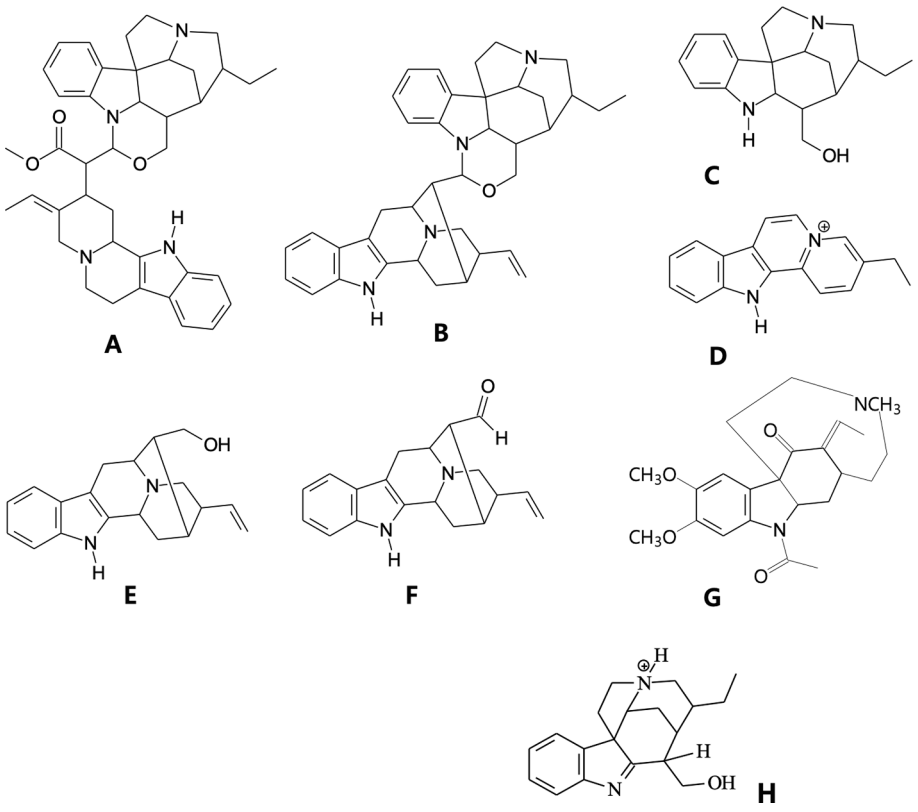


Fig. 1. Known alkaloids of Pao Pereira: Geissospermine (A), Geissolosimine (B), Geissoschizoline (C), Flavopereirine (D) Vellosiminol (E), Vellosimine (F), Geissovelline (G) and 1,2-dehydrogeissoschizoline (H).

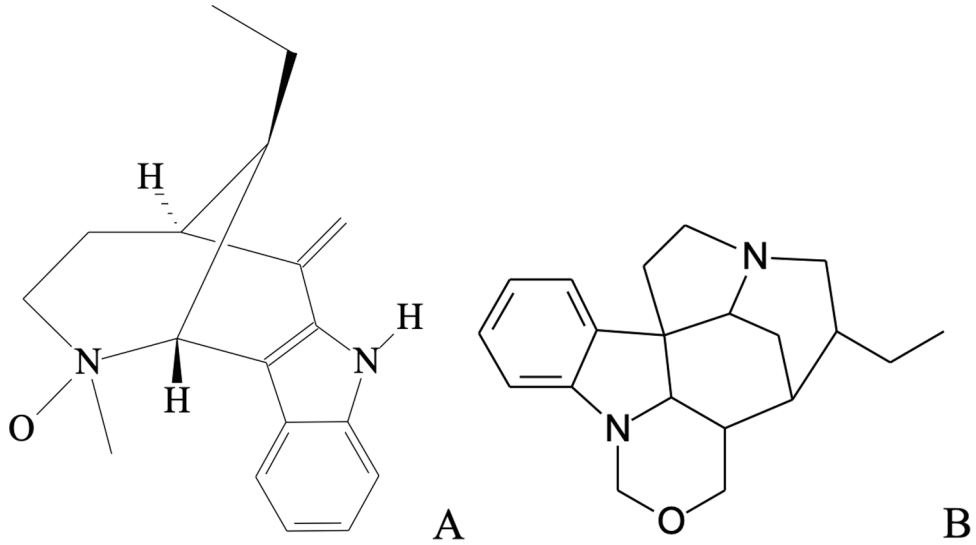


Fig. 2. Pausperadine (A) and Geissoschizone (B) structures.

after very laborious extraction and purification via crystallization steps.

More specifically, seven indole alkaloids - Geissospermine, Geissolosimine, Geissoschizoline, 1.2-dehydrogeissoschizoline, Vellosiminol, Vellosimine and Flavopereirine - were purified and their chemical structure elucidated by Rapoport and his coworkers at the California University [13–16]. Subsequently, the same research group characterized a further mono-indole species in the stem-bark extract, as reported in a work published in the early 1970s [17]. This molecule was named Geissovelline. The respective formulae are reported in Fig. 1 in the aim to visualize the very close structural connection between the bis-indole alkaloid molecules, Geissospermine and Geissolosimine, and the other mono-indole species (only Geissovelline shows some structural differences). In fact, the structures of the mono-indole species are easily recognizable within the more complex structures of the bis-indole molecules suggesting a pattern of biosynthetic routes very homogeneous. A structural aspect that will be confirmed in the following.

No further analytical studies into the chemical characterization of *Geissospermum vellosii* bark extracts were accomplished until recently. Two works have been reported [18,19]; one that focuses on the characterization of a new alkaloid, named Pausperadine (Fig. 2A), and a

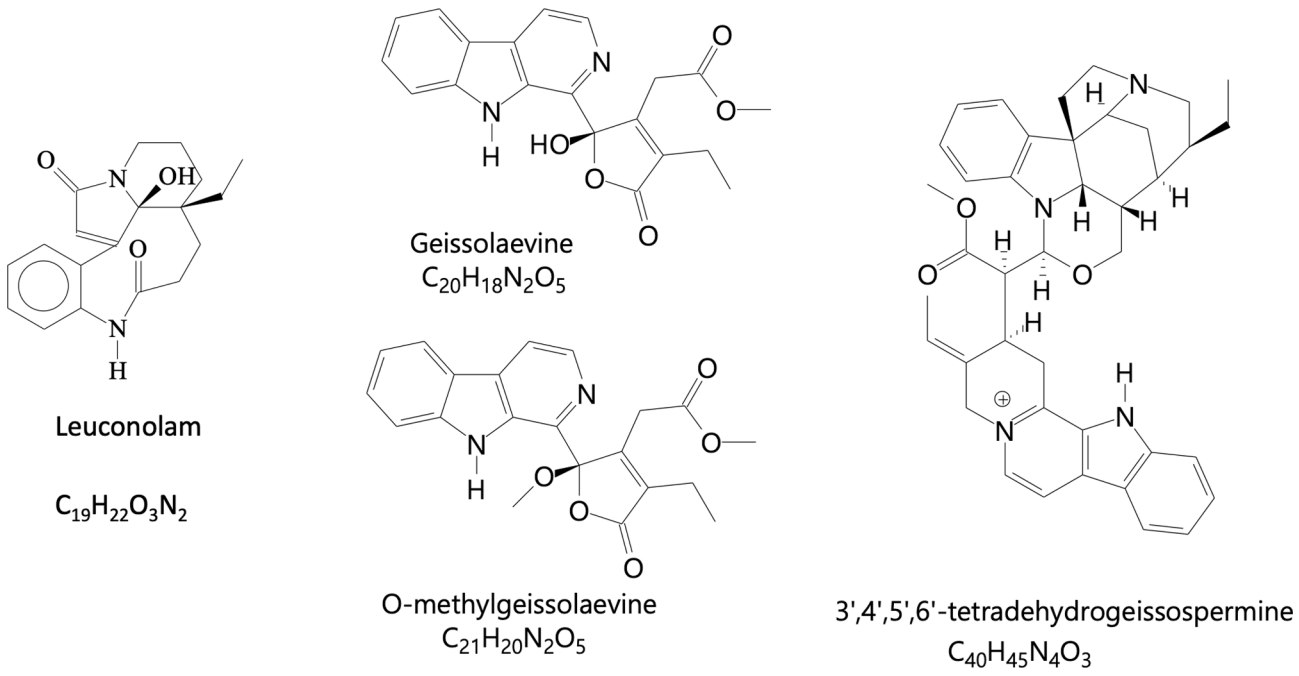


Fig. 3. Four new Geissospermum vellosii alkaloids whose structures were recently elucidated using NMR techniques[20].

second where the preliminary extraction step was based on High-Performance Countercurrent Chromatography (HPCCC) and flash column chromatography, and several fractions containing single pure alkaloids were obtained. Preliminary identification was achieved using various NMR techniques, and subsequent HPLC-MS analyses were used to confirm the identification on the basis of MS^2 high-resolution spectra. Five indole alkaloids, four of which were previously identified species, and a new one, named Geissoschizone (Fig. 2B), were identified. However, the extraction step was quite laborious as it aimed to recover enough substance to perform NMR analyses and to study antiplasmodial activity.

Although NMR is a very powerful identification technique, it requires high purity substances in substantial quantities. Chemical manipulation is therefore inevitably high and even high-performance counter-current chromatography [19], which is based on the partition coefficient between immiscible solvents, only allows a limited number of constituents to be isolated and chemically characterized. In fact, constituents that are present in lower concentrations or possessing very similar physical-chemical properties (e.g. log P values) can be often missed or neglected.

A molecular networking-based study on *Geissospermum laeve* (syn. *Vellosii*) has recently been published [20], in which four new indole alkaloids (Fig. 3) were isolated and their structures elucidated by NMR and HR-MS. Moreover, five previously known compounds (Raubasine, Ibogamine, Quebrachamine, Alstonine and Yohimbine) that had not yet been described in the *Geissospermum* genus, were dereplicated, using a computer-based approach [21], from the *G. laeve* alkaloid extract network, and were assigned various levels of identification confidence.

However, MS^2 spectra databases recorded on a QTOF -MS/MS instrument provided series of product ions whose genesis changed upon using different collision energies. Otherwise, an ion trap has the capability to provide MS^n spectra, allowing to achieve more spectral information useful for structural interpretation. *Geissospermum vellosii* (*syn. laeve*) bark extract was never analyzed as a whole via chromatographic separation and qualitative characterization of as many constituents as possible. This type of analytical approach is important from the point of view of quality control because the bark samples available on the market are often sold at wildly varying prices and there are many different *Geissospermum* species that, even if are sharing some alkaloid molecules, are not the *vellosii* species. A second aspect is that a better knowledge of the bark content may improve the carrying on of further clinical studies concerning the therapeutic effects of this plant.

In this work, we have adopted a Soxhlet extraction of the stem bark with a 70:30 ethanol-water mixture, and a cold extraction, with the same solvent mixture, with ultrasound. Furthermore, we have performed HPLC separations in satisfactory conditions after testing several stationary and mobile phases of different selectivity. The results obtained from the analysis of the samples from the two different extraction techniques completely overlapped.

The complexity of the obtained chromatographic profiles clearly highlights that it is impossible for a single purification technique to provide pure fractions useful for the NMR analysis of all constituents. The strategy for the structural identification of alkaloid molecules that we adopted was therefore based on the entire set of interpretation tools offered by high-resolution mass spectrometry – elemental composition with relative accuracy, ring plus double-bond values, and MS^2 and MS^3 high-resolution spectra that are also thoroughly interpreted using neutral losses numerically evaluated in high resolution - all in combination with the indispensable contribution of UV–Vis Diode Array absorption spectra. Accurate fragmentation patterns have been elaborated and proposed as an important support to the formulation of proposals of the constituent structures. This last aspect was of particular relevance as each single fragmentation step ought to be not only chemically coherent, per se, but also reasonably compatible with the other steps that define the global fragmentation pattern.

The presence of the majority of the previously described alkaloids has been confirmed, and to10 yet-to-be-described species a hypothesis of structure was assigned. Moreover, we have obtained a great deal of experimental data to support the hypothesis that six known species that had never before been described in the Geissospermum genus may be indeed present, as has recently been suggested [20].

2. Experimental

2.1. Reagents

Ethanol for use as an extraction solvent, and acetonitrile for use as an organic modifier of HPLC mobile phases were LC-MS-grade solvents

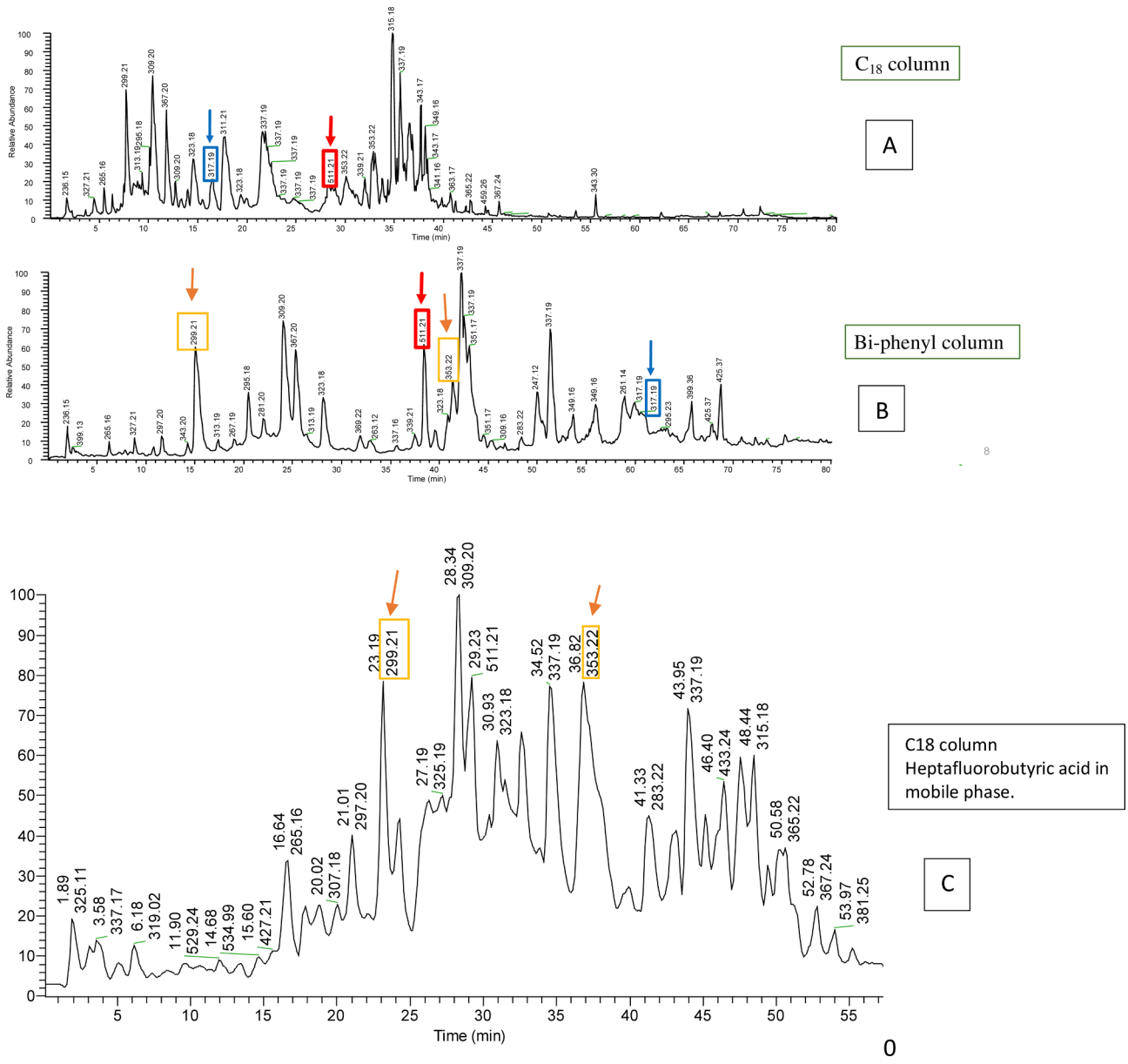


Fig. 4. Chromatographic separations of bark-extract constituents obtained using different mobile-phase/stationary- phase combinations (see experimental section). The chromatographic runs obtained using the biphenyl column, the C18 column, and the C18 column with heptafluorobutyric acid as a counter ion in the mobile phase are illustrated in A, B, and C, respectively. The numerical values above each peak correspond to their *m/z* values. Blue and red rectangles marked two peaks with different retention times obtained on C18 and biphenyl column and yellow rectangles marked two other peaks with different retention times obtained respectively in case of biphenyl column and C18 column eluted with heptafluorobutyric acid in the mobile phase. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

(Sigma Aldrich, now Merck KGaA). Ultrapure water (MilliQ, Millipore Merck, Darmstadt, Germany) was acidified (0.1%) either with extra pure formic acid HCOOH (>98%, Sigma Aldrich now Merck KGaA) or heptafluorobutyric acid (Sigma Aldrich, now Merck KGaA). Pure standards of Alstonine and Serpentine were purchased from ChemFaces (Wuhan, China). The bark sample was purchased from Tropilab (Surinam).

2.2. Extraction

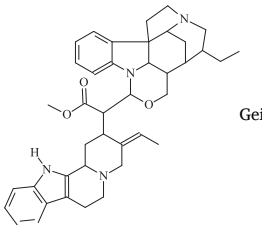
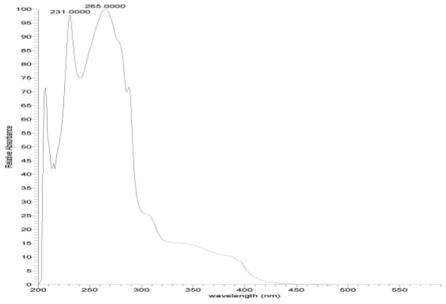
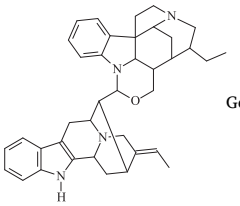
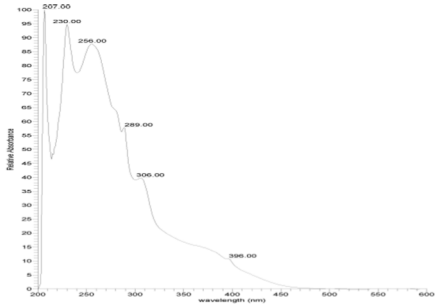
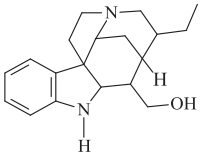
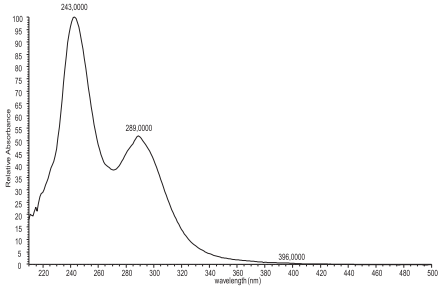
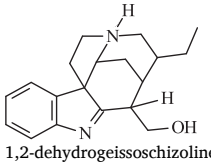
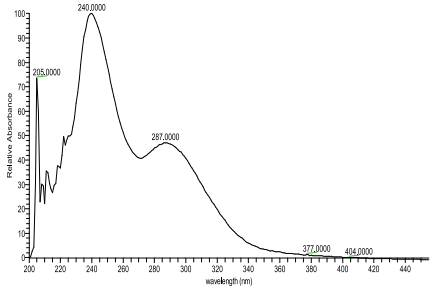
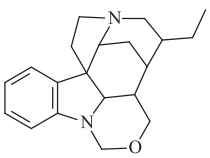
Geissospermum bark was frozen and subsequently finely ground using an Ultra-Turrax blender (IKA, Staufen, Germany). 6.5 g of the obtained powder were extracted using a Soxhlet system with 200.0 mL of a 70:30 mixture Ethanol/0.1% HCOOH in ultra-pure water. The water acidification had the double function of imparting a charge to the basic molecules of alkaloids and contemporarily make up pH conditions less prone as possible to oxidation phenomena. The oil-bath temperature was set at 93 °C ± 3 °C for the entire duration of the extraction step. The mean time of a single extraction cycle was about 45 min. The extraction procedure was stopped after 24 cycles after verification that more cycles were not providing better extraction yield. The extraction solution was centrifuged for 10 min at 5000 rpm. The supernatant was 1:3 diluted with a CH_3CN/H_2O 15:85 mixture before HPLC analysis.

2.3. Chromatographic conditions

The separation properties of the two columns that had different selectivities, Gemini® NX-C18 (Phenomenex, Bologna, Italy) and Pinnacle® DB Biphenyl (Restek, Cernusco sul Naviglio, Milan, Italy) were checked.

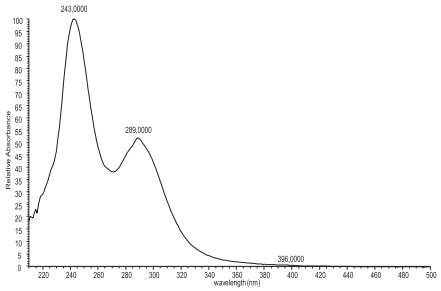
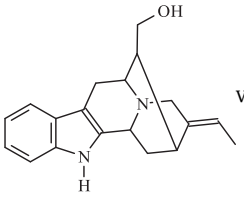
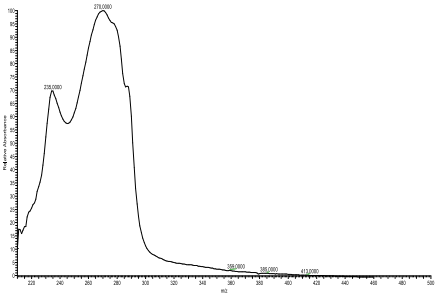
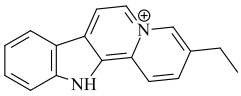
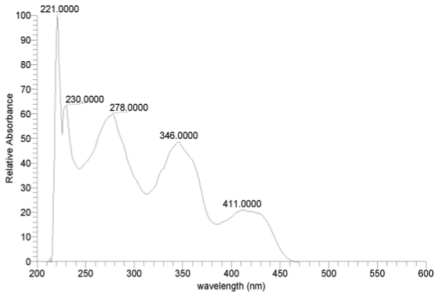
The Gemini® NX-C18 column 150 × 2.0 mm, d_p 3.0 µm. Eluent A:

Table 1
UV absorption spectra and mass spectrometric data of the detected alkaloids whose structure has previously been described in the literature.

Compound Elemental Formula Molecular Weight	Precursor ion *m/z* value	MS^2 product ion *m/z* values (percent signal intensity)	MS^3 Product ion *m/z* values (percent signal intensity)	UV absorption spectra
 Geissospermine $C_{40}H_{48}O_3N_4$ 632.834 u	$m/z_{(z=2)}$ = 317.194 **$C_{40}H_{50}O_3N_4$** **Δppm = 1.290**	490.305(100) 144.080 (71)325.192 (7)309.197 (15)	317.94 → 490.305383.123 (100)281.202 (91)309.197 (72)351.207 (51)349.192 (27)194.097 (14)	
 Geissolosimine $C_{38}H_{44}ON_4$ 572.351 u	$m/z_{(z=2)}$ = 287.183 **$C_{38}H_{46}ON_4$** **Δppm = 1.141**	309.197(100) 265.169 (74)	287.183 → 265.169144.081 (100)122.096 (87) 287.183 → 309.197281.202 (100)182.097 (97)253.134 (61)210.092 (35) 897)	
 Geissoschizoline $C_{19}H_{26}ON_2$ 298.204 u	m/z = 299.212 **$C_{19}H_{27}ON_2$** **Δppm = 0.610**	299.212 (100) 194.096 (49)281.201 (49)206.154 (33)238.159 (26)236.143 (25)256.17 (20)144.080 (11)	299.212 → 281.201162.128 (100)238.160 (33) 299.212 → 236.143194.096 (100) 299.212 → 238.159196.112 (100)209.120 (33)182.097 (21)	
 1,2-dehydrogeissoschizoline $C_{19}H_{24}ON_2$ 296.188 u	m/z = 297.197 **$C_{19}H_{25}ON_2$** **Δppm = 0.550**	234.128 (100) 279.186 (72)250.156 (42)144.080 (27)	297.279 → 279.186236.144 (100)250.156 (33) 297.197 → 234.128219.105 (100)205.089 (85)206.097 (43)192.081 (19)	
 Geissoschizone	m/z = 311.212 **$C_{20}H_{27}ON_2$** **Δppm = 0.460**	281.201 (100) 250.159 (85)293.201 (59)208.111 (33)	311.212 → 281.201250.159 (100)208.111 (38)	

(*continued on next page*)

Table 1 (*continued*)

Compound Elemental Formula Molecular Weight	Precursor ion *m/z* value	MS^2 product ion *m/z* values (percent signal intensity)	MS^3 Product ion *m/z* values (percent signal intensity)	UV absorption spectra
$C_{20}H_{26}ON_2$ 310.203 u				
 Vellosiminol $C_{19}H_{22}ON_2$ 294.172 u	*m/z* = 295.180 **$C_{19}H_{23}ON_2$** **Δppm = 0.570**	277.169 (100	295.180 → 277.169146.096 (100)248.131 (42)262.147 (37) 144.087 (22	
 Flavopereirine $C_{17}H_{15}N_2$ 247.123	*m/z* = 247.123 **$C_{17}H_{15}N_2$** **Δppm = 0.455**	247.123 (100) 232.099 (59)245.107 (8)		

aqueous formic acid (0.01%) or alternatively aqueous heptafluorobutyric acid 5.0 mM, eluent B acetonitrile + 0.01% formic acid or heptafluorobutyric acid 5.0 mM. Gradient conditions: 0–5 min from 10 to 15% B, an isocratic step at 15% of B for 20 min, 25–60 min from 15 to 50% B, 60–75 min from 50 to 100% B. Injection volume 10 µL, flow rate 0.200 mL/min.

Pinnacle® DB Biphenyl column 150 × 2.1 mm, d_p 3.0 µm. Eluent A: aqueous formic acid (0.01%), eluent B acetonitrile + 0.01% formic acid. Gradient conditions: an isocratic step at 15% of B for 12 min, 12–15 min from 15 to 21% B, an isocratic step at 21% of B for 15 min, 30–109 min from 21 to 100% B. Injection volume 10 µL, flow rate 0.200 mL/min.

2.4. UV DAD detection and mass spectrometry conditions

The Diode array PDA Plus Surveyor (Thermo Scientific, Rodano, Italy) was used in the wavelength range 220–500 nm.

Multistage HR-MS experiments were performed using an ESI ionization source that was connected to a linear ion trap equipped with a high-resolution analyzer (LTQ-Orbitrap, Thermo Scientific, Bremen Germany).

The LTQ-Orbitrap analyzer was used in the *m/z* range 220–1200. Capillary temperature: 270 °C, sheath gas flow rate: 35 (arbitrary units), auxiliary gas flow rate: 15 (arbitrary units), source voltage: 4.5 kV, capillary voltage: 16 V, tube lens: 55 V. Resolution: 30,000 (FWHM). Mass accuracy: 5 ppm (without internal calibration). MS^2 and MS^3 collision energy: 30 (arbitrary units).

3. Results and discussion

The first important analytical goal was to obtain a good separation of the constituents of the stem-bark extract. Satisfactory chromatographic resolution was necessary because the UV-absorption properties of the molecules constitute indispensable interpretation support to the mass spectrometric results. It was therefore useful, when possible, to obtain UV spectra that could be traced back to the chemical class of the compounds.

We tested two reverse phase stationary phases with different selectivity, a totally lipophilic C18 phase and a diphenyl phase in which π-π interactions predominate. Various mobile-phase compositions and gradient-elution conditions were also tested.

There was no combination of stationary and mobile phase that was able to provide the base-to-base resolution of all of the peaks present in the various chromatographic runs. Nevertheless, satisfactory separations were obtained, and the corresponding chromatograms are illustrated in Fig. 4 (A, B), in which each peak is denoted by its *m/z* value. The separations obtained with the two stationary phases showed some complementarity, which was useful, in some cases, to obtain a clearer connection between the UV absorption properties and mass spectra. Moreover, useful alternative chromatographic results were also obtained from the C18 column by adding heptafluorobutyric acid as a

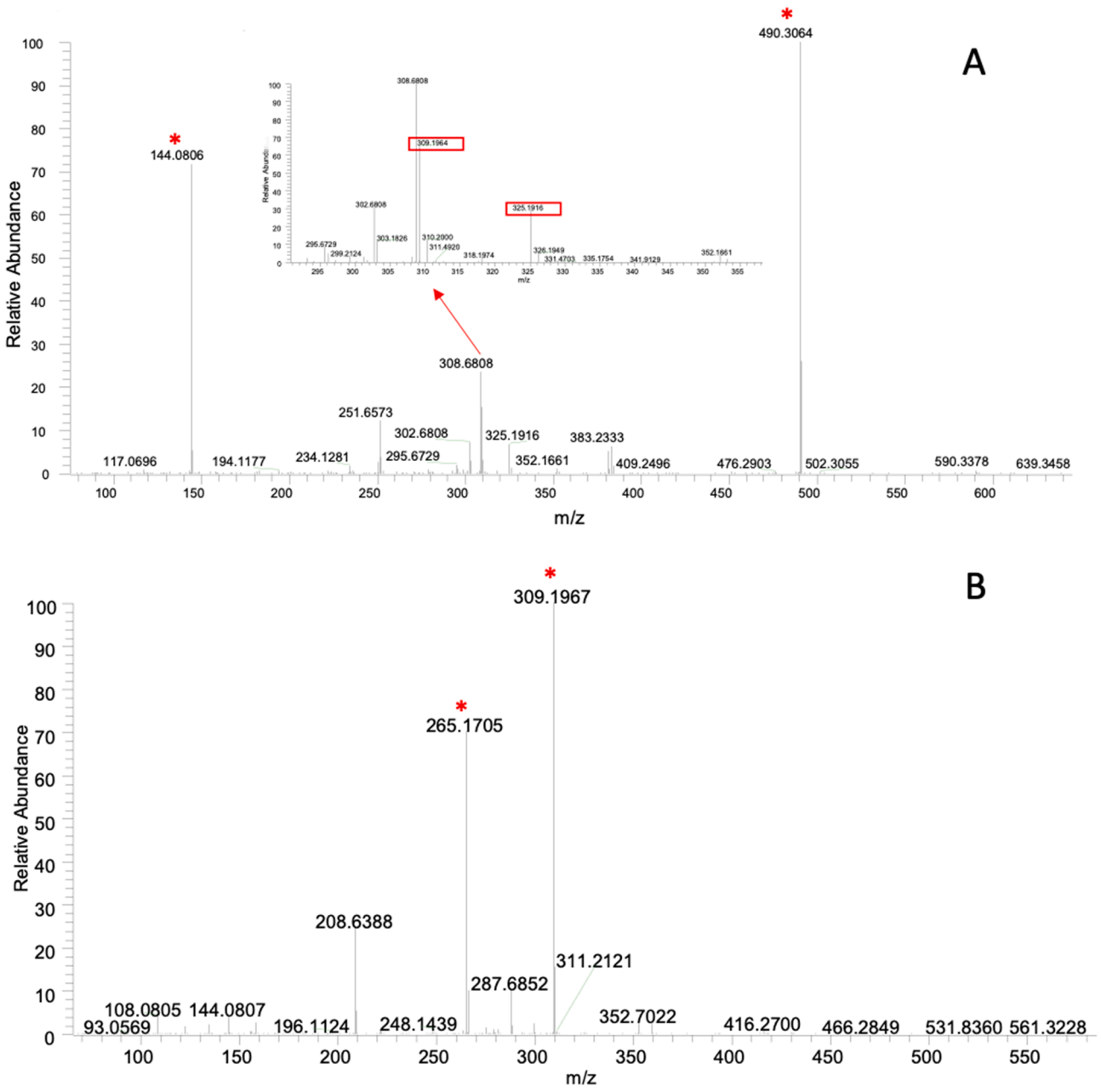


Fig. 5. MS^2 spectra of Geissospermine (A) and Geissolosimine (B) obtained using a collision energy of 30 (arbitrary scale).

counter ion in the mobile phase (Fig. 4, C).

The previously described indole alkaloids were preliminarily identified using the accurate *m/z* values that make reference to their molecular weight, and this was further supported by the estimation of their elemental composition thanks to the high resolution (5 ppm accuracy) and ring plus double bonds values. A connection was then established to their UV absorption properties, which was another important preliminary classification step. In fact, the UV spectra indicated the presence of two main chromophores. The indole chromophore, typical of Vellosiminol (E), and the indoline chromophore, typical of Geissoschizoline (C). Flavopereirine (D) (Fig. 1) was characterized by a different UV spectrum due to its higher π-electron conjugation. The bis-indole alkaloids that bear both indole and indoline groups exhibited mixed absorption spectra.

The second important investigation step was a thorough inspection of the multistage MS spectra. This was an important step because it can prepare the mass spectrometric interpretation tools of this type of molecules by taking advantage of the knowledge of their previously described structures. In some cases, the MS^2 spectra were sufficient for a clear confirmation of molecular structure, whereas, in other cases, the MS^3 mode was necessary for the structural information to have a sounder basis. Table 1 reports the UV and High-Resolution MS data in the MS^2 and MS^3 modes.

The detailed fragmentation pattern was deduced by analyzing the MS^2 spectra of the precursor ion and MS^3 of a chosen product ion. As expected, they were complex, as the structures involved bear many rings and competitive fragmentation pathways. We will describe, in detail, some examples of the analytical procedures adopted as a significant paradigm of the interpretation strategy used throughout the present work as it is hoped that the reader will find it useful.

The full mass spectra of the more complex structures of bis-indole alkaloids, Geissospermine and Geissolosimine, exhibited double charged quasi-molecular precursor ions at $m/z_{(z=2)}$ values 317.194 and 287.183, respectively. So, as expected, their first fragmentation event

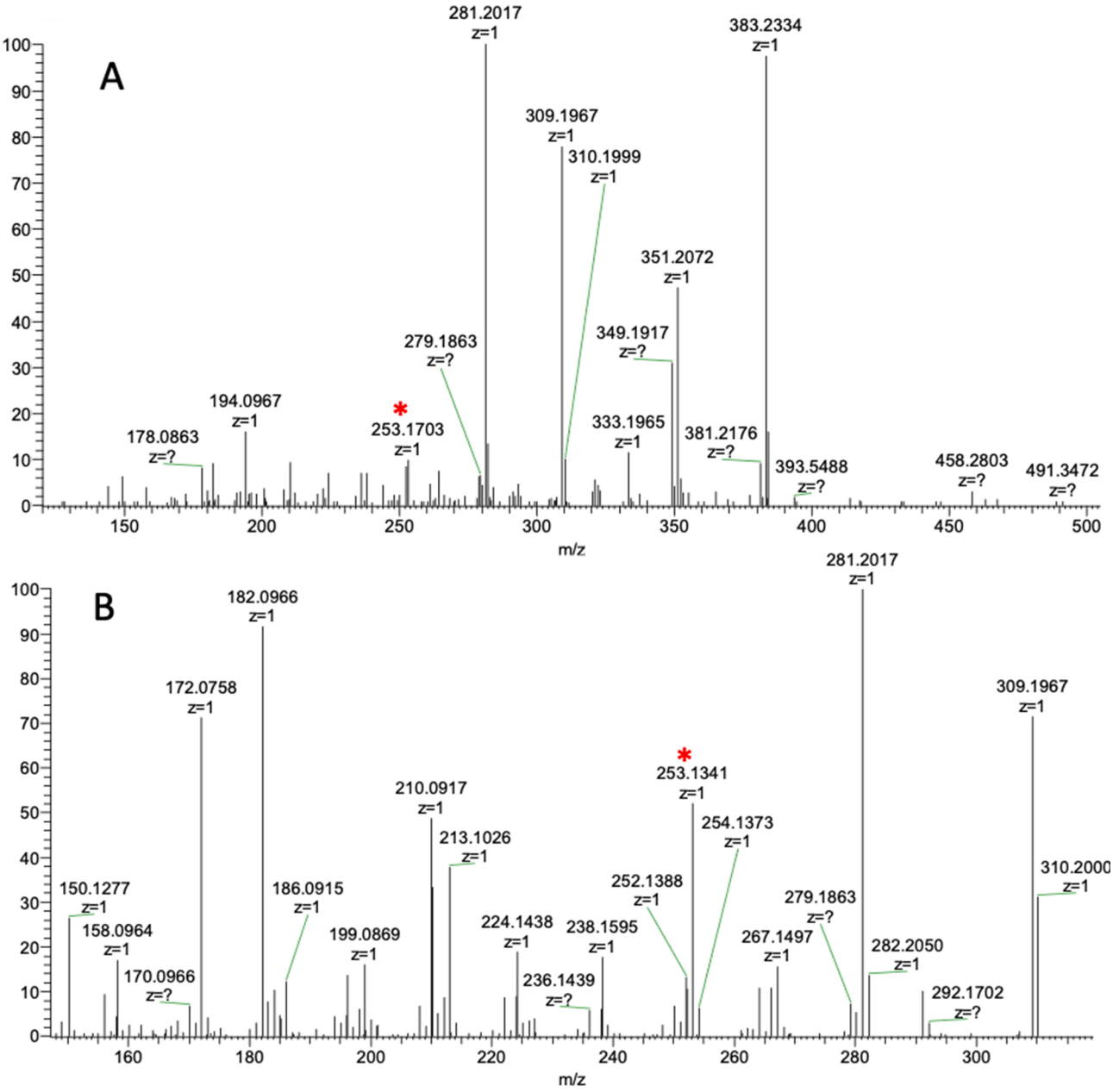


Fig. 6. MS^3 spectrum of the MS^2 product ion of m/z = 490.306 (A) of Geissospermine and of the MS 2 product ion of m/z = 309.197 (B) of Geissolosimine.

provided couples of singly charged ions whose sum of m/z values corresponded to the molecular weight of the bis-indole molecule plus two hydrogen ions. Geissospermine showed two couples of these product ions 325.191 + 309.197, and 490.305 + 144.081 (the members of each couple are marked by a star of the same color) and the second couple was largely predominant (Fig. 5A). On the other hand, Geissolosimine showed a main fragmentation mechanism that provided the couple of mono-charged product ions at m/z values of 309.197 and 265.169, respectively (Fig. 5B).

The different structures of the indole moiety of Geissospermine and Geissolosimine, justifies the difference in fragmentation behavior.

A detailed MS^2 and MS^3 of the precursor ions at $m/z_{(z=2)}$ = 317.194 and $m/z_{(z=2)}$ = 287.183 and of the respective diagnostic product ions at $m/z_{(z=2)}$ = 490.305 and $m/z_{(z=2)}$ = 309.197 derived from the bis-indole products Geissospermine and Geissolosimine can be found in Schemes 1 and 2 of the Supplementary Information section.

The evidence for the higher intensity of the couple of mono-charged product ions, with m/z values of 490.306 and 144.081 in the MS^2 spectrum of Geissospermine, can be justified by the high stability of the product ion of m/z = 144.081, whose electronic configuration is highly conjugated (Scheme 1 of Supplementary Information section, cleavage A).

The MS^3 experiments provided several other molecular details that were able to strengthen the characterization of the structures. The MS^3 spectra of the mono-charged product ions of m/z = 490.306 of Geissospermine (**A**) and m/z = 309.197 of Geissolosimine (**B**) are reported In Fig. 6.

The MS^3 spectrum (Fig. 6A) of the ion of m/z = 490.306 coming from the MS^2 of the doubly charged precursor ion of $m/z_{(z=2)}$ = 317.194 shows a series of product ions that are due to successive neutral losses and not due to competitive alternative pathways (Scheme 1 of Supplementary section, fragmentation pathway A). It is worth paying special attention to the intermediate product ion of m/z = 309.196. This ion fragments further by twice losing a nominal mass of 28 u and producing the ion of m/z = 253.170. The first neutral loss corresponds to the accurate mass of 27.99 u (CO molecule) and the second one to 28.031 u

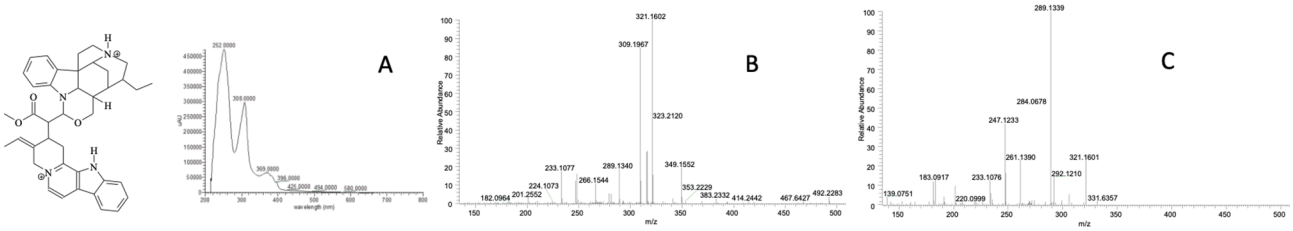


Fig. 7. Structure of 3′,4′,5′,6′-tetradehydrogeissospermine, UV absorption spectrum (A), MS 2 (B) and 315.178, 321.1602 MS 3 (C) spectra.

($CH_2 = CH_2$ molecule).

The same ion of $m/z = 309.196$ is present as the base peak in the MS^2 spectrum of Geissolosimine (Fig. 5B), and, by comparing the MS^3 spectra of Geissospermine (A) and Geissolosimine (B) in Fig. 6, it is possible to note that the product ions with m/z values that are lower than 309.196 are different in number and intensity. As a matter of fact, there is a base peak of $m/z = 182.097$ in the Geissolosimine MS^3 spectrum that is scarcely present in the case of Geissospermine, while the common product ion of $m/z = 253$, in terms of nominal mass, has distinct values in terms of accurate mass, specifically $m/z = 253.170$ in the fragmentation scheme of Geissospermine and $m/z = 253.134$ in the fragmentation scheme of Geissolosimine (both denoted by red stars). In the last case, there is no significant numerical relationship (evaluated in high resolution) between this product ion and the immediately preceding one ($m/z = 281.201$), meaning that it must necessarily derive from the precursor ion of $m/z = 309.196$.

The proposed dissociation mechanism is illustrated in Scheme 2 of Supplementary Information, which hypothesizes an alternative fragmentation step, for the product ion of $m/z = 253.134$, that involves the energetically more demanding loss, from the species of $m/z = 309.196$, of a neutral butene molecule (M = 56.06 u) to give the ion of $m/z = 253.134$, instead of the simple loss of CO to give the ion of $m/z = 281.201$. This different behavior may be justified by the fact that the collision energy is only just residual in the case of the ion of the $m/z = 309.196$ intermediate product of the MS^3 fragmentation of Geissospermine, whereas it is totally absorbed in the case of the Geissolosimine MS^3 experiment, in which the same ion is the precursor one.

Furthermore, the base peak of $m/z = 182.096$ in MS^3 spectrum of Geissolosimine (Fig. 6 B) is also unlikely to derive from the numerically preceding product ions because there is no significant numerical relationship (evaluated in high resolution) that can justify its derivation. As illustrated in Scheme 2 (Supplementary Information), the species from which it may derive is the ion of $m/z = 210.091$ after a loss of 27.99 u (CO molecule). In turn, this last species can be derived from the ion of $m/z = 253.134$ after a loss of a $CH_2 = CH\text{-}NH_2$ molecule (43.042 u).

The presence of alternative fragmentation pathways involving the same species is then important support for the confirmation of the molecule structure.

Analogous inspections of the MS, MS^2 and MS^3 spectra of the other already known indole alkaloids allowed us to propose fragmentation pathways that can support the proposal of their molecular structure. Assessing the ability of high-resolution multistage mass spectrometric data to offer structural information about this type of molecule is an important achievement. All fragmentation schemes are reported in the Supplementary Information section [Schemes 1–7].

Turning to species that have more recently been described in *Geissospermum vellosii* (syn. *laeve*) - Pausperadine [18], Geissolaevine, O-methylgeissolaevine, Leuconolam and 3′,4′,5′,6′-tetradehydrogeissospermine [20] - we have not found any evidence to confirm the presence of the first four. However, the last one was an important presence in our extract. In fact, we found a species of $m/z_{(z=2)} = 315.178$ for which the elemental composition $C_{40}H_{44}N_4O_3$ was calculated (Δppm = 1.39) and likely corresponds, in the first instance, to 3′,4′,5′,6′-tetradehydrogeissospermine. As its structure was elucidated using the NMR technique [20], and the double bonds that are caused by dehydrogenation are accurately positioned, we verified the ability of the obtained UV absorption spectrum, MS^2 and MS^3 spectra to agree with the elucidated structure (Fig. 7) .

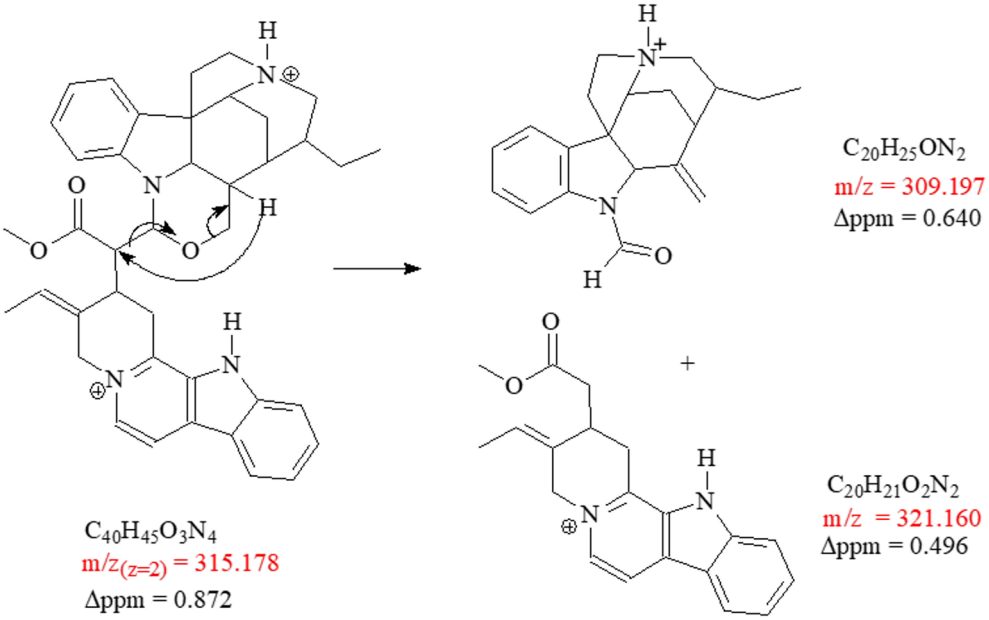


Fig. 8. MS^2 fragmentation mechanism of 3′,4′,5′,6′-tetradehydrogeissospermine.

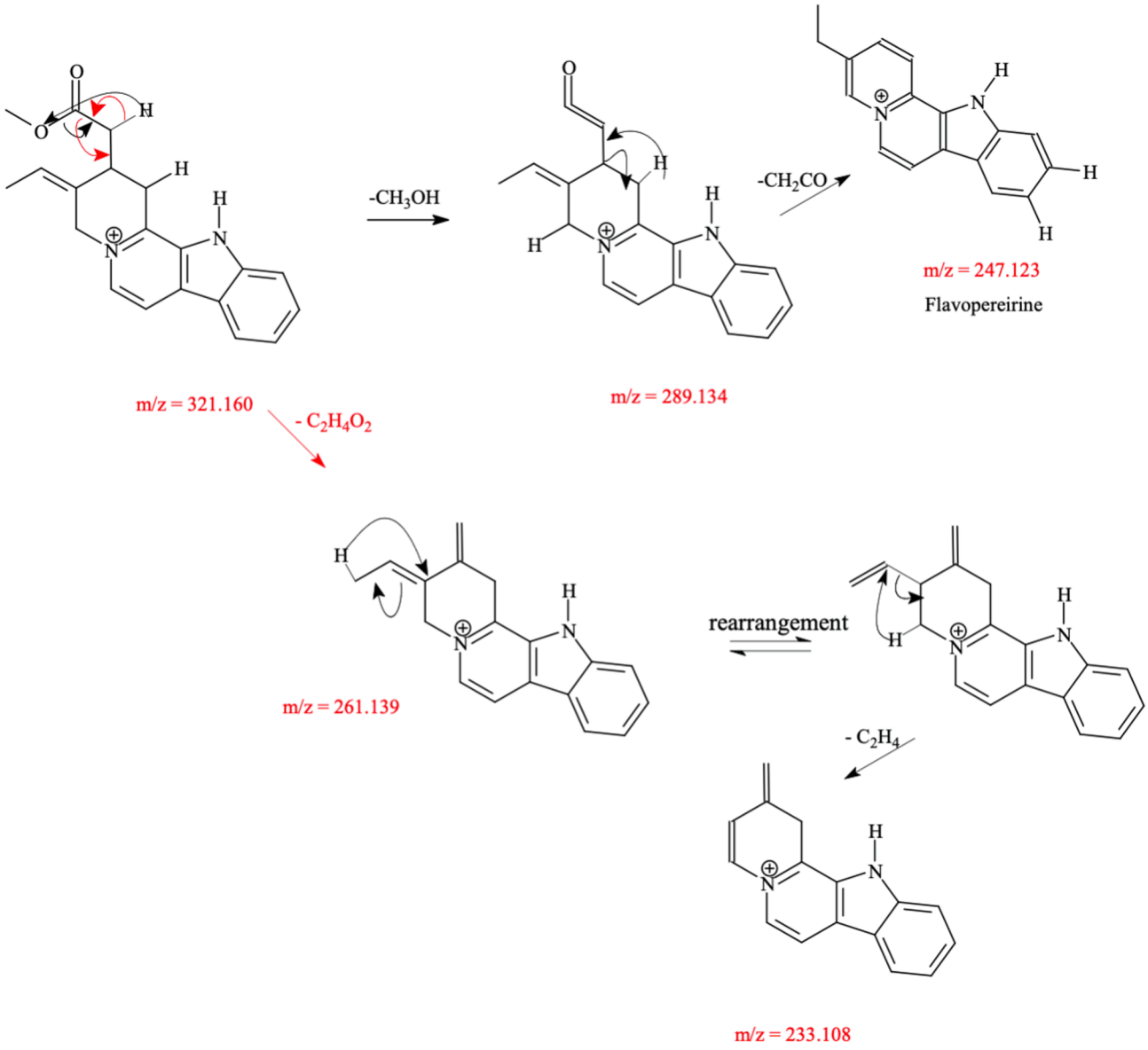


Fig. 9. m/z (z = 2) = 315.178 → 321.160 MS^3 fragmentation mechanism.

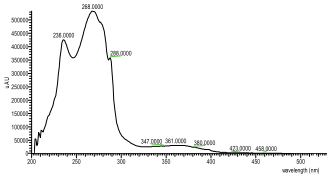
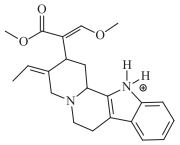
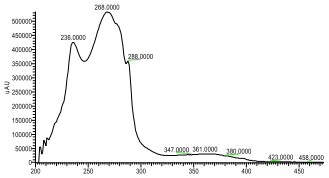
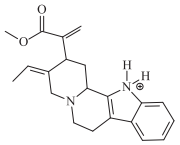
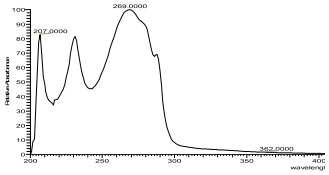
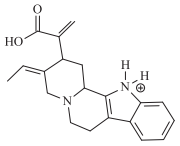
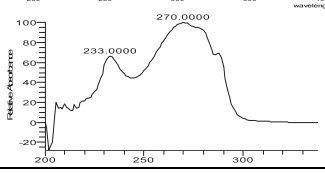
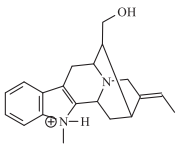
As can be seen, the UV spectrum of 3′,4′,5′,6′-tetradehydrogeissospermine is quite different to that of Geissospermine (Table 1) as a different chromophore system is present, and the MS^2 spectrum of the doubly charged precursor ion of $m/z_{(z=2)}$ = 315.178 shows that two singly charged ions of m/z = 309.197 and m/z = 321.160 are principally formed; these can be traced back to a fragmentation pathway that is different to the one previously described for Geissospermine (compare with Supplementary Information Scheme 1). The higher π-conjugation of the indole moiety of the molecule and the presence of a quaternary ammonium ion favors a different product ions pattern. The proposed mechanism is reported in Fig. 8 where the species of m/z = 309.197 is the indoline moiety of the molecule, as already found in the fragmentation schemes of Geissospermine and Geissolosimine (Supplementary Information Schemes 1, 2). The species of m/z = 321.160 was the object of a successive fragmentation (MS^3 spectrum in Fig. 7 C) and the proposed product ions pattern is reported in Fig. 9.

It is worth noting that Flavopereirine ($C_{17}H_{15}N_2$, m/z = 247.123) is an important product ion as a consequence of the high π-electron conjugation of the indole moiety.

The same investigation protocol was applied to many other species that are present in the obtained chromatographic separation of the Geissospermum bark extract. A doubly charged quasi-molecular ion of $m/z_{(z=2)}$ 294.192 was identified, and other singly charged, so far undescribed, substances were detected at m/z values of 367.201, 337.191, 323.176, 309.197, 515.239, 511.207, 349.155, 351.171, 363.170 and 353.223. The best way to preliminary categorize all these species was to place them together in terms of similarity to their UV spectra, which provide information about the chromophore system present. From this point of view, the four singly charged species in Table 2 may be subdivided into two couples: the first is made up of the species of m/z = 367.201 (compound **1**, $C_{22}H_{27}O_3N_2$) and 337.191 (compound **2**, $C_{21}H_{25}O_2N_2$) with perfectly overlapping UV spectra (identical to that of Vellosiminol, see Table 1); and the second is made up of the species of m/z 323.176 (compound **3**, $C_{20}H_{23}O_2N_2$) and 309.197 (compound **4**, $C_{20}H_{25}ON_2$), which also have mutually overlapping UV spectra, but slightly different from those of the first couple. Nevertheless, it appears that the chromophore system can be essentially traced back to the presence of an indole group.

The obtained MS^2 and MS^3 data confirm the structural correlation among the constituents of the first two groups, and show that their precursor ions share several common diagnostic product ions. In particular, the m/z value of 337.191 (**2**) is both the first product ion of

Table 2
UV absorption spectra, mass spectrometry data and proposed structures for the new species that are strictly related to the known bis-indole molecules (compounds **1**, **2**, **3**, **4**).

COMPOUND NUMBER Elemental Formula	$[M + H]^+$ *m/z* value Elemental formula Accuracy	UV–VIS SPECTRUM	FRAGMENT ION MS^2 (RELATIVE INTENSITY %)	FRAGMENT ION MS^3 (RELATIVE INTENSITY %)	PROPOSED STRUCTURE
1 **Geissospermine related** $C_{22}H_{26}O_3N_2$	*m/z* = 367.201 $C_{22}H_{27}O_3N_2$ Δppm= 1.28		337.191 (100)305.165 (26)236.128 (20)349.192 (19)	367.201 → 337.191 180.102 (100)305.165 (20)	
2 **Geissospermine related** $C_{21}H_{24}O_2N_2$	*m/z* = 337.191 $C_{21}H_{25}O_2N_2$ Δppm= 1.29		180.102 (100)305.165 (49)	337.191 → 305.165 277.170 (100)287.154 (40)174.901 (20)	
3 **Geissospermine related** $C_{20}H_{22}O_2N_2$	*m/z* = 323.176 $C_{20}H_{23}O_2N_2$ Δppm= 1.62		166.086 (100)279.186 (51)305.165 (33)148.111 (23)144.080 (6)	323.176 → 279.186 148.111 (100)122.096 (74)136.111 (57)	
4 **Vellosiminol related** $C_{20}H_{24}ON_2$	*m/z* = 309.197 $C_{20}H_{25}ON_2$ Δppm= 0.70		291.186 (100)152.107 (17)	309.197 → 291.186 160.112 (100)261.239 (54)276.161 (48)148.111 (24)	

the precursor at *m/z* = 367.201 (**1**), and the precursor ion of an independent species, with both showing several common product ions (Supplementary Information Schemes 8, 9). The species of *m/z* = 323.176 (**3**) differs from the species of *m/z* = 337.191 (**2**) by 14 u, and, nevertheless, provides the product ion of *m/z* = 305.165 by losing a water molecule, meaning that it must have a free –OH group in a suitable position. A structure is proposed in Scheme 10 of the Supplementary Information, together with fragmentation behavior that justifies the obtained product ions and supports the similarity with the species of *m/z* = 337.191. The species of *m/z* = 309.197, despite being isobar with the obtained product from the fragmentation of Geissospermine and Geissolosimine (Fig. 6 A, B and scheme 1,2 in Supplementary Information) and differing from the above species of *m/z* = 323.176 (**3**) by 14 u, has an elemental composition that is incompatible with this apparent similarity and different MS^2 and MS^3 spectra. In fact, it has a more significant similarity to Vellosiminol (*m/z* = 295.181), from which it equally differs by 14 u. The line of reasoning that led to the proposal of a molecular structure rested on the observation that this numerical difference from Vellosiminol may be related to a possible substitution of a hydrogen with a methyl group. By comparing the fragmentation pathways formulated for Vellosiminol (Supplementary Information Scheme 6) with the analogous ones (all differing by 14 u) of the present unidentified molecule (Supplementary Information Scheme 11), it is possible to identify the probable position of the methyl substitution. In fact, in principle, the methyl substitution may be on the heterocyclic nitrogen atom or on the carbon atom of the lateral chain, but the couple of product ions at *m/z* values 146.096 and 160.112, in Schemes 6 and 11 (Supplementary information), respectively, settles the question, positioning the methyl substitution on the heterocyclic nitrogen atom. It is worth noting that in the context of the bark stem composition of the plant in study the tools provided by high resolution mass spectrometry together with the support of UV–Vis spectra are able to discriminate a case of isobar structure. All the described data and the consequent proposal of plausible structures are summarized in Table 2.

In order to insert these new species into the context of the known bark components, we deliberated on the fact that the majority of the previously described mono-indole alkaloids of *Geissospermum vellosii* show a strict structural connection to bis-indole Geissospermine and Geissolosimine (see formulae in Fig. 1). This is likely because the biosynthetic routes that start from the amino acid tryptophan initially form mono-indole molecules and successively assembles them into the bis-indole structure. Assuming that this biosynthetic behavior is also valid for these newly detected species, we examined whether they may be mono-indole precursors or, otherwise, hydrolysis or degradation products of the bis-indole molecules. A reasonable structural relationship between the proposed structures of the species of *m/z* values 323.176, 337.191, 367.201 (compounds **3**, **2** and **1**, respectively) and the bis-indole Geissospermine molecule is illustrated in Fig. 11 (A, B, C). The double arrows used to connect the separated mono-indole species to the bis-indole ones derive from the hypothesis that they may be either biosynthesis intermediates or metabolic products. The plausibility of this connection is reinforced by the fact that the complementary structures corresponded to previously identified species in two out of three cases (Fig. 10, Geissoschizone pathway A, 1,2-dehydrogeissoschizoline pathway B.).

There was evidence of the presence of a second species at *m/z* = 337.191, for which, in spite of being isobar and exhibiting the same elemental composition and the same ring plus double value of the previously described compound **2** ($C_{21}H_{25}O_2N_2$), reported in Table 2, we had difficulty to hypothesized an isomerism relationship because there

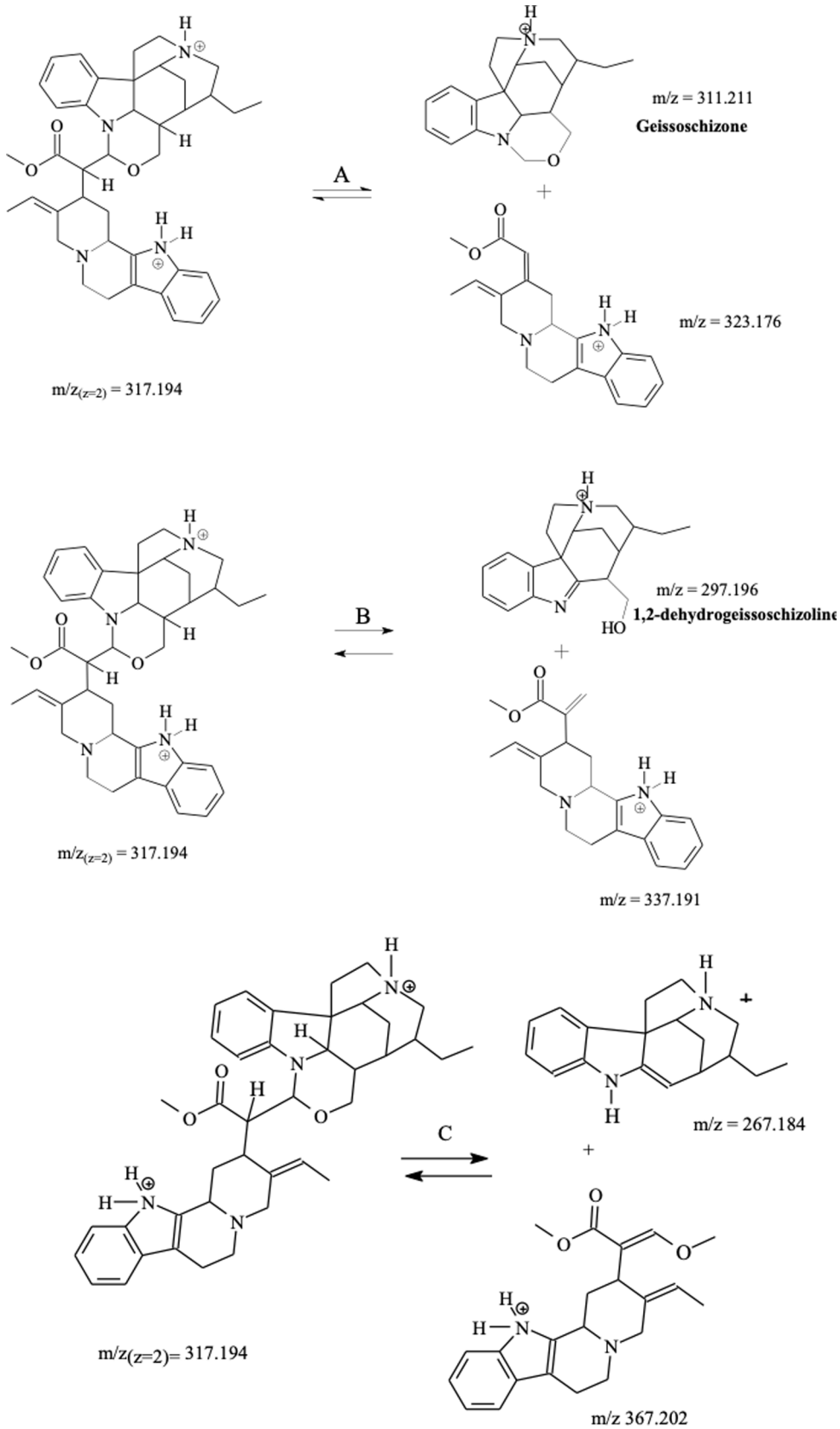


Fig. 10. Possible structural relationship between the newly described species and the bis-indole structure of Geissospermine.

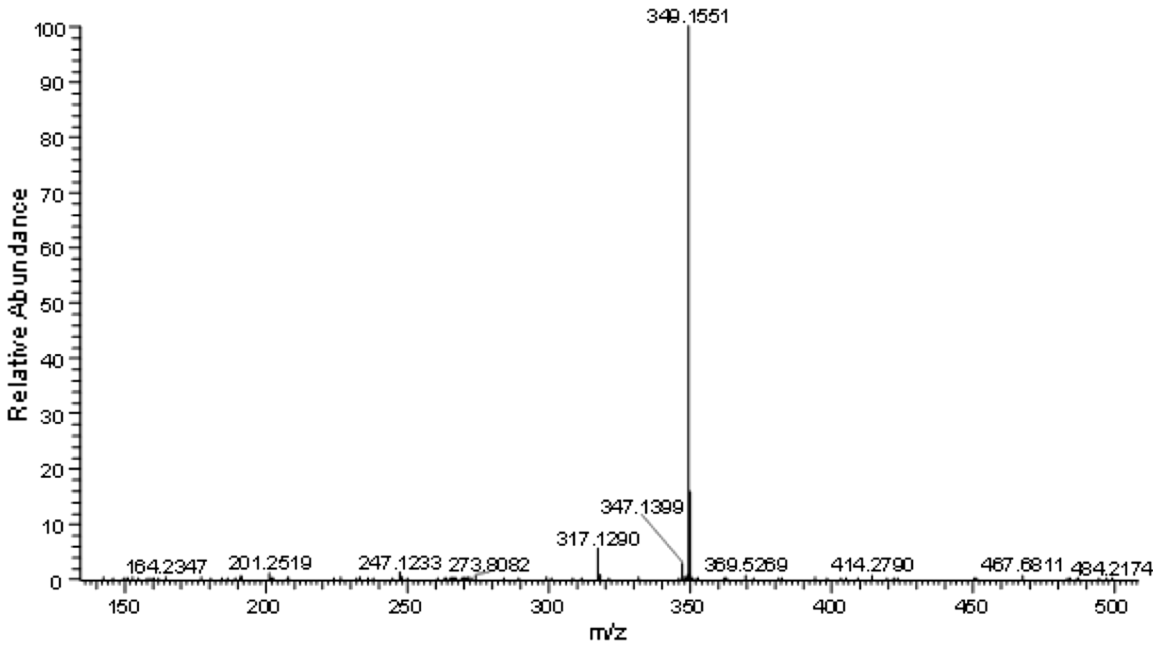
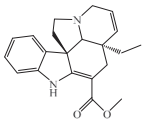
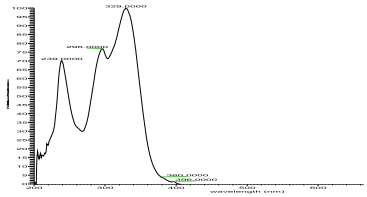


Fig. 11. MS^2 spectrum of the precursor ion of m/z = 511.207.

Table 3
UV spectrum and mass spectrometry data of the isobar species of m/z = 337.191.

Structure	$[M + H]^+$ **m/z value** **Elemental formula** **Accuracy**	UV–Vis spectrum	MS^2 Product ion (relative intensity %)	MS^3 Product ions (relative intensity %)
 **Tabersonine** $C_{21}H_{24}O_2N_2$	m/z = 337.191 $C_{21}H_{25}O_2N_2$ Δppm = 1.410		305.165(100)	337.191 → 305.165 196.099 (88)277.170 (62)234.128 (45)

are some significant MS^2 and MS^3 spectra differences. Besides the UV spectrum was significantly dissimilar from that of compound **2** in Table 2, suggesting the presence of a different chromophore system.

All these experimental evidences, summarized in Table 3, are in line with the monoterpene indole alkaloid Tabersonine, which is present in the database reported in a previous work [20].

The plausibility of the identification was supported by the proposal of a product ions pattern, in agreement with MS^2 and MS^3 data, reported in Supplementary Information Scheme 12.

On the other hand, the other singly charged species of m/z values 511.207 (compound **5**), 349.155 (two species, one of which is the unknown compound **6**), 351.171 (compound **7**) and 363.170 (compound **8**) have UV absorption spectra that are very similar to that of the species of $m/z_{(z=2)}$ = 315.178 (3′,4′,5′,6′-tetradehydrogeissospermine Fig. 7A), suggesting that the same chromophore, which is an indole moiety with a high conjugation degree, is present.

First of all, it is worth discussing, in detail, the fragmentation behavior of the ion of m/z value 511.207 (**5**), as it is quite new, peculiar and, in addition, has a strict structural connection with the species at m/z = 349.155 (**6**).

Its MS^2 spectrum shows a single product ion of m/z = 349.155 (Fig. 11).

The fact that, in spite of its high molecular weight, the molecule only bears a single charge suggests that there are some important structural differences with regards to the other bis-indole alkaloids present in the plant. As a matter of fact, the neutral loss of m/z 162.05 u from the precursor ion at m/z 511.207 suggested that it was highly compatible with an O-linked hexose structure (Δppm = -0.49). This is not a surprising result as glycosylated alkaloids are largely described in the literature as being products of the action of glycosyltransferase enzymes [22–24]. In order to obtain further structural details about this molecule, a MS^3 experiment was performed and the obtained product ions of the series 511.207 → 349.155 are reported in Fig. 12.

While the obtained elemental composition ($C_{21}H_{21}O_3N_2$, m/z = 349.155, Δppm = 0.21) for the MS^2 product ion is compatible with that of Serpentine or its epimer Alstonine, the accuracy of this numerical comparison is not sufficient as neither epimer (molecular structure in Fig. 13 A) has a free –OH group able to bear an O-linked sugar molecule that would justify the neutral loss illustrated in Fig. 12. We therefore assumed an isobar molecular structure that is modified for this product ion with respect to that of Alstonine/Serpentine, and that involves the opening of the heterocyclic ring with the oxygen. A comparison between

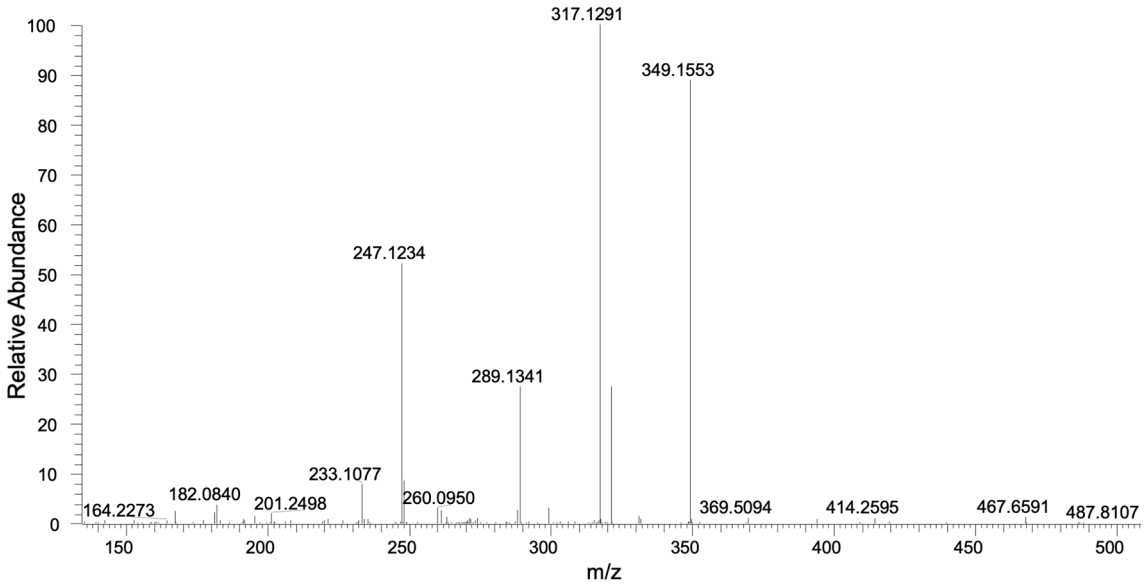


Fig. 12. MS^3 spectrum of the series 511.207 → 349.155.

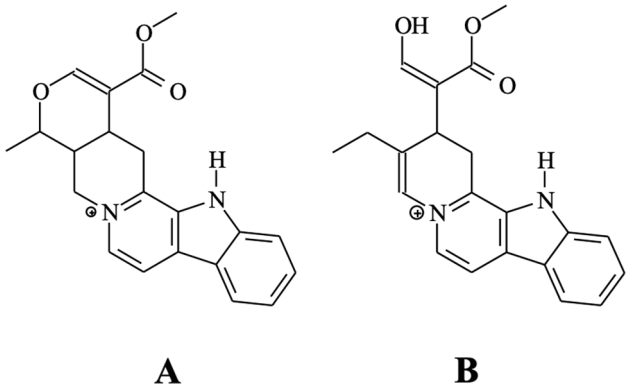


Fig. 13. Comparison of the structure of Serpentine/Alstonine (A) and of the proposed open ring isobar structure (B).

the structures of Serpentine/Alstonine and the proposed isobar structure with the open heterocyclic ring conformation is illustrated in Fig. 13.

Supplementary Information Scheme 13 illustrates the proposed fragmentation paths (based on MS^2 and MS^3 data) of the species of m/z = 511.207 (**5**), that terminate with the formation of the very stable structure of Flavopereirine (m/z = 247.123).

The fact that the two independent species elute at different retention times, but have the same m/z = 349.155 value (Fig. 11) and identical UV spectra must be of some significance.

The species that elutes first is characterized by a product ions pattern coming from the MS^2 precursor ion at m/z 349.155 that overlaps somewhat with the product ions pattern coming from the same species at m/z 349.155, MS^3 precursor ion of the glycosylated molecule (511.207 → 349.155, Fig. 12).

The species that elutes second shows a MS^2 fragmentation pattern quite similar, but exhibits an additional product ion (m/z = 263.082, squared in red) of consistent intensity, as illustrated in Fig. 14.

The high-resolution data show that the product ion of m/z = 263.082

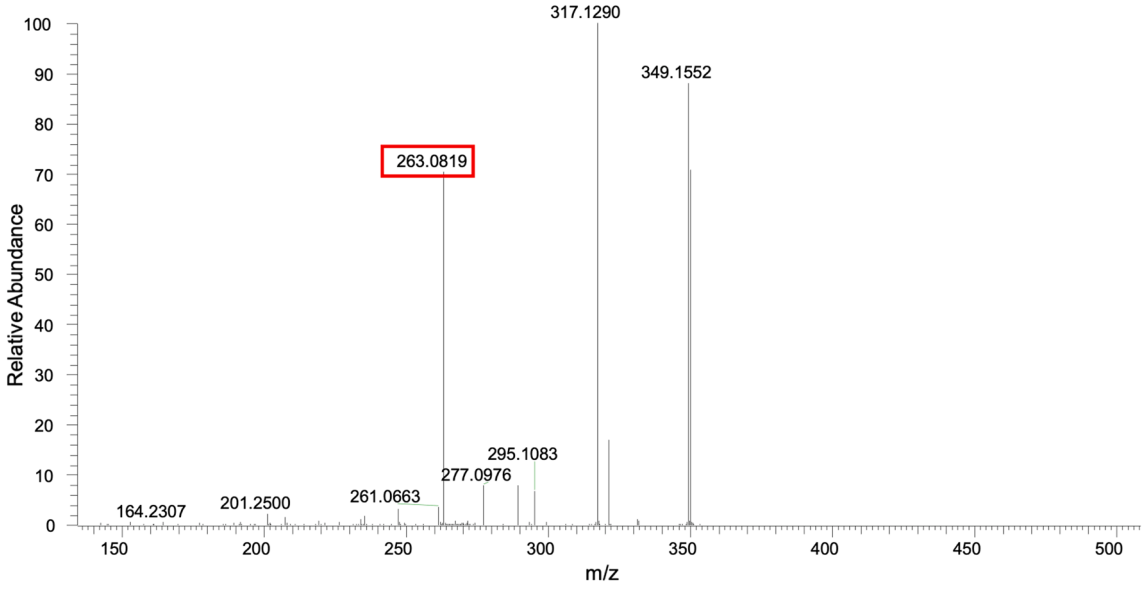


Fig. 14. MS^2 spectrum of the species of m/z = 349.155 with a slightly different fragmentation pattern.

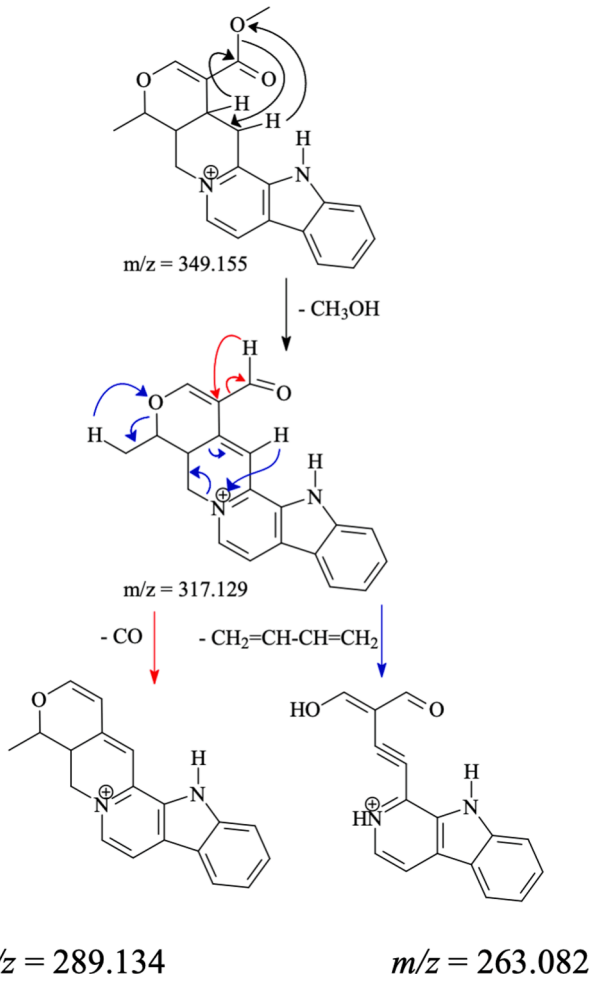


Fig. 15. Fragmentation mechanism of the Serpentine/Alstonine molecule.

comes from the ion of *m/z* 317.129 through a neutral loss of 54.047 u, which corresponds to C_4H_6 (butadiene, Δppm = 0.10). It is also evident that the formation of this product ion occurs at the expense of the lowered production of product ions of *m/z* values 289.134 and 247.123 (Fig. 12). This is justified by the presence of the closed heterocyclic ring that is typical of the structure Serpentine or Alstonine (Fig. 13A). In fact, this is the moiety of both the epimers from which the butadiene structure most likely fragments. Based on this assumption, we can propose a fragmentation mechanism, reported in Fig. 15, in which the fragmentation pathway in blue, which is predominant because of the presence of the heterocyclic ring rather than the open structure, allows the formation of the product ion with the highest π-π electron conjugation system, and therefore most stable..

As a consequence, we hypothesized that the two singly independent molecules of *m/z* = 349.155 were: one that coincides with the product ion from the fragmentation of the glycosylated molecule of *m/z* = 511.207 (**5**), with the heterocyclic ring in an open conformation; and the other with the structure of the epimers Serpentine and Alstonine.

To verify this structural hypothesis, we reanalyzed the bark extract, but spiked it with a known quantity (20 ppm) of Serpentine and Alstonine pure standards. The result was that Serpentine showed an earlier retention time than both of the species of *m/z* = 349.155 (**6**), present in the bark extract, whereas Alstonine enhanced the chromatographic peak of the species that elutes later (Fig. 16).

The coherence of the experimentally obtained results confirmed the structural relationship that exists between the product ion of the glycosylated molecule and the two independently present species, which have, at high resolution, the same *m/z* value. They are most likely involved in a common biosynthetic route. The presence of Alstonine was never previously observed in *Geissospermum* bark extract, although the presence of its epimer Serpentine had been suggested in a previous work [20]. This is another example of the fact that many molecules that come from the MS^2 fragmentation of molecules of a more complex structure (bis-indole or glycosylated) were also present as distinct molecular species in our extract. This further example reaffirms the uniformity of the plant's biosynthetic pathways and helps to define a highly coherent global molecular pattern.

The multistage high-resolution MS spectra of the two other species in this group (*m/z* values 351.171 and 363.170) showed many common product ions that indicate the presence of similar molecular structures.

In particular, the species at *m/z* = 351.171 (**7**) may be considered either an intermediate or a metabolic product of 3′,4′,5′,6′-tetradehydrogeissospermine, as proposed in Supplementary Information Scheme 14.

The species of *m/z* = 363.170 (**8**) shows the same product ions pattern as the species of *m/z* = 349.155, from which it differs by 14 u, which corresponds to a methyl substitution. The methyl substitution is easy to locate as both of the species give the product ion of *m/z* = 317.129 via a neutral loss of methanol and ethanol respectively (Compare Supplementary Information Scheme 13 and Scheme 15).

It is worth noticing that all of these species that have a UV spectrum characterized by the high π-electron conjugation of the indole moiety (compounds **5**, **6**,**7**,**8** and Alstonine reported in Table 4) shows the common final product ion corresponding to Flavopereirine ($C_{17}H_{15}N_2$, *m/z* = 247.123).

All these results are summarized in Table 4.

We paid special attention to the identification of molecules whose presence was suggested, but not with enough reliability, in a previous work [20]; specifically Raubasine ($C_{21}H_{24}O_3N_2$, 352.178 u), Ibogamine ($C_{19}H_{24}N_2$, 280.194 u), Yohimbine ($C_{21}H_{26}O_3N_2$, 354.194 u) and Quebrachamine ($C_{19}H_{26}N_2$, 282.209 u) whose structures are reported in Fig. 17.

The case of the Raubasine molecule is particularly intriguing. We found two important chromatographic peaks of *m/z* = 353.223 (likely epimers, both noted as compound **9**) that do not correspond to the $[M + H]^+$ *m/z* value of Raubasine (353.186) in terms of high resolution. In fact, the calculated elemental composition was $C_{22}H_{28}O_2N_2$, instead of $C_{21}H_{24}O_3N_2$. The UV spectrum (indole chromophore), the elemental composition and its accuracy, the MS^2 and MS^3 data are summarized in the following Table 5. In Supplementary Information Scheme 16, we propose a yet undescribed molecular structure that is in agreement with the detailed product ions pattern that was provided by the experimental mass spectra.

However, although we did not find any signal that could be traced back to Raubasine as such, we did find evidence of its presence as a glycosylated molecule (*m/z* = 515.239, compound **10**). The UV spectrum was typical of an indole group, and the MS^2 data indicated the presence of three product ions the most intense of which was characterized by an elemental composition, evaluated in high resolution ($C_{21}H_{25}O_3N_2$, Δppm = 1.39), compatible with Raubasine's structure. There is, in this case, an analogy to the case of glycosylated Alstonine, as described previously. Moreover, also Raubasine is not in its original conformation, but its heterocyclic ring must be in an open conformation in order to allow bond formation with the hexose molecule to occur. The comparison between the two isobar conformations is illustrated in Fig. 18.

All high-resolution MS data are reported, in Supplementary Information Scheme 17, together with the proposal of detailed product ions formations that confirms the plausibility of the identification of Raubasine in its open conformation. Furthermore, it should be noted that the two product ions, of *m/z* values 323.176 and 337.191 derived from Raubasine fragmentation, are isobars with the independently present species that were discussed, and reported, in Table 2. In particular, the species with the same *m/z* = 337.191 value exhibited identical product

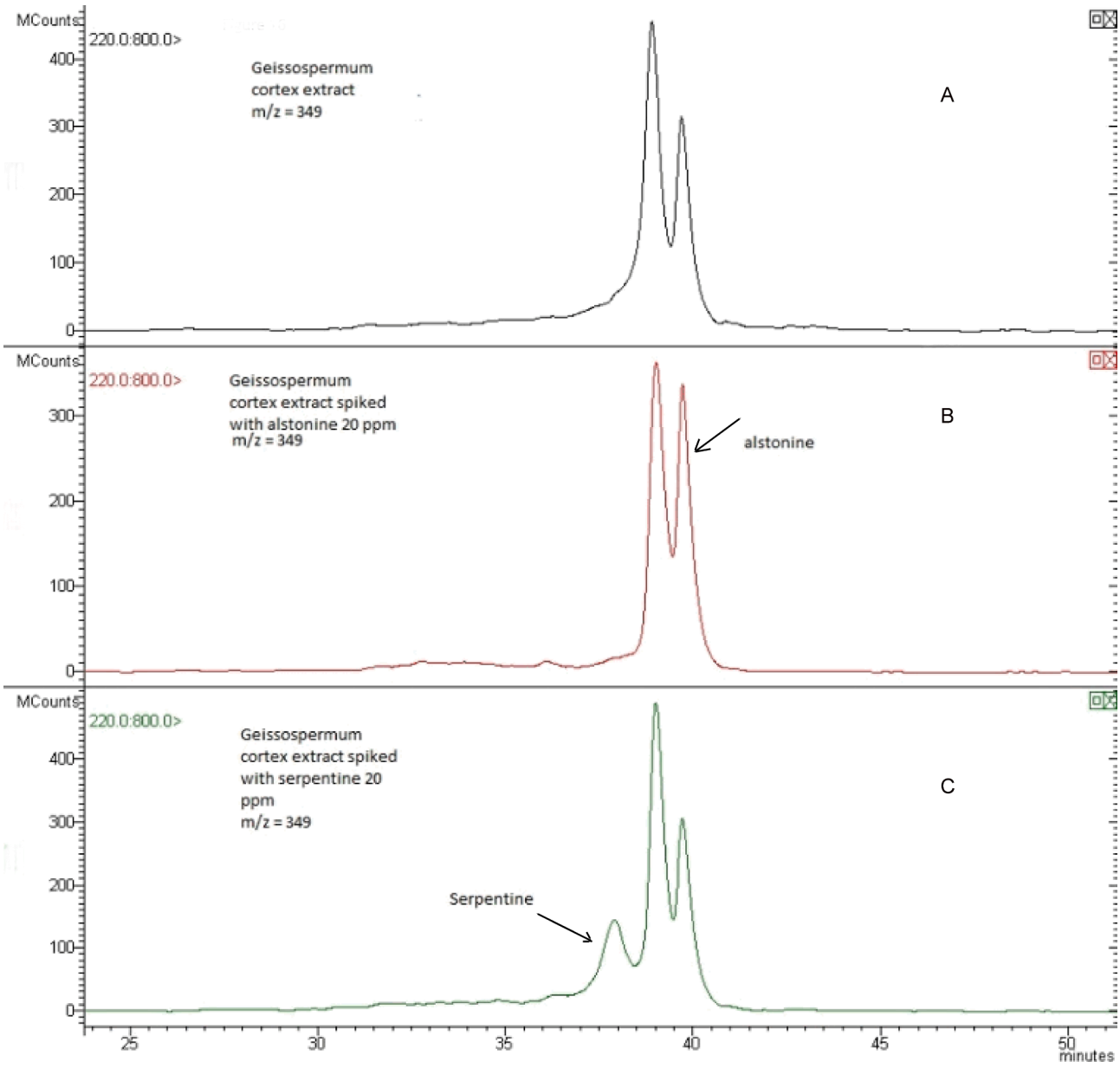


Fig. 16. Extracted ion chromatogram ($m/z = 349$), zoomed in at the time interval in which the peaks of interest are eluting, of Geissospermum bark extract analyzed as such (A), spiked with 20 ppm of Alstonine (B) and 20 ppm of Serpentine (C).

ions formation, whereas the species with the same $m/z = 323.176$ value showed different product ions formation, which is likely due to the different position of the –OH group (Supplementary Information Schemes 10 and 11).

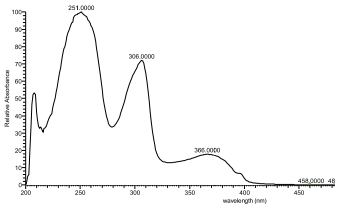
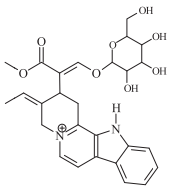
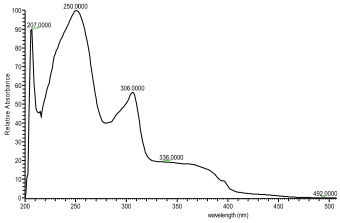
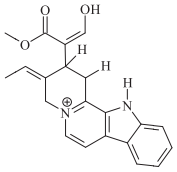
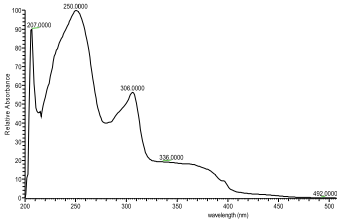
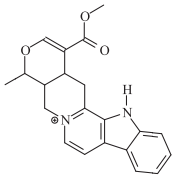
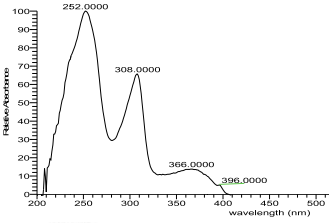
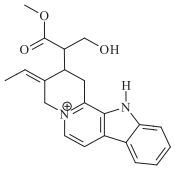
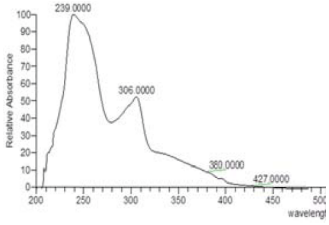
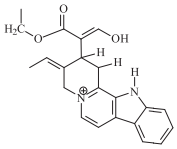
The high-resolution evaluation of the elemental composition of $[M + H]^+$ precursor ions of Ibogamine, Yohimbine and Quebrachamine were satisfactorily accurate (Δppm values were 2.079, 2.423 and 0.685 respectively). The molecular structure, the elemental composition and the accuracy of their evaluation, and the MS^2 and MS^3 data are reported in Table 6.

A thorough interpretation of these data (Supplementary Information Schemes 18–23) further strengthens the preliminary identification that was based on elemental composition and substantially increases the likelihood of identification. The global fragmentation patterns are complex and articulate, and MS^3 spectra play a fundamental role; first, in rationally organizing several MS^2 product ions, and, secondly, in introducing other product ions that are often useful for further confirming the hypothesis of the molecular structure.

Finally, we tried to characterize the molecular structure of the doubly charged precursor ion of $m/z_{(z=2)}$ value 294.192. Its UV absorption spectrum has a shape quite similar to that of a bis-indole species. MS^2 experiments provided a couple of singly-charged product ions of m/z values 309.197 and 279.186, whose sum corresponds to the doubly charged molecular ion $[M + 2H]^{2+}$ of $m/z = 588$. The first product ion was already found in the MS^2 experiments of both Geissospermine and Geissolosimine as illustrated above (Fig. 4), and has been already characterized. The product ion at $m/z = 279.186$ ($C_{19}H_{27}N_2$, $\Delta ppm = 1.199$) is a new entry and some clues as to its structure may come from the similarity of the MS^3 spectrum $294.192(z = 2) \rightarrow 279.186$ and the MS^2 spectrum of Ibogamine ($m/z = 281.202$). In fact, product ions are present that differ from those obtained for Ibogamine by two units (more specifically the m/z values 250.195, 248.144, 136.112 for comparison with those reported in Table 6). The relative proposed product ions patterns are reported in Supplementary Information Scheme 24.

On this basis, we can propose the molecular structure of the bis-

Table 4
UV absorption spectra, mass spectrometry data and proposed structures for the newly described species (except Alstonine) that are strictly related to the 3′,4′,5′,6′-tetradehydrogeissospermine molecule.

COMPOUNDS NUMBER Elemental formula	$[M+H]^+$ *m/z* value Elemental formula Accuracy	UV–VIS SPECTRUM	MS^2 PRODUCT IONS (RELATIVE INTENSITY %)	MS^3 PRODUCT IONS (RELATIVE INTENSITY %)	PROPOSED STRUCTURE
5 Tetradehydrogeissospermine related $C_{27}H_{30}O_8N_2$	*m/z* = 511.207 $C_{27}H_{31}O_8N_2$ Δppm= -0.36		349.155 (100)	511.207 → 349.155317.129 (100)247.123 (53)289.134 (29)321.160 (27)	 Δppm= 1.62
6 Tetradehydrogeissospermine related $C_{21}H_{20}O_3N_2$	*m/z* = 349.155 $C_{21}H_{21}O_3N_2$ Δppm= 1.29		317.129 (100) 247.123 (58)289.133 (30)	349.155 → 317.129289.133 (100)247.123 (99)299.118 (16)261.138 (13)	
Alstonine Tetradehydrogeissospermine related $C_{21}H_{20}O_3N_2$	*m/z* = 349.155 $C_{21}H_{21}O_3N_2$ Δppm= 1.12		317.129 (100) 263.082 (73)	349.155 → 317.129263.082 (100)289.133 (44)	
7 Tetradehydrogeissospermine related $C_{21}H_{22}O_3N_2$	*m/z* = 351.170 $C_{21}H_{23}O_3N_2$ Δppm= 1.14		321.160 (100) 247.123 (23)233.740 (23)289.134 (20)	351.170 → 321.160289.134 (100)247.123 (26)261.139 (16)	
8 Tetradehydrogeissospermine related $C_{22}H_{22}O_3N_2$	*m/z* = 363.170 $C_{22}H_{23}O_3N_2$ Δppm= 1.68		335.139 (100) 317.129 (87)247.123 (38)291.149 (22)289.134 (16)307.144 (10)	363.170 → 335.139317.129 (100)291.149 (80)247.123 (51)307.144 (40)289.134 (30)	363.170 → 313.17247.123 (100)289.134 (80)	

indole species of $m/z_{(z=2)} = 294.192$ as a union of Ibogamine and the species of $m/z = 309.197$, which has already been well characterized. Its fragmentation can lead to the formation of dehydro-ibogamine, according to the scheme in Fig. 19.

In this case the indoline moiety seems to participate in the building up of a possible bis-indole molecular structure not in the form that was already found in Geissospermine and Geissolosimine (Fig. 1. A, B, Supplementary Information 1, 2). Two clues seem to favor this molecular structure: the first is the impossibility of justifying the formation of the obtained MS^2 product ions using the indoline moiety in the form present in the cited bis-indole molecules; the second is the fact that the estimated ring plus double bond value is 19, as can be verified from the elemental composition, whereas it would be 18 if the combination was as identified in the other bis-indole molecules.

All these results are summarized in Table 7.

4. Conclusions

The chromatographic separation of *Geissospermum vellosii* bark extract has been satisfactorily performed. It is the first time that a

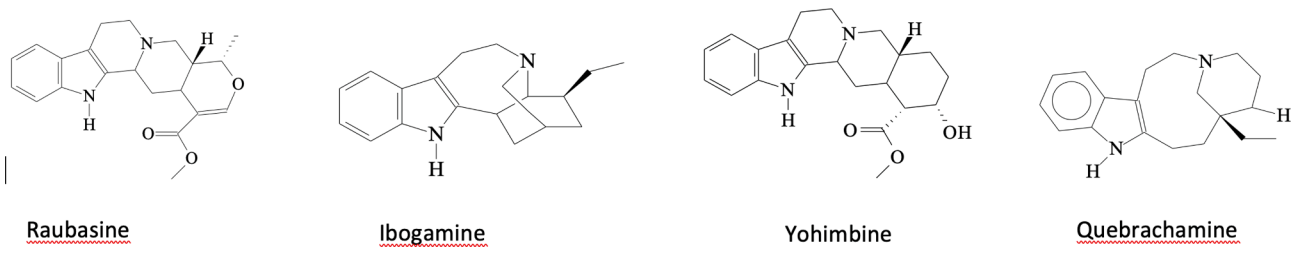
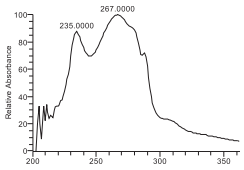
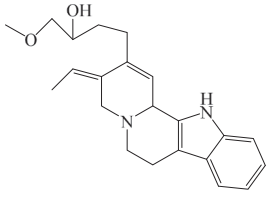


Fig. 17. Structure formulae of alkaloids suggested as possibly being present in Geissospermum bark.

Table 5
UV absorption spectra, mass spectrometry data and proposed structure for the newly described structure.

COMPOUND NUMBER Elemental formula	$[M + H]^+$ *m/z* value Elemental formula Accuracy	UV–VIS SPECTRUM	MS^2 PRODUCT IONS (RELATIVE INTENSITY %)	MS^3 PRODUCT IONS (RELATIVE INTENSITY %)	PROPOSED STRUCTURE
9 $C_{22}H_{28}O_2N_2$	$C_{22}H_{29}O_2N_2$ $m/z = 353.223$ Δppm= 1.28		MS^2 product ions335.212 (100)307.181 (59)150.091 (51)279.186 (46)289.171 (38)	MS^3 product ions 353.223 → 335.212289.171 (100)150.091 (50)178.123 (19) 353.223 → 307.181150.091 (100)176.107 (25)144.081 (21) 180.102 (100)305.165 (20)	

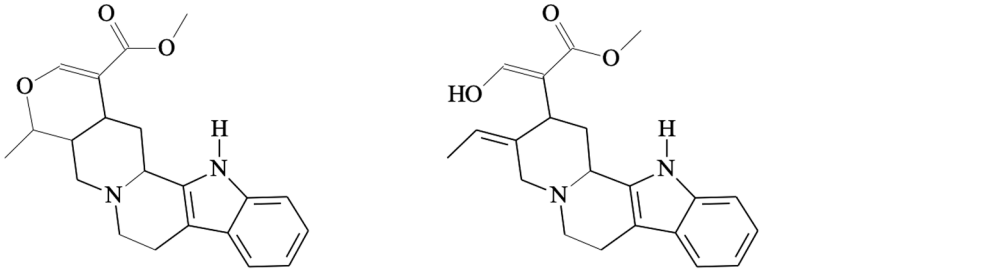


Fig. 18. Comparison between the two isobar conformations.

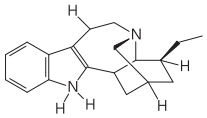
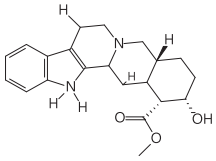
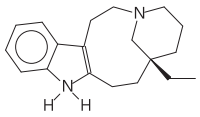
phytochemical profile of this plant has been obtained after a relatively simple extraction procedure and starting from a small amount of raw material. It was evident that the obtained chromatographic resolution was not compatible with a purification process that could allow the subsequent use of NMR to further confirm the proposed structure of all the constituents.

So we had to face the general question of the ability of high resolution mass spectrometry, in suitable and well defined conditions, to provide information about molecular structures. The suitable and well defined conditions may be represented by the fact that we are dealing with a vegetal material whose composition in indole alkaloids is the final effect of very homogeneous, genetically ruled, biosynthetic routes producing molecules structurally highly interrelated.

Starting from the known structures of a number of *Geissospermum vellosii* alkaloids, we could verify that the application of multistage HRMS and UV–Vis Diode Array spectrophotometry allowed to obtain data according in all details with them. The extension, on this basis, of the cited detection techniques to the unknown alkaloid species present in the bark extract, in view of the assumption (later confirmed) of their close structural relationship, allowed us to propose for them plausible structures.

It is worth noting that the MS^3 spectra were important investigation tools which had the double role to select between possible alternative hypothesis of MS^2 fragmentation pathways and to provide new product

Table 6
Mass Spectrometry data obtained for the known structures of Ibogamine, Yohimbine and Quebrachamine, which had never been reported in the *Geissospermum* genus.

	MS^2 PRODUCT ION (% RELATIVE INTENSITY)	MS^3 PRODUCT ION (% RELATIVE INTENSITY)		
 Ibogamine $C_{19}H_{25}N_2$ $m/z = 281.202$ $\Delta ppm = 2.079$	138.128 (100)236.144 (54)252.175 (25)250.159 (19)264.175 (11)	281.202 → 236.144182.0967 (100)194.097 (57)221.103 (46)208.125 (25)	281.202 → 252.175196.112 (100)236.144 (95)144.081 (74)210.128 (69)182.0967 (60)170.096 (43)	281.202 → 250.159221.120 (100)235.136 (94)208.112 (73)194.097 (46)
 Yohimbine $C_{21}H_{26}N_2O_3$ $m/z = 355.202$ $\Delta ppm = 2.079$	355.202 (100)251.155 (91)212.128 (74)224.128 (63)338.176 (50)144.081 (37)326.176 (34)337.191 (27)	355.203 → 338.176234.128 (100)308.165 (66)320.165 (53)276.139 (41)260.144 (28)	355.203 → 251.155144.081 (100)108.081 (45)	355.203 → 224.128192.102 (10)206.118 (55)122.096 (38)164.107 (30)
 Quebrachamine $C_{19}H_{27}N_2$ $m/z = 283.218$ $\Delta ppm = 0.685$	238.159 (100)240.175 (73)184.112 (66)144.081 (40)210.128 (33)255.186 (29)252.175 (25)	283.218 → 238.159196.112 (100)182.097 (91)209.120 (39)144.081 (11)	283.218 → 240.175170.097 (100)198.128 (49)	283.218 → 255.186238.160 (100)184.112 (47)182.097 (37)212.144 (16)

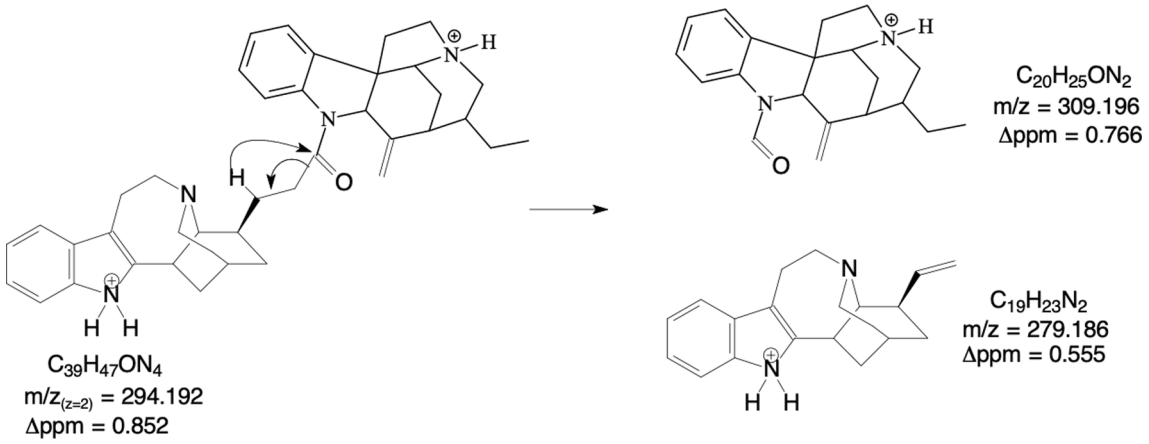


Fig. 19. Proposed structures for the doubly charged species of m/z (z = 2) = 294.192 and its MS2 product ions.

ions useful to support the molecular structure hypotheses. The accurate elemental composition and ring plus double bonds values together with UV–Vis Diode Array spectra completed the set of data whose informational content was converging towards the identification of 10 not yet described alkaloid molecules.

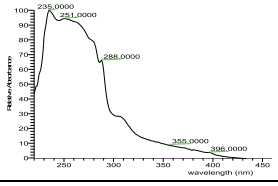
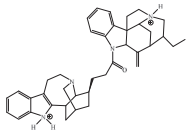
The presence of six other substances (Alstonine, Tabersonine, Raubasine, Ibogamine, Yohimbine and Quebrachamine), not previously reported in the *Geissospermum* genus (even if suggested as being present [21]), was confirmed using a detailed match between spectrometric and spectrophotometric experimental data and the known molecular structure of these molecules.

From this point of view, the successful identification of Alstonine was paradigmatic. Although this molecule had never previously been reported as a component of *Geissospermum vellosii* bark extract, all of the experimental data we accumulated could be traced back to the structure of the known epimers Alstonine/Serpentine. The use of pure standards allowed us to confirm the accurate identification and overcome the ambiguity.

As a matter of fact the correct identification of epimers does depend exclusively on the disposal of pure standards, often not easily available on the market.

In conclusion, all of the proposed molecular formulae are the results

Table 7
UV and MS data of the identified double charged bis-indole species of $m/z_{(z=2)} = 294.192$.

COMPOUND NUMBER ELEMENTAL FORMULA	ACCURATE $m/z_{(z=2)}$ VALUE	UV–VIS SPECTRUM	PRODUCT IONS MS^2 (RELATIVE INTENSITY %)	PRODUCT IONS MS^3 (RELATIVE INTENSITY %)	PROPOSED STRUCTURE
10 $C_{39}H_{46}ON_4$	$m/z_{(z=2)} = 294.192$ $C_{39}H_{48}ON_4$ $\Delta ppm = 0.956$		309.197 (78)279.186 (100)	294.192 → 309.197201.398 (100)182.096 (93)281.201 (93)172.075 (74)253.134 (46)	

of inference from a large amount of data, including UV spectra and multistage high-resolution mass data. As we have shown in a detailed manner, there was a congruence in the high-resolution mass spectrometry results, also in terms of common product ions from different precursors and the plausibility of neutral losses.

It must be underlined that we did not obtain reliable evidence of the presence of some previously characterized species such as Geissolaevine, Methyl-Geissolaevine, Pausperadine and Geissovelline. However, they exhibit structures that are quite different to those of the other species present, particularly in terms of oxygen-atom number and type of bonds involved (see Fig. 1G and Fig. 3). Their absence is not justifiable in terms of difference in solubility as all of the substances that had already been identified in literature were found in our extract, and the polarity of these species is quite comparable. One experimental aspect we may underline is the difference in the extraction techniques used in previous bark composition studies and this work. We have used a very soft (ethanol–water 70–30) extraction system that was able to maintain the integrity of the original composition. The majority of the previous works used, at least in some step, lipophilic solvents and heavy extract basification [14–17], conditions that may induce the oxidation or hydrolysis of the more labile bonds. Another point of uncertainty is the lack of information on the possible differences in the harvest time of Geissospermum bark and its possible influence on constituent composition.

The alkaloid rich bark of *Geissospermum vellosii* is surely an important reservoir of potentially bioactive substances, as moreover demonstrated by studies of the efficacy of single purified constituents in cancer and other pathologies therapy [2–12]. The fingerprinting of the bark detailed in the present work is improving the chemical knowledge of the bark content. In the light of the fact that selected purified fractions could show important bioactivity features or that the entire phytocomplex could demonstrate enhance effects in respect to single purified substances we hope that the results here presented might be of help in the deepening of this type of investigations.

CRediT authorship contribution statement

Riccardo Aigotti: Methodology, Investigation, Formal analysis. **Valentina Santoro:** Investigation, Formal analysis, Visualization. **Daniela Gastaldi:** Investigation, Methodology, Writing – review & editing. **Michael Zorzi:** Formal analysis. **Federica dal Bello:** Formal analysis, Investigation. **Maurizio Grandi:** Resources, Supervision, Conceptualization. **Claudio Baiocchi:** Supervision, Conceptualization, Writing – review & editing.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary material

Supplementary data to this article can be found online at https://doi.org/10.1016/j.jchromb.2022.123307.

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