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Characterization of the variation in the imidazole alkaloid profile of *Pilocarpus microphyllus* in different seasons and parts of the plant by electrospray ionization mass spectrometry fingerprinting and identification of novel alkaloids by tandem mass spectrometry

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Pilocarpus microphyllus* (Rutaceae), popularly known as jaborandi, is the only commercial source of an imidazole alkaloid named pilocarpine. In the present study, the variation in the profile of imidazole alkaloids in different seasons and in different parts of the *P. microphyllus* plant during the summer was analyzed by electrospray ionization mass spectrometry in the positive ion mode [ESI(+)-MS]. The fingerprints of these extracts repeatedly presented similar ions which were mass-selected and studied by tandem mass spectrometry (ESI-MS/MS and ESI-MS/MS/MS) and high-resolution mass spectrometry, resulting in the characterization of eight imidazole alkaloids. The data from the ESI(+)-MS fingerprints were analyzed by principal component analysis (PCA), showing that pilocarpine was present mainly in the summer, whereas in the autumn mainly pilosine and winter anhydropilosine were found. Three alkaloids, reported for the first time in extracts of *P. microphyllus*, were found. Analysis of the distribution of alkaloids in different parts of the plant during the summer showed that, although pilocarpine was present throughout the plant, 13-nor-8(11)-dihydropilocarpine was found mainly in the stem, pilosine and anhydropilosine were present mainly in the intermediary leaves, and the three new alkaloids were mainly found in the leaflets and petioles. Based on the dissociation patterns of these alkaloids, we observed that there were three structurally related groups of alkaloids differing in their distribution in the plant tissues and responding differently to seasonal variations. These results also indicate that these three groups of alkaloids could belong to intermediate, parallel or competitive pathways for pilocarpine formation biosynthesis. Copyright © 2007 John Wiley & Sons, Ltd.

Pilocarpus microphyllus (Rutaceae) is widely distributed in the northern region of Brazil,[1] where it is popularly known as jaborandi. Pilocarpine, an important imidazole alkaloid,[2] is extracted from the leaves of jaborandi, and has been used mainly for the treatment of glaucoma since it reduces intraocular pressure;[3] however, it also stimulates sweat and lachrymal glands and controls xerostomia.[4,5] Jaborandi is the main commercial source of pilocarpine; however, exploitation of native plants without reforestation between 1975 and 1994 has brought this species to the brink of extinction.[6]

Jaborandi leaves also contain other imidazole alkaloids, such as pilosine, pilosinine, pilocarpidine, anhydropilosine and 13-nor-8(11)-dihydropilocarpine, whose pharmacological and physiological properties are still unknown.[7–10] The presence of pilocarpine and pilosine in jaborandi was first described by Felter and Lloyd in 1898,[11] although their complete configuration was determined many years later.[7,8,12] Brochmann-Hanssen *et al.*[13] and Cordell[14] suggested that the biosynthesis of pilocarpine might have histidine and histamine as precursors, although this hypothesis has not yet been confirmed. Since pilocarpine is of great interest to pharmaceutical industries, knowledge of the biosynthetic pathways leading to its production would allow us to study its metabolic regulation in the plant and to act trying to favor pilocarpine production over the other alkaloids. Information about possible biosynthetic pathways could ideally be obtained by characterizing other pilocarpine-related

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alkaloids produced by this plant and the factors that affect their production. This profiling could indicate important correlations between these alkaloids and the establishment of their place in pilocarpine biosynthesis.

In our studies we observed that the pilocarpine content in jaborandi varies in response to abiotic factors[15,16] and with the time of year (I.N. Abreu and co-workers, unpublished data). Nevertheless, it is not known if the other imidazole alkaloids found in this plant species also vary throughout the year, as no commercial standards of the alkaloids are available. The seasonal variation in the alkaloid content in other plant species has been previously demonstrated[17] as has the influence of factors which are inherent to the age of the tissues, the part of the plant as well as genetic factors.[18,19]

Direct infusion electrospray ionization mass spectrometry (ESI-MS) has been previously and successfully applied as a fast fingerprinting method for complex mixtures such as plant extracts,[20] propolis,[21] beer,[22] whisky,[23] wine,[24] and vegetable oils.[25] High-resolution ESI-MS and tandem mass spectrometry (MS/MS) have both been used for the direct, on-line characterization and identification of compounds in propolis.[26] We therefore tested the ability of direct infusion ESI-MS to provide a detailed snapshot of the alkaloid composition of jaborandi during different seasons and in different parts of the plant, and tandem mass spectrometry (ESI-MS/MS and ESI-MS/MS/MS) for the characterization of the structures of these alkaloids.

EXPERIMENTAL

Mass spectrometry

Approximately 10 μL of the extracts were dissolved in 1 mL of a solution of equal parts of water and CH_3CN containing 0.1% of HCO_2H for mass spectrometric analysis.

All ESI mass spectra and tandem mass spectra were acquired in the positive ion mode on an Applied Biosystems Q-trap mass spectrometer (Foster City, CA, USA) comprising a triple quadrupole and a linear ion trap, over a mass range of *m*/z 50–400. The operational parameters used for acquiring the ESI mass spectra were: capillary 5000 V, temperature 200°C, declustering potential 70 V, and entrance potential 6 V. For the ESI-MS/MS and ESI-MS/MS/MS experiments the above conditions were maintained and the collision energy was optimized, in the range between 15 and 30 V, for each protonated alkaloid or product ion analyzed. Nitrogen was used as the curtain gas (20 psi), nebulizing gas (20 psi) and collision gas (medium – arbitrary setting). Standards and extracts were injected directly into the ESI source by a syringe pump at a flow rate of 10 $\mu L.min^{-1}$.

High-resolution positive ion ESI mass spectra were acquired on a Micromass Q-Tof mass spectrometer (Manchester, UK), over a mass range of *m*/*z* 50–400, under the following operational parameters: capillary 3000 V, cone 30 V, extractor 0 V, temperature 120°C, with nitrogen as nebulizing gas. Standards and extracts were injected directly into the ESI source by a syringe pump at a flow rate of 10 $\mu L.min^{-1}$.

Plant material and alkaloid extraction

Intermediary leaves (third leaf from the apex) of *P. microphyllus* (in adult stage of development, kept in greenhouse conditions) were collected in different seasons: spring (October), summer (January), autumn (May) and winter (July). As the studies with these samples indicated that in the summer there was a greater production of pilocarpine, different parts of the plant were collected at this period of the year: flower bud, flower, leaflet, young leaf, adult leaf, petiole of adult leaf, apical stem, intermediary stem, basal stem and root. In all cases, samples were taken from three different plants and extractions performed separately. The plant material was extracted according to the method of Avancini *et al*.[15] Dried leaves (50 mg) were wetted with two drops of 10% NH_4OH, and then a 15 min extraction was carried out with $CHCl_3$ (0.3 mL) with vigorous shaking for 1 min. The $CHCl_3$ was recovered by centrifugation and the precipitate re-extracted with $CHCl_3$. The $CHCl_3$ fractions were pooled and extracted with 2% H_2SO_4 (0.3 mL × 2). The pooled acid extracts were neutralized to pH 12 with NH_4OH and extracted twice with 0.3 mL $CHCl_3$. The organic fractions were pooled, dried in a Speed-vac, dissolved in purified water and analyzed by mass spectrometry.

Chemicals

Pure pilocarpine was purchased from Sigma (St. Louis, MO, USA) and a standard of pilosine was obtained from the Merck Pharmaceutical Company (Rio de Janeiro, Brazil). Solvents used for the extraction of the leaves of *P. microphyllus* were of analytical grade (Merck, Darmstadt, Germany). For the ESI-MS analyses the solvents used were HPLC-grade CH_3CN (Tedia, Fairfield, OH, USA), analytical-grade formic acid (Merck, Darmstadt, Germany), and water was purified using a Milli-Q water purification system (Millipore S.A.S., Molsheim, France).

Statistical analysis

Principal component analyses (PCAs) were performed with the SIMCA – P software (version 11.0, Umetrics, Umeå, Sweden). The mass spectra were expressed as the signal intensities of individual $[M+H]^+$ ions, for all ions with intensities of 10% or more in the spectrum of each sample.

RESULTS AND DISCUSSION

Comparison of ESI-MS fingerprints of samples from different seasons and plant parts

Variation in the pilocarpine content in jaborandi has been observed at different times of the year and in different parts of the plant. However, as no commercial standards of the other alkaloids present in the extracts of this species are available it was not known if the other alkaloids also vary throughout the year, in the same way as pilocarpine (**1**). Therefore, in the present study, the profiles of all the alkaloids found in the foliar extract of *P. microphyllus*, in different seasons of the year, were studied by ESI-MS fingerprinting, followed by fingerprinting of different parts of the adult plant in the summer; examples are presented in Fig. 1.

Figure 2 presents the PCA of the fingerprints of foliar extracts in different seasons of the year. PC1 is responsible for 62% of the observed variation and separates the leaves

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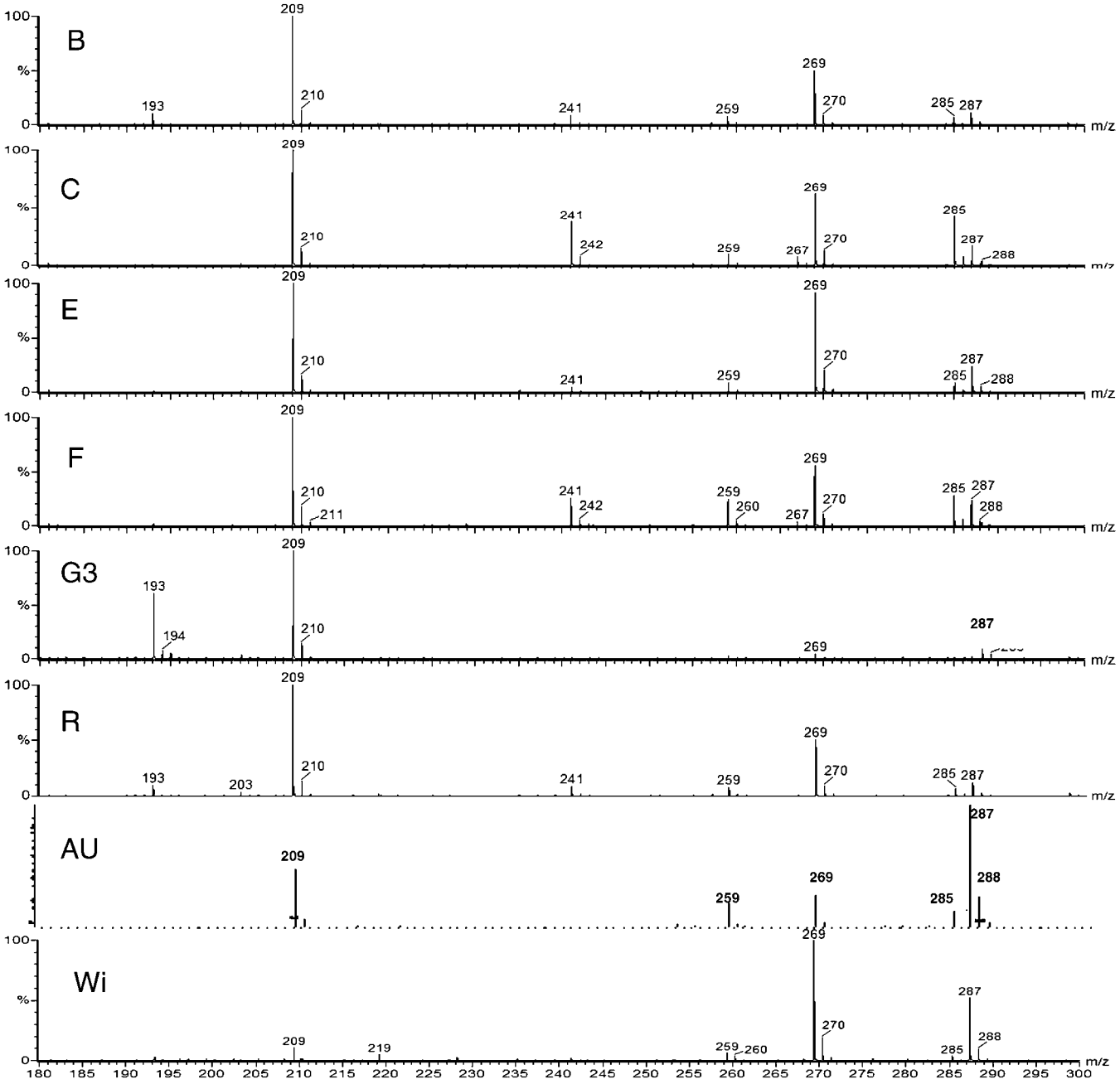

Figure 1. ESI(+)-MS fingerprints of extracts of *P. microphyllus* during the summer: (B) flower; (C) leaflet; (E) adult leaf; (F) petiole of adult leaf; (G3) basal stem; (R) root. *P. microphyllus* adult leaf during the autumn (AU) and winter (Wi).

collected in the summer, when pilosine (**2**, *m*/*z* 287) and anhydropilosine (**3**, *m*/*z* 269) are not predominant, from leaves collected in the winter with high content of anhydropilosine (**3**, *m*/*z* 269). PC2 accounts for 27% of the variation and separates the autumn samples, due to the presence of pilosine (**2**, *m*/*z* 287), from the other samples. Spring samples are placed in an intermediate position between winter and summer samples. Furthermore, the fingerprints of the summer samples contain more intense ions corresponding to 13-nor-8(11)-dihydropilocarpine (**4**) or its isomer, 13-nor-7(11)-dihydropilocarpine (*m*/*z* 193), **1** (*m*/*z* 209), and *m*/*z* 241 (**5**), 259 (**6**) and 285 (**7**), corresponding to compounds not previously found in jaborandi leaves.

These results indicate that, depending on the season, distinct biosynthetic pathways predominate. The structures of the pilocarpine-related alkaloids found in these extracts were determined by comparison of their tandem mass spectrometry dissociation patterns with those of standards of pilocarpine and pilosine and confirmed by high-resolution mass measurements, as detailed later in this discussion.

P. microphyllus is a species originally from the Amazon region in Brazil (latitude approximately −3°), where the seasons are not well defined, with very small oscillations in temperature (with an annual average of 28°C) and high rainfall. The present study, however, was carried out in the southeastern region of Brazil (latitude −23.54°), where there are well-defined seasonal changes, with great differences in the temperature and rainfall regime during the year. Therefore, we do not exclude the possibility that the results presented might be partially influenced by the regional

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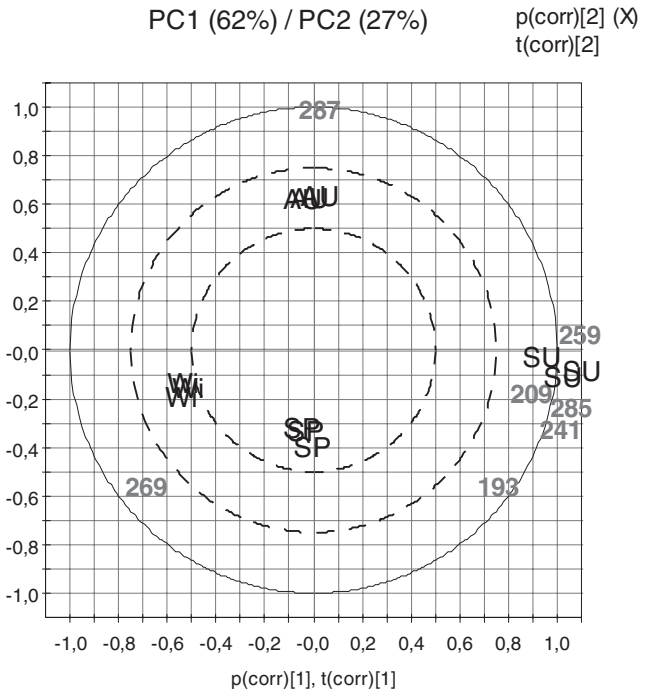

Figure 2. PCA analysis of the data from ESI(+)-MS fingerprints of the extracts of intermediary leaves of *P. microphylus* collected in the spring (SP), summer (SU), autumn (AU) and winter (Wi). Score plot and loading plot of PC1 × PC2. Data of the three replicates are displayed.

climate conditions and further comparative studies with plants collected in the Amazon region could clarify this point. The genus, *Pilocarpus*, contains approximately ten species with ample distribution throughout Brazil,[27] whose pilocarpine contents vary widely. Of course, genetic and environmental factors could influence the production of pilocarpine in each species in several ways that extend beyond the scope of this study, presenting an interesting new line of research.

After determining that the production of **1** occurs mainly in the summer, different parts of adult plants (flower bud, flower, leaflet, petiole, young leaf, intermediary leaf, apical stem, medial stem, basal stem and root) were collected in the summer and extracted for alkaloids. These extracts were analyzed by ESI-MS (Fig. 1) and the results compared by PCA, as shown in Fig. 3. PC1 shows that the presence of **4** (*m*/*z* 193) in the stem samples (G1, G2 and G3) was responsible for separating these samples from the other parts of the plant (Figs. 3(A) and 3(B)). PC2 separated the intermediary leaves (E) from the rest of the plant due to the presence of **3** (*m*/*z* 269) and **2** (*m*/*z* 287). In Fig. 3(B), one observes that the presence of **5** and **7** (*m*/*z* 241 and 285) were characteristic of the leaflets (F) and petioles (C). **1** (*m*/*z* 209) was present in all the parts of the plant and therefore was not a differential factor in this analysis.

Studies of the cellular localization of alkaloids in different plant species have demonstrated that biosynthetic enzymes may occur in distinct types of cells, suggesting that the transport of biosynthetic intermediates occurs.[28] Based on

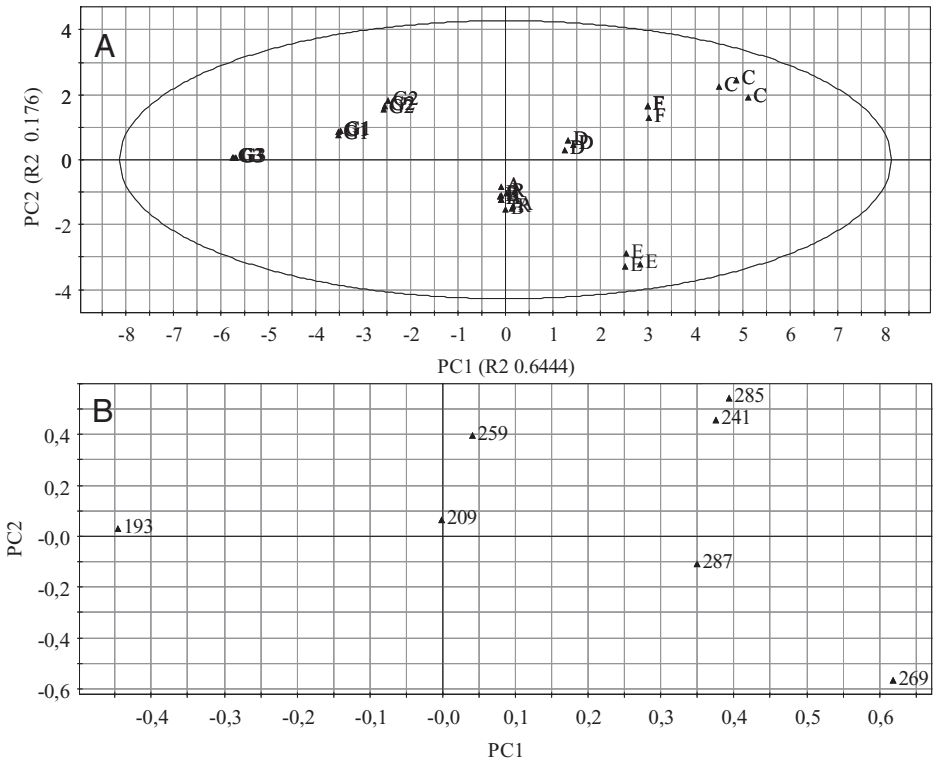

Figure 3. Score plot (A) and loading plot (B) of the PCA analysis of the data from ESI(+)-MS fingerprints of extracts of various parts of *P. microphyllus* during the summer*:* A, flower bud; B, flower; C, leaflet; D, young leaf; E, adult leaf; F, petiole of adult leaf; G1, G2 and G3, stems (apical, intermediary, basal); R, root. Data of the three replicates are displayed.

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these premises, it was essential to know the structure of all the alkaloids present in the plant extracts studied in order to determine how they are related and if they belong to the same or related biosynthetic pathways. For the characterization of their structures, each protonated molecule was therefore mass-selected and subjected to collision-induced dissociation (CID) MS/MS.

ESI-MS^n analysis of alkaloids found in the extracts

Initially, we dissolved the standards of **1** and **2** in the same solution used for the extracts, infused these solutions into the ESI source, and selected their protonated molecules for CID using the Q-trap mass spectrometer. The analyses of the dissociation patterns of the other alkaloids are based on key information obtained from the ESI-MS/MS data of these two standards. Note that the product ion spectra of protonated **1** and **2** obtained directly from the plant extract were identical to those of the standards, and can be observed in Figs. 4(A) and 4(B).

Figure 4(A) shows the product ion spectrum of $[\mathbf{1}+H]^+$. The main product ion at *m/z* 95 is important since it reveals the presence of the 1-methyl-5-imidazoylmethyl group. This diagnostic ion is seen in all product ion spectra of the protonated alkaloids extracted from pilocarpine that contain this group. The *m/z* 95 ion is also observed as the main product ion in the dissociation of epiisopilosine (an isomer of pilosine), as reported by Tedeschi *et al.*[12] Other important

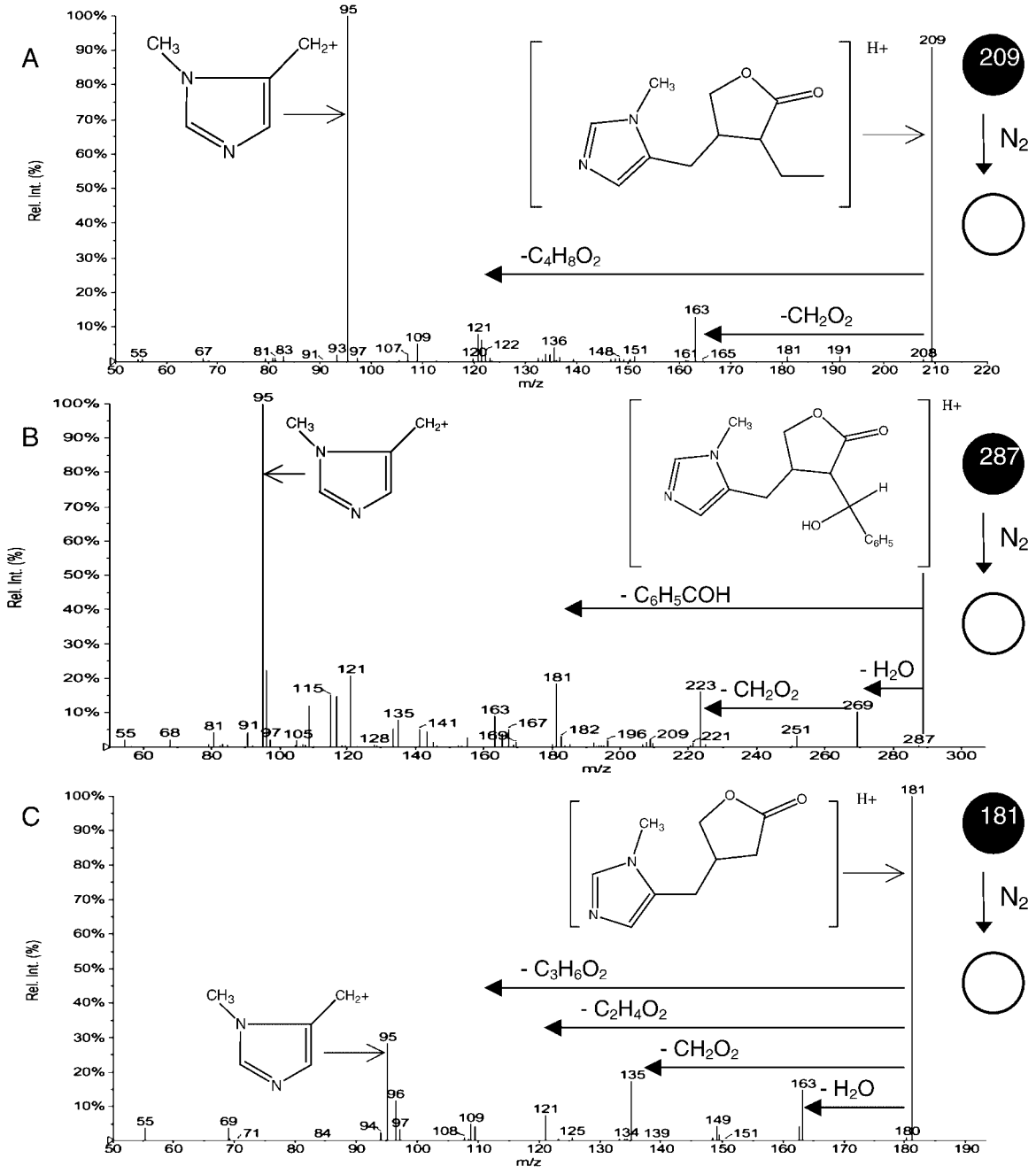

Figure 4. ESI product ion spectra of (A) protonated pilocarpine (**1**, *m/z* 209); (B) protonated pilosine (**2**, *m/z* 287); and (C) protonated pilosinine (**8**, *m/z* 181).

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product ions in Fig. 4(A) result from neutral loss of CH_2O_2 (*m/z* 163) and $C_4H_8O_2$ (*m/z* 121). The dissociation pattern of $[\mathbf{1}+H]^+$ is similar to that reported by Van der Merbel *et al.*[29]

Figure 4(B) shows the product ion spectrum of $[\mathbf{2}+H]^+$ and the main product ion is, again, the diagnostic 1-methyl-5-imidazoylmethyl ion of *m/z* 95. Protonated pilosine also loses C_6H_5COH to form *m/z* 181. This ion was also mass-selected and further dissociated via ESI-MS/MS/MS, and comparison of its spectrum with the product ion spectrum of *m/z* 181 found in the extract (Fig. 4(C)) indicates that both have the same structure, which is compatible with the structure of the alkaloid pilosinine (**8**). In addition to the diagnostic *m/z* 95 ion, the product ions observed in both cases result from neutral losses of water (*m/z* 163), CH_2O_2 (*m/z* 135), $C_2H_4O_2$ (*m/z* 121) and $C_3H_6O_2$ (*m/z* 109). These ions can also be observed in the product ion spectrum of $[\mathbf{2}+H]^+$.

The dissociation patterns of other protonated alkaloids detected in the ESI-MS fingerprinting of the jaborandi extract were then compared with the dissociation patterns of protonated **1**, **2** and **8**. Note that in aqueous solutions pilocarpine can also epimerize to isopilocarpine, which hydrolyzes to isopilocarpic acid.[29] Using direct infusion ESI-MS we cannot distinguish between diastereoisomers in the plant extract. Therefore, when we refer to a specific compound identified in the plant extract, the prevalence or co-existence of another stereoisomer is not excluded.

Another dissociation route of $[\mathbf{2}+H]^+$ results from neutral loss of water, forming *m/z* 269, followed by the loss of CH_2O_2 (*m/z* 223). The product ion of *m/z* 269 was therefore mass-selected and dissociated further via ESI-MS/MS/MS. When compared with the product ion spectrum of *m/z* 269 found in the extract (Fig. 5(A)), great similarity is observed indicating that both ions have the same structure, which is compatible with the alkaloid anhydropilosine (**3**). Important product ions observed in both cases result from the neutral losses of water (*m/z* 251) and CH_2O_2 (*m/z* 223), as well as the diagnostic *m/z* 95 ion. Note that the product ion of *m/z* 181 is not formed, which is logical for the structure proposed. Furthermore, ESI-MS/MS/MS of the *m/z* 223 ion obtained from both $[\mathbf{2}+H]^+$ and $[\mathbf{3}+H]^+$ gave similar spectra with the main ions resulting from the opening of the imidazolic ring (spectrum not shown) that favors the neutral losses of CH_3NC (*m/z* 182) and CH_3NHCN (*m/z* 167).

The dissociation pattern of protonated **4** (*m/z* 193), shown in Fig. 5(B), is also similar to that of $[\mathbf{1}+H]^+$. Protonated **4** dissociates to the diagnostic *m/z* 95 ion but, possibly owing to the double bond on the lactam ring, does not lose water and CO, but rather loses a molecule of HCN (*m/z* 166) followed by another HCN (*m/z* 139), which indicates the opening of the imidazolic ring. We propose therefore that compound **4** is probably the imidazole alkaloid, 13-nor-8(11)-dihydropilocarpine, and/or its isomer, 13-nor-7(11)-dihydropilocarpine,

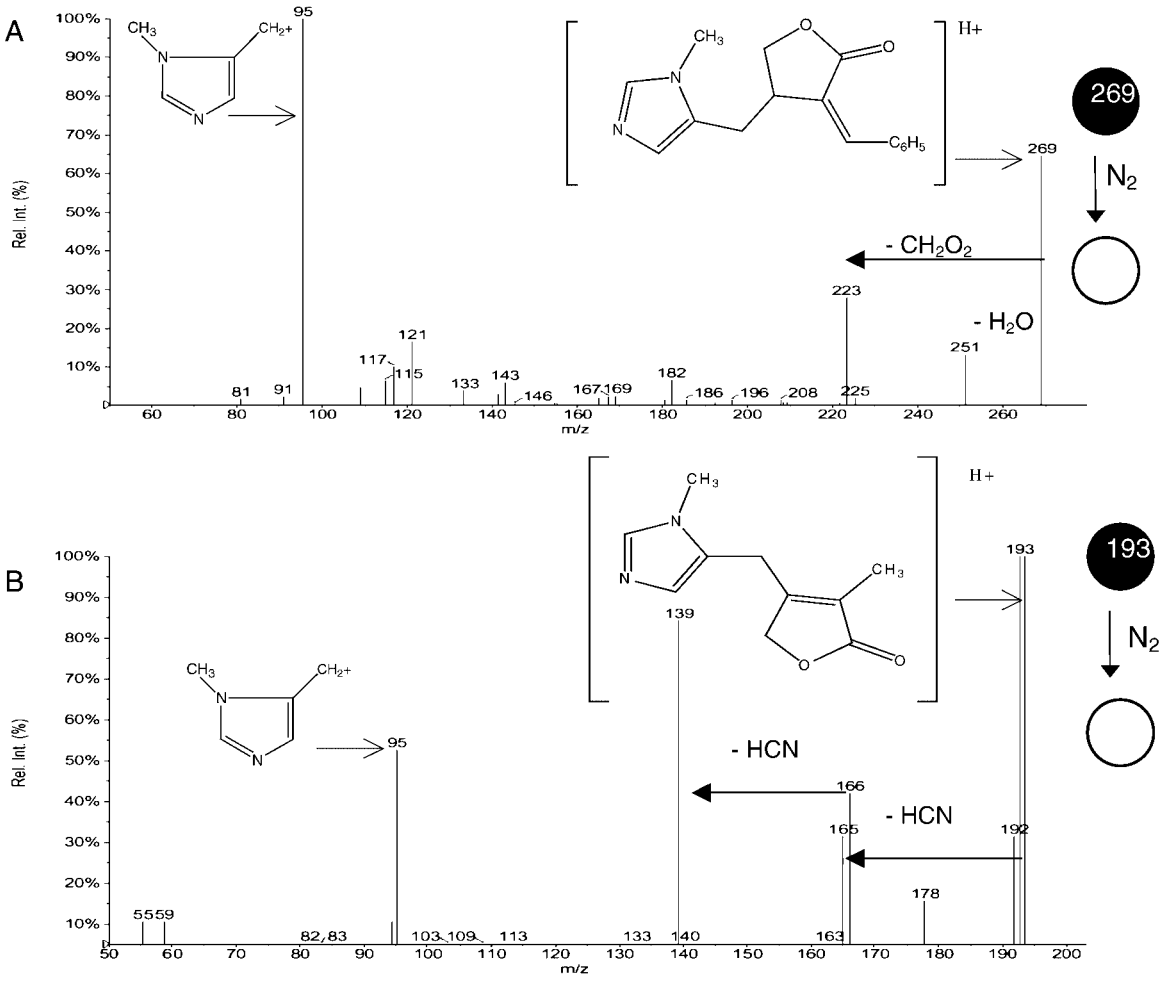

Figure 5. ESI product ion spectra of (A) protonated anhydropilosine (**3**, *m/z* 269) and (B) protonated 13-nor-8(11)-dihydropilocarpine or isomer (**8**, *m/z* 193).

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both previously extracted from the roots of *Pilocarpus trachyllophus*, a plant of the same genus as jaborandi.[10]

Up to this point we have identified known imidazole alkaloids found previously in extracts of the *Pilocarpus* genus. However, the alkaloids **5**, **6** and **7** detected in the ESI-MS fingerprints as the protonated molecules, *m/z* 241, 259 and 285, cannot be attributed to any previously reported imidazole alkaloid from this genus. The structures proposed for two of these compounds are based on compounds identified as intermediates in the synthesis of isopilosine by Link and Bernauer.[7] An important common characteristic of these compounds is that the ESI-MS/MS spectra of these three alkaloids display a common and new diagnostic ion of *m/z* 105 (Figs. 6(A)–6(C)), attributed to the benzoyl ion, $C_6H_5CO^+$.

The product ion spectrum of $[\mathbf{7}+H]^+$ (Fig. 6(A)) shows, in addition to the diagnostic benzoyl ion, *m/z* 105, the *m/z* 95 ion, diagnostic for the 1-methyl-5-imidazoylmethyl group, indicating that both groups are present in its structure. The ions of *m/z* 109 and 121 are similar to those found for $[\mathbf{1}+H]^+$. Neutral losses of CO_2 (*m/z* 241) followed by loss of water (*m/z* 223) indicate that there are at least three oxygen atoms in the compound formula. The ESI-MS/MS/MS spectrum of *m/z* 223 (resulting in product ions of *m/z* 182 and 167) is similar to that of the *m/z* 223 product ion originating from $[\mathbf{2}+H]^+$ and $[\mathbf{4}+H]^+$, indicating similar structures. Based on

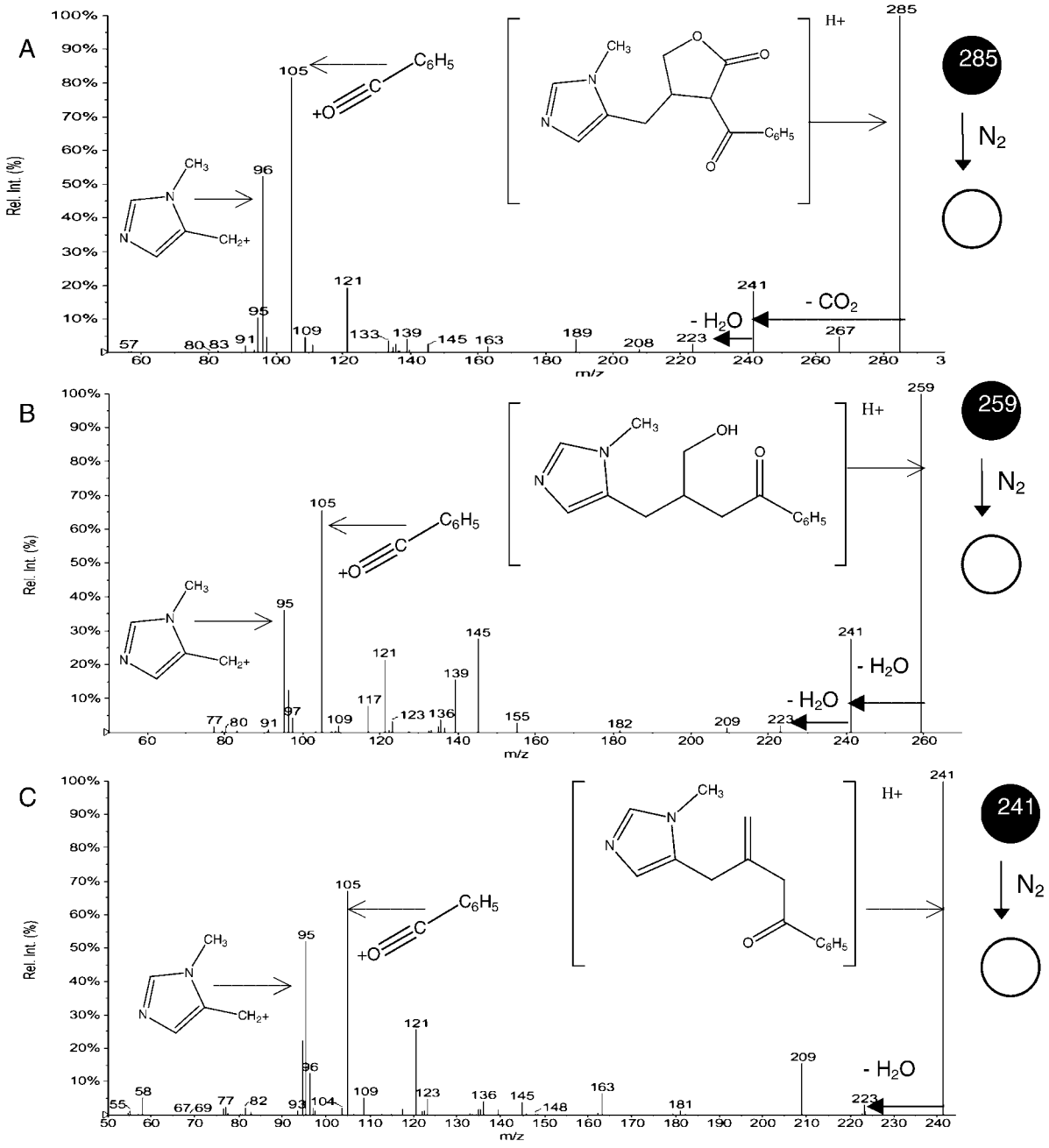

Figure 6. ESI product ion spectra of (A) protonated compound **7** (*m/z* 285); (B) protonated compound **6** (*m/z* 259); and (C) protonated compound **5** (*m/z* 241).

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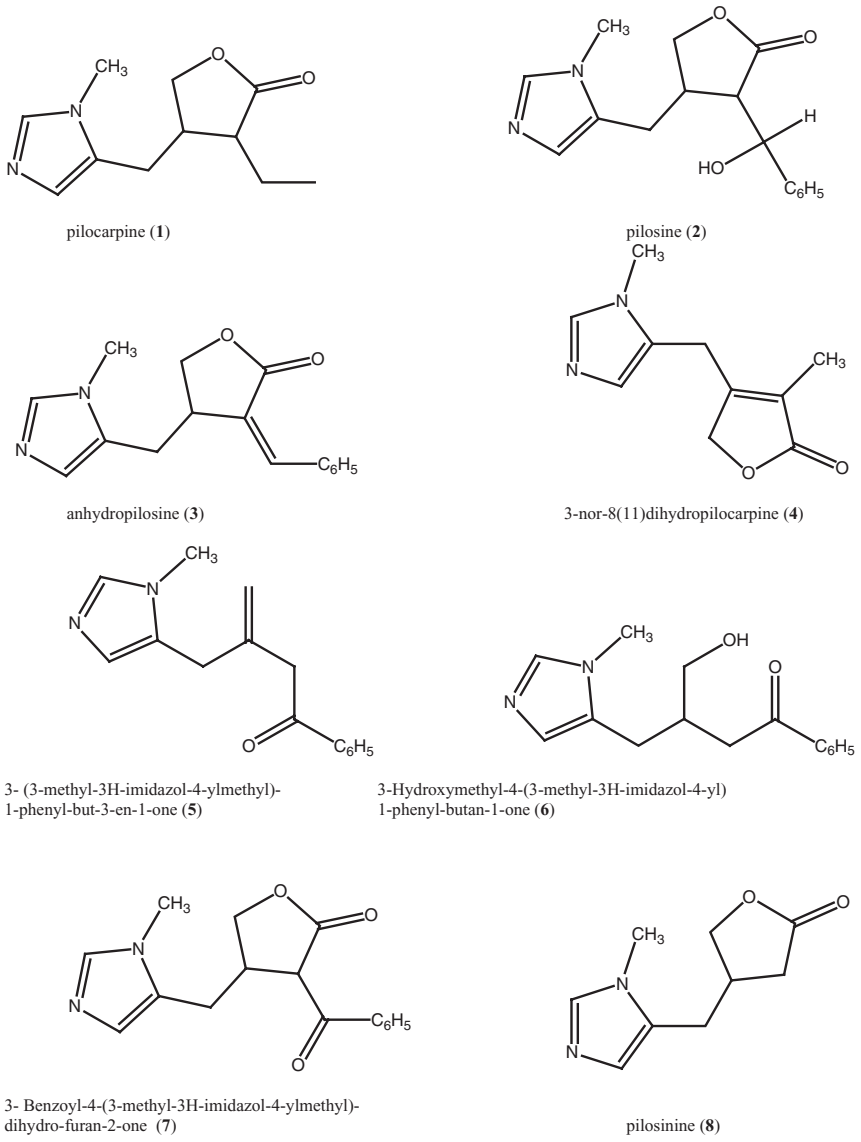

Scheme 1.

this information, we propose for **7** the structure shown in Scheme 1. This compound was identified as an intermediate in the synthesis of isopilosine by Link and Bernauer.[7]

The product ion spectrum of $[\mathbf{6}+H]^+$ (Figure 6B) shows two diagnostic ions of *m*/*z* 95 and 105 as well as *m*/*z* 241 formed by loss of water. This compound was also found as an intermediate in the synthesis of isopilosine by Link and Bernauer.[7] The ESI-MS/MS/MS spectra of *m*/*z* 241 from $[\mathbf{7}+H]^+$ and $[\mathbf{6}+H]^+$ (not shown) are similar to the product ion spectrum of $[\mathbf{5}+H]^+$ (Fig. 6(C)), also found in the extract. The low signal intensity of this ion made it impossible to mass select its product ions for more detailed structural

Table 1. High-resolution positive ion ESI-MS values of alkaloids found in the extract of *P. microphyllus* leaves

Number	Name of compound	Formula	Calculated monoisotopic monoprotonated mass	High-resolution mass	Difference (ppm)
1	pilocarpine	$C_{11}H_{16}N_2O_2$	209.1290	209.1251	18.6
2	pilosine	$C_{16}H_{18}N_2O_3$	287.1395	287.1338	19.8
3	anhydropilosine	$C_{16}H_{16}N_2O_2$	269.1290	269.1237	19.6
4	3-nor-8(11)-dihydropilocarpine	$C_{10}H_{12}N_2O_2$	193.0977	193.0939	19.6
5	3-(3-methyl-3*H*-imidazol-4-ylmethyl)-1-phenyl-but-3-en-1-one	$C_{15}H_{16}N_2O$	241.1341	241.1328	5.3
6	3-hydroxymethyl-4-(3-methyl-3*H*-imidazol-4-yl) 1-phenyl-butan-1-one	$C_{15}H_{18}N_2O_2$	259.1446	259.1397	18.9
7	3-benzoyl-4-(3-methyl-3*H*-imidazol-4-ylmethyl)-dihydro-furan-2-one	$C_{16}H_{16}N_2O_3$	285.1239	285.1185	18.9
8	pilosinine	$C_9H_{12}N_2O_2$	181.0977	181.0942	19.3

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analysis. For all three compounds of this group, the product ion of *m/z* 121 is formed, confirming further similarity in their structures, as well as with **1**. The structures proposed for compounds **6** and **5** can also be seen in Scheme 1.

The high-accuracy and high-resolution mass spectrum of the jaborandi extract (Table 1) confirms the elemental composition of the alkaloids identified herein.

CONCLUSIONS

All the compounds observed in their protonated forms with a signal intensity of over 10% in the ESI(+)-MS fingerprint of the extract were analyzed and structures have been proposed based on their elemental composition, dissociation patterns and diagnostic product ions as well as by comparison with the dissociation patterns of standards of known jaborandi alkaloids. Furthermore, as the plant leaves were extracted using a method selective for alkaloids, the extract has been found to contain imidazole alkaloids with subtle variations in structure. Ion suppression during ESI is therefore expected to be minimal, because these components belong to the same class of alkaloids with similar structures and ionization efficiencies upon ESI. The relative intensity of the ions detected in the ESI-MS fingerprint is therefore expected to be proportional to the relative concentrations of each alkaloid in the extract. Hence, we have been able to structurally characterize via direct infusion ESI-MS/MS/MS the main imidazole alkaloids in the extracts of *P. microphyllus* samples.

Based on the dissociation patterns of the main compounds found in the extracts, we observed that there were three structurally related groups of compounds. Group A comprising compounds **1**, **4** and **8** (*m/z* 209, 193 and 181, respectively); group B comprising compounds **2** and **3** (*m/z* 287 and 269, respectively); and group C comprising compounds **5**, **6** and **7** (*m/z* 241, 259 and 285, respectively) The results of the PCAs (Fig. 2) show how these groups varied with the seasons, with groups A and C occurring mainly in the summer and group B in the autumn and winter. The results of the PCAs shown in Fig. 3 show how these groups vary within the plants during the summer, with group A being widely distributed in the plant, while group B is restricted to the intermediary leaves and group C mainly in leaflets and petiole.

These results indicate that these three groups of alkaloids could belong to intermediate, parallel or competitive pathways for pilocarpine biosynthesis. Further studies using isotopically labelled precursors and the systematic analysis of their incorporation into pilocarpine may reveal the relationship between these pathways and their contribution for pilocarpine biosynthesis.

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