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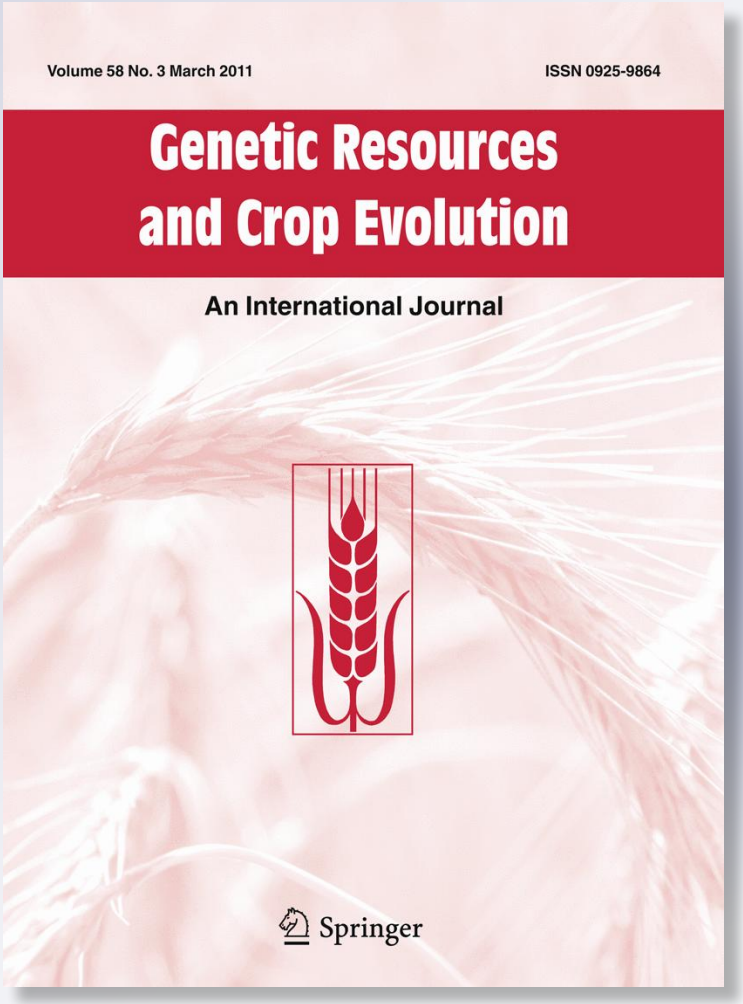

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NOTES ON NEGLECTED AND UNDERUTILIZED CROPS

Screening species of *Pilocarpus* (Rutaceae) as sources of pilocarpine and other imidazole alkaloids

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Abstract Plants of the *Pilocarpus* genus (Rutaceae) are popularly known as *jaborandi* and are the only source of pilocarpine, an imidazole alkaloid used in eye-drops for the treatment of glaucoma as well as for the stimulation of sweat and lachrymal glands. Alkaloid extracts from leaf samples of seven species of *Pilocarpus*, from the states of São Paulo and Maranhão in Brazil, were analyzed using HPLC–ESI–MS/MS. The samples contained between 0.88 ± 0.04 and 1.00 ± 0.14% of alkaloids in relation to the dry weight of their leaves, with significant differences in results ($P \leq 0.05$) found only between *Pilocarpus microphyllus* planted in the state of Maranhão and *Pilocarpus spicatus, Pilocarpus trachyllophus, Pilocarpus pennatifolius* and *Pilocarpus jaborandi*; as well as between *Pilocarpus spicatus* and *Pilocarpus racemosus.* Pilocarpine was not found in *P. spicatus,* whereas in the other species it ranged from 2.6 ± 0.1 to 70.8 ± 1.2% of total alkaloids. *P. microphyllus* planted in the state of Maranhão for pilocarpine extraction had the highest total alkaloid content, but it had only 35% of pilocarpine in relation to total alkaloids. Three other species contained more pilocarpine in relation to total alkaloids: *P. jaborandi* (70.8%), *P. racemosus* (45.6%) and *P. trachyllophus* (38.7%); and could be candidates for pilocarpine extraction. Differences in alkaloid content were significant for all these samples ($P \leq 0.05$). Imidazole alkaloids were observed and partially characterized based on their retention times and high resolution mass. The seven species analyzed had different imidazole alkaloid profiles, but only one did not present quantifiable pilocarpine contents in its leaves. The *Pilocarpus* genus shows potential for the prospection of novel alkaloids.

Keywords High performance liquid chromatography mass spectrometry · Imidazole alkaloids · Jaborandi · Pilocarpine · *Pilocarpus* · Pilosine

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Introduction

The genus *Pilocarpus* Vahl (Rutaceae) was first presented to the European medical community through leaf samples from the state of Pernambuco in Brazil. The sample was brought by Symphronio O. C. Coutinho in 1873 when he arrived to study medicine in Paris, France (Holmstedt et al. 1979) That sample was at first incorrectly identified as

Pilocarpus pennatifolius Lemaire by Baillon in 1873, who later correctly identified it to be material from *Pilocarpus selloanus* Engl. (Kaastra 1982). Several species of *Pilocarpus* are popularly known in Brazil as *jaborandi,* a term which derives from the name of these plants in the Brazilian Indian Tupi-Guaraní language (ya-mbor-endi) meaning "the one who causes mouth dripping" (Holmstedt et al. 1979). This common name led to classification mistakes in the late nineteenth century, when even piperaceous plants received this name (Holmstedt et al. 1979). As recently as the 1900s, the names *Pilocarpus jaborandi* Holmes and *Pilocarpus microphyllus* Stapf ex Wardleworth were used as synonyms, giving rise to doubts about the correct identity of the species investigated (Kaastra 1982). The name of the genus, *Pilocarpus*, is probably based on the shape of the mericarps, as *pilos* means felt hat in Greek and *carpos* means fruit.

Pilocarpus is a neotropical genus comprising shrubs and trees, with species distributed from southern Mexico, throughout Central America and the Antilles, as far as Argentina (Kaastra 1982). The genus includes 17 species and 14 of them are distributed in the Brazilian territory, with predominance in the oriental part of the country identified as the genetic diversity center of the genus (Oliveira 2007). Their distribution is an important aspect, as the leaves used for pilocarpine extraction were (until some years ago) exclusively obtained from native plants growing in the wild (Sawaya et al. 2010). However, only two species, *P. microphyllus* and *P. jaborandi*, are cited as agricultural crops in Mansfeld′s Encyclopedia of Agricultural and Horticultural Crops (Kruse 2001).

This taxon belongs to the *Pilocarpinae* sub-tribe (*Cuspariae* tribe, *Rutosidae* sub-family). The members of this taxon are usually trees or small shrubs, with long racemes of small flowers. The flower buds are globose, with chordate anthers. The flowers are actinomorphic tetramers or pentamers. The pistil has carpels fused at the base resulting in mericarps (Skorupa 2000).

Pilocarpus is a typical neglected genus as very little is known about important botanical aspects. Most information on its species is focused on the content of pilocarpine, an imidazole alkaloid exclusively found in plants of this genus. Pilocarpine is mainly used in eye-drops for the treatment of glaucoma (an eye-disease that can cause blindness) but is also for the stimulation of sweat and lachrymal glands (Goodman and Gilman 2001). Its use in ophthalmology was a secondary discovery, as the Brazilian Indians used these leaves to induce sweating and salivation (Pinheiro 1997) which are due to its cholinergic activity (Valdez et al. 1993). It has been documented that the Indians took this plant with caution, for a series of diseases including catarrh, asthma and malaria (Holmstedt et al. 1979).

Because the chemical synthesis of pilocarpine is expensive, native *jaborandi* plants were intensively exploited in the sub-Amazonian forest between 1975 and 1990. The leaves were harvested by people living in or near the forest and Merck pharmaceutical company was the only buyer. The collectors stripped the leaves by hand during the dry season; air dried them in the sun by spreading on plastic sheets on the ground to a moisture content of approximately 10–12% before selling. Excessive collection determined high plant mortality and the collection of *jaborandi* leaves in the state of Maranhão was reduced by half between 1975 and 1992 (Pinheiro 1997). Consequently *P. jaborandi*, *P. microphyllus*, *Pilocarpus alatus* Joseph ex Skorupa and *Pilocarpus trachylophus* Holmes were included in a list of Brazilian endangered species (Pinheiro 2002). These species form a unique clade and their phylogenetic relationships are associated with their geographical distribution (Oliveira 2007).

The unsustainable exploitation accelerated actions for the domestication of wild plants, which went through the basic steps of prospection, collection and evaluation of the wild material for the cultivation and adaptation to human use of a native jaborandi species. In 2002, the Centroflora Group (Vegeflora Extrações do Nordeste Ltda—www.centroflora.com.br) began to cultivate one species, *P. microphyllus* and became responsible for the pilocarpine extraction from the jaborandi leaves, but Merck still carries out the purification and commercialization of the alkaloid. Living germplasm collections of *Pilocarpus* in Brazil are maintained in the northern state of Maranhão by Empresa Brasileira de Pesquisa Agropecuária, a federal Brazilian research organization and by Merck Company (Pinheiro 2002; Sawaya et al. 2010).

P. microphyllus is considered to have the highest concentration of pilocarpine in its leaves. Other species are considered to have less pilocarpine and

varied concentrations of other alkaloids in their leaves (Pinheiro 2002), whereas one species (*Pilocarpus spicatus* St. Hilaire) was reported to contain no pilocarpine (Kaastra 1982). After the domestication and large-scale cultivation of *P. microphyllus* by Centoflora, interest in the other species of *jaborandi* waned.

The main alkaloids isolated from this genus are imidazole alkaloids similar to pilocarpine (Kaastra 1982). Different species present varied alkaloid profiles: pilocarpine is reported in six species, whereas seven other imidazole alkaloids are reported in only three species (Santos and Moreno 2004). Of these, only epiisopilosine has been screened for pharmacological and toxicological activity. At high doses it is a stimulant of the parasympathetic system, similar to pilocarpine. However, its DL_{50} is twice that of pilocarpine, indicating that it is less toxic (Lucio et al. 2002). Other, non-imidazole, alkaloids have also been found in this genus and show hallucinogenic and antifungal activity (Santos and Moreno 2004).

Most reports on the isolation of imidazole alkaloids from *Pilocarpus* species date from the initial studies between 1875 and 1912 when species identification was controversial. Other studies between 1959 and 1996 were performed on single species using classical isolation and identification techniques (Santos and Moreno 2004). No comparative studies of the imidazole alkaloids found in different *Pilocarpus* species using modern chromatographic techniques have been reported. These studies would be important to search for alternative species for pilocarpine production, as well as to discover other bioactive alkaloids.

Recently, a high performance liquid chromatography method compatible with electrospray ionization tandem mass spectrometry (HPLC–ESI–MS/MS) was developed and used to study alkaloids in *P. microphyllus* (Sawaya et al. 2008). This method reduces the possibility of degradation, isomerisation and artifacts which haunt classical phytochemical techniques. Initial studies with direct insertion ESI–MS fingerprinting allowed the characterization of previously undetected alkaloids from this species (Abreu et al. 2007). The purpose of the present study was to compare the alkaloid profile of eight samples of leaves (from seven different species) of *Pilocarpus* grown in two regions of Brazil and to determine their pilocarpine content. The results were then compared to those of previous reports of the alkaloid profiles of these species. So far, this is the most complete study on imidazole alkaloids in *Pilocarpus* and can indicate other potential species for the extraction of pilocarpine and other alkaloids.

Materials and methods

Plant material

Leaves of *P. microphyllus* (150310), *Pilocarpus carajaensis* Skorupa (150311), *P. spicatus* (150312), *Pilocarpus trachyllophus* (150313), *P. jaborandi* (150314) and *Pilocarpus racemosus* Vahl (150315) were collected in January 2009 on the farm of Merck in Barra do Corda, State of Maranhão, (northern Brazil). Leaves were taken from six individual plants of each species and air-dried to a humidity content of approximately 10%. The mixed leaves of each species were considered as one sample. These samples were kindly supplied by Renato C. Rocha and Jose Sena from Merck Company. The species were taxonomically identified by Carlos Toledo Rizzini (senior researcher at Rio de Janeiro Botanical Gardens) and planted as a living collection in Barra do Corda, Maranhão. A second group of leaf samples of *P. microphyllus* (150316) and *P. pennatifolius* (15317) were collected in February on the Campus of UNICAMP, Campinas, São Paulo (southeastern Brazil) and dried under the same conditions. The mixed leaves of each species were considered as one sample These samples were identified by Jorge Tamashiro, Researcher of the Department of Plant Biology at UNICAMP, and are part of a living collection at this University. The eight leaf samples were ground, freeze dried and kept at a temperature below 4°C until extraction.

Solvents and chemicals

HPLC grade acetonitrile was purchased from Tedia (Fairfield, OH, USA) and purified water obtained from a Mille-Q water purification system (Millipore, Molsheim, France). Analytical grade formic acid, ammonium acetate, sulfuric acid and chloroform were purchased from Merck (Darmstadt, Germany). Pilocarpine standard was obtained from Sigma (St. Louis, MO, USA).

Extraction procedure

Alkaloid extraction was carried out according to the method developed and validated by Avancini et al. (2003), which consists of the following steps: the sample is moistened with 10% NH_4OH; after 15 min extraction is carried out 3 times with $CHCl_3$; the pooled organic extracts are re-extracted twice with 2% H_2SO_4; the pooled acid extracts are adjusted to pH 12 with NH_4OH and extracted twice with $CHCl_3$. The solvent was extracted under vacuum and the dry extracts weighed and frozen until analysis. Extraction was carried out in triplicate from 100 mg aliquots of each sample.

HPLC–ESI–MS/MS equipment and conditions

Chromatographic separation was achieved at 24°C on an Agilent (Palo Alto, CA, USA) 1100 HPLC system, using a 5 μm Inertisil ODS-2 column (250 × 4.6 mm I.D.) obtained from Varian (Middleburg, The Netherlands) and a flow rate of 1 mL min^{-1}. A binary gradient was performed starting with 95:5, v/v (solvent A: solvent B). Solvent A was 0.01 M ammonium acetate buffer adjusted to pH 4 with formic acid and Solvent B was acetonitrile. The gradient was ramped to 90:10 v/v (A: B) in 8 min, and then to 75:25, v/v (A: B) at 20 min, and held until 22 min, returning to the initial condition at 25 min.

The ESI–MS and ESI–MS/MS were acquired in the positive ion mode on an Applied Biosystems API 5000 mass spectrometer (Foster City, CA, USA) with a triple quadrupole system, ranging between 100 and 400 m/z. The operational parameters used were: capillary 5,000 V, temperature 350°C, declustering potential 70 V and entrance potential 6 V. For the ESI–MS/MS experiments, the above conditions were maintained and collision energy was set at 30 V. Nitrogen was used as curtain gas (20 psi), nebulizing gas (20 psi) and collision gas (medium).

The calibration curve of pilocarpine was prepared using a stock solution of pilocarpine in water, diluted to concentrations ranging from 1 to 100 μg mL^{-1} in water prior to injection. The dry alkaloid extracts were diluted in water at a concentration of 100 μg mL^{-1}. Injections of 10 μL were used for the samples and standard solutions. Identification of the alkaloids was based on their elution order and ESI–MS/MS data in comparison with the pilocarpine standard and those from the literature.

High resolution mass spectrometry

The extract solutions above were mixed (1:1) with acetonitrile containing 0.1% formic acid and analyzed in the positive ion mode in a Thermofischer Scientific high resolution mass spectrometer (7.2 tesla, FT-LQT-MS ultra) using a Nanomate automatic injector (Advion, Manchester, England) under the following conditions: capillary 1.5 kV and pressure 0.3 psi.

Statistical analysis

Data are expressed as the mean ± standard deviation (SD) of three determinations. Statistical significance between results was pinpointed by unpaired Student's *t*-test. An associated probability (*P* value) of less than 5% was considered significant.

Results and discussion

Total alkaloids and pilocarpine contents

The yield of alkaloids from the *Pilocarpus* samples varied between 1.08 and 0.88% in relation to dry weight of freeze dried leaves (Table 1). As the percentage of water in fresh leaves and even in air-dried leaves varies considerably, the samples were freeze-dried before extraction, resulting in slightly higher percentages of alkaloids than usually reported for these species. For *P. microphyllus* the alkaloid content of air dried leaves was said to be of roughly 1% (Pinheiro 1997) and the pilocarpine content of fresh leaves was below 0.03% (Sandhu et al. 2006). The dried leaves of *P. pennatifolius* were reported to have 0.5% of alkaloids or 0.2% of pilocarpine (Lucio et al. 2002). Total alkaloid contents was quite similar for the species analyzed, with significant differences in results ($P \leq 0.05$) found only between PmM and samples Ps, Pt, Pp, and Pj; the difference between Ps and Pr was also significant (Table 1).

The concentration of pilocarpine, the pharmacologically important alkaloid found in this genus, was determined by HPLC in all the samples, in comparison with a calibration curve of pilocarpine standard;

Table 1 Percentage of alkaloids extracted in relation to the dry mass of leaves and the percentage of pilocarpine in the extracts of the samples of *Pilocarpus* analysed

Sample	Grown in	% alkaloids extracted	% of Pilocarpine in extract
P. microphyllus (PmM)	Maranhão	1.08 ± 0.06	34.5 ± 0.4
P. microphyllus (PmS)	São Paulo	0.88 ± 0.22	23.6 ± 0.3
P. carajaensis (Pc)	Maranhão	1.00 ± 0.14	2.6 ± 0.1
P. spicatus (Ps)	Maranhão	0.88 ± 0.04	Not quantified
P. trachyllophus (Pt)	Maranhão	0.92 ± 0.04	38.7 ± 0.4
P. pennatifolius (Pp)	São Paulo	0.94 ± 0.06	14.2 ± 0.3
P. jaborandi (Pj)	Maranhão	0.94 ± 0.01	70.8 ± 1.2
P. racemosus (Pr)	Maranhão	0.96 ± 0.02	45.6 ± 0.6

and varied significantly between species (Table 1). The differences in concentration of pilocarpine for all samples were all significant ($P \leq 0.01$). The highest pilocarpine content was found in *P. jaborandi* (70.8 μg in 100 μg of alkaloids, or 70.8%) and the lowest that could be quantified was found in *P. carajaensis* (2.6 μg/100 μg of alkaloids, or 2.6%). *P. microphyllus* is considered to have the highest concentration of pilocarpine in this genus (Pinheiro 1997) and is the species that is planted for this purpose. However, three other species presented higher percentages of pilocarpine in relation to total alkaloids extracted: *P. jaborandi*, *P. racemosus* and *P. trachyllophus*. These three species presented pilocarpine as the main alkaloid in their composition. The sample of *P. microphyllus* grown in the state of Maranhão showed higher pilocarpine content than the sample from the state of São Paulo, possibly due to the effect of environmental conditions on alkaloid production. However, a large genetic variability in this species has been described, which may also lead to variations in pilocarpine content (Sandhu et al. 2006). Only traces of pilocarpine (identified by the ion of m/z 209) were observed in the HPLC–ESI–MS chromatogram of *P. spicatus* using the extracted ion mode (XIC), they were too low to be quantified (less than 1% of total alkaloids). This result is in line with the affirmation that pilocarpine is not found in *P. spicatus* (Kaastra 1982).

Alkaloid profile in different species

In the chromatograms (Fig. 1) the alkaloids are numbered as in Table 2. The molecular formula, name and structure of known alkaloids are shown in Fig. 2. Nine novel alkaloids were detected and their molecular formula determined by high resolution mass spectrometry. The samples of *P. microphyllus* from the states of São Paulo and Maranhão showed a similar alkaloid profile, with small qualitative differences, which may be due to environmental and/or genetic differences. *P. microphyllus* from the state Maranhão however, had the highest percentage of total alkaloids. *P. jaborandi*, *P. racemosus* and *P. trachyllophus* also had similar profiles, which are quite different from *P. microphyllus.* The pilocarpine (**4**) peak was the most intense in the chromatogram of these three species and few peaks of higher retention times were observed, indicating that they produced mainly pilocarpine and less of the other imidazole alkaloids. These species could also be interesting candidates for pilocarpine extraction. *P. pennatifolius* had a reasonably high concentration of pilocarpine (**4**) and some compounds with higher retention times, but pilosine (**6**) and its isomers were not present. *P. carajaensis* had a lower concentration of **4** and several alkaloids with higher retention times. Although **6** was not present, three of its isomers (**9**, **10**, and **12**) were detected. Although *P. spicatus* displayed some imidazole alkaloids, its profile was quite different from the other species analyzed herein. In a previous study of *P. microphyllus,* three groups of imidazole alkaloids were observed and it was suggested that they might belong to different pathways for pilocarpine biosynthesis (Abreu et al. 2007). The present results corroborate the previous hypothesis, as most of the species produce pilocarpine, but also present other imidazole alkaloids, which vary between species. These alkaloids and their presence in different species will be discussed below.

Pilocarpine (**4**) was identified by comparison with a standard. A small peak directly before **4** (Fig. 1) is

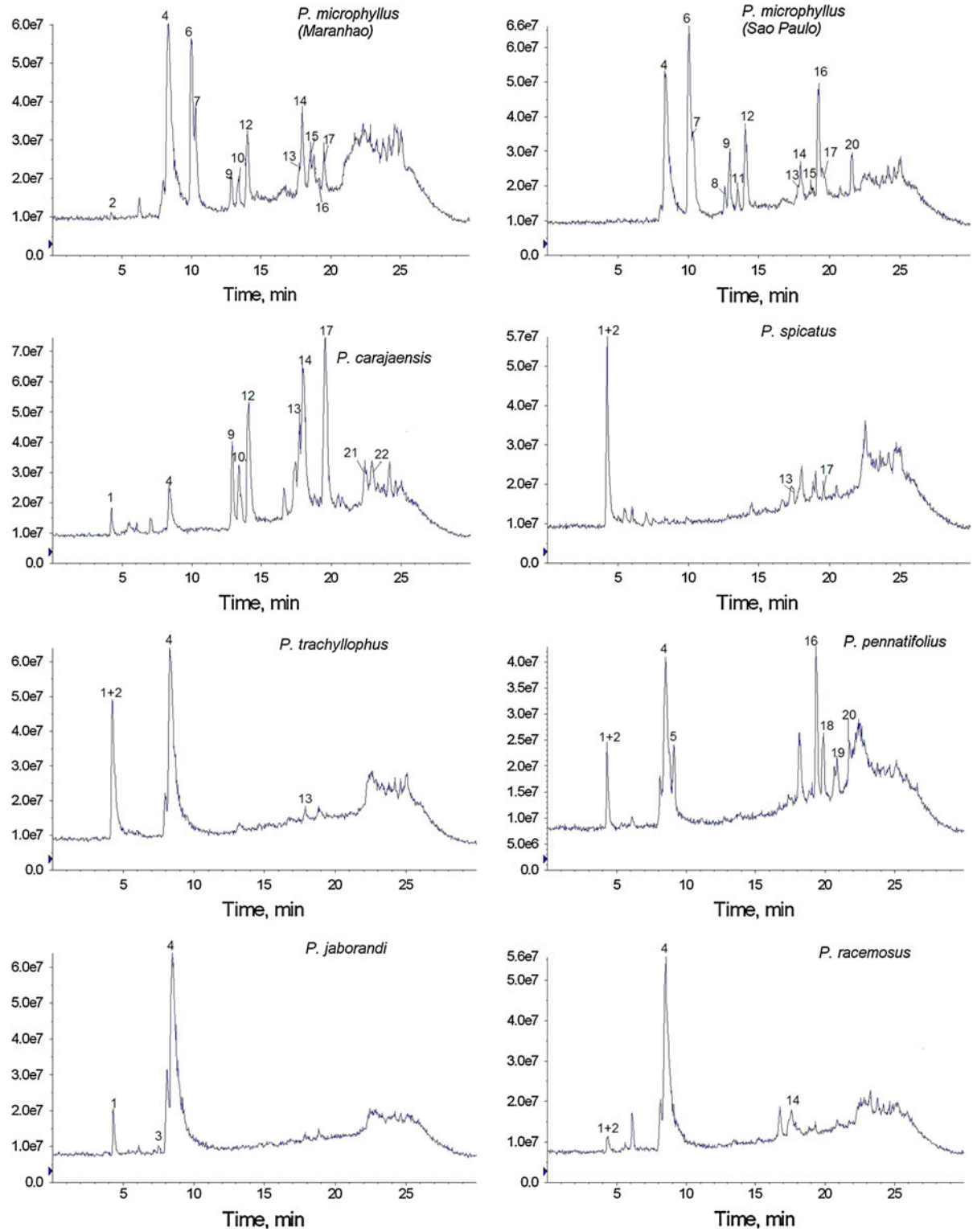

Fig. 1 HPLC-MS of the extracts of 8 samples of *Pilocarpus*. Alkaloids are numbered as in Table 2

Table 2 Alkaloids found in samples of *Pilocarpus*, characterized by the high resolution mass of their protonated molecules and retention time. Species abbreviated as in Table 1

No.	High resolution $[M+H]^+$ *m/z*	Rt min.	Samples							
			PmM	PmS	Pc	Ps	Pt	Pp	Pj	Pr
1	179.08150	4.3			p	p	p	p	p	p
2	193.09712	4.3	p			p	p	p		p
3	195.11281	8.1							p	
4	209.12836	8.4	p	p	p		p	p	p	p
5	273.12348	9.1						p		
6	287.13882	10.0	p	p						
7	287.13882	10.3	p	p						
8	273.12348	12.5		p						
9	287.13882	12.8	p	p	p					
10	287.13882	13.4	p		p					
11	273.12348	13.5		p						
12	287.13882	14.1	p	p	p					
13	259.14442	17.7	p	p	p	p	p			
14	259.14442	18.0	p	p	p					p
15	285.12352	18.5	p	p						
16	255.11291	19.3	p	p				p		
17	269.12834	19.5	p	p	p	p				
18	257.12854	20.0						p		
19	257.12854	21.0						p		
20	255.11291	21.7		p				p		
21	269.12834	22.4	p		p					
22	269.12834	22.9			p					

p present in extract

isopilocarpine, an epimer possibly produced as an artifact during the extraction procedure due to the hydrolysis of the lactone ring.

Pilosine (**6**) was also identified by comparison to a standard (Abreu et al. 2007). The next four peaks in the sample of *P. microphyllus* from Maranhão state (**7**, **9**, **10** and **12**) have the same mass and molecular formula and are isomers of pilosine, found in a previous study (Sawaya et al. 2008), with slight differences in their ESI–MS/MS. Several epimers and isomers have been reported (Tedeschi et al. 1974; Voigtländer et al. 1978) with the same molecular formula. Of these isomers; pilosine has been reported in *P. microphyllus* and pilosine, isopilosine, epiisopilosine and epiisopiloturine in *P. jaborandi* (Santos and Moreno 2004). This is nearly the opposite of the present results, as *P. jaborandi* did not present any of these alkaloids. Of the seven species analyzed, only *P. microphyllus* contained **6** and several of its isomers while *P. carajaensis* did not have **6**, but had three of its isomers **9**, **10** and **12**. The sample of *P. microphyllus* from São Paulo did not present isomer **10**, which is in line with previous results (Sawaya et al. 2008).

Anhydropilosine (**17**) was reported previously (Voigtländer et al. 1978) and was detected in samples of *P. microphyllus* from São Paulo and Maranhão states as well as in previous studies (Abreu et al. 2007; Sawaya et al. 2008). Here it was also observed in *P. spicatus* and in *P. carajaensis,* along with two other isomers (**21**, **22**) with the same high resolution mass but with longer retention times. These isomers were observed for the first time.

Two isomers of 258 Da, identified as protonated molecules of m/z 259 (**13**, **14**), were observed previously in *P. microphyllus* leaves (Sawaya et al. 2008) and in this study one isomer or both were observed in *P. microphyllus, P. carajaensis, P spicatus, P. trachyllophus* and *P. racemosus*. Figure 2 shows the general structure and name tentatively proposed for these isomers (**13**, **14**), and of another alkaloid (**15**) of m/z 285; **15** was observed only in the leaves of *P. microphyllus* in previous studies of alkaloids (Abreu et al. 2007) and in this study.

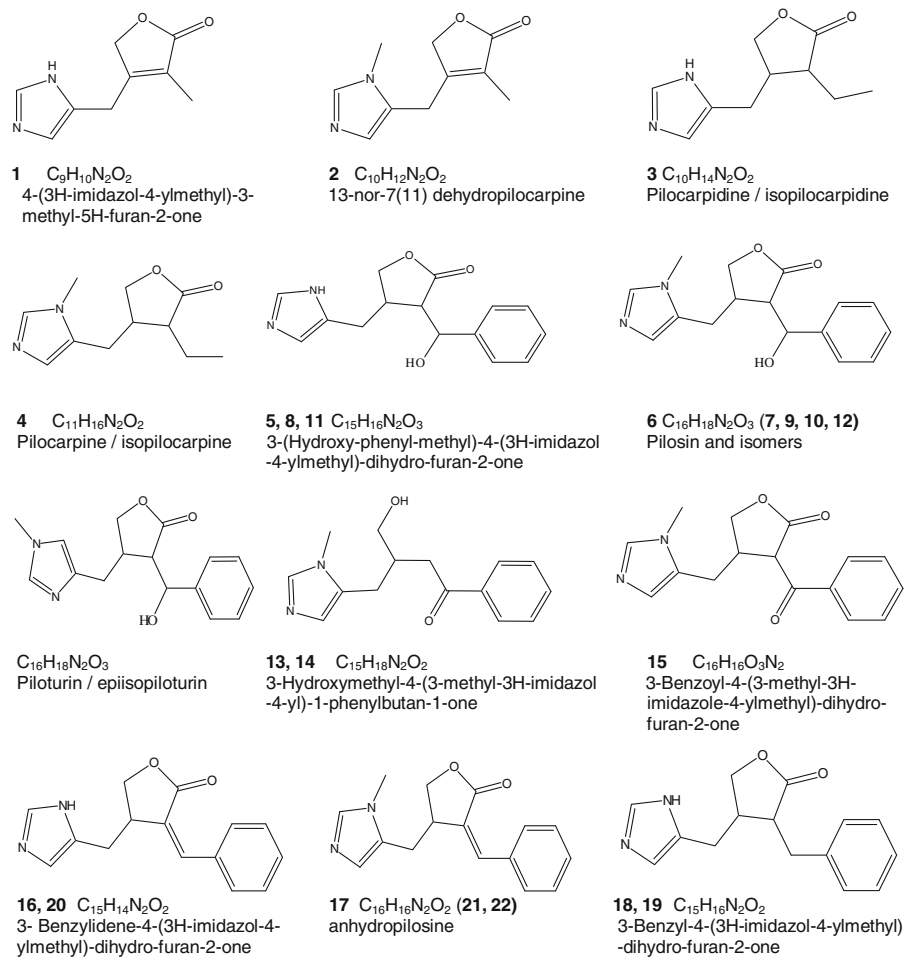

Fig. 2 Molecular formulas, structures and names of alkaloids previously identified in species of *Pilocarpus,* numbered as in Table 2

The alkaloid, 13-nor-7(11)-dehydro-pilocarpine (**2**), was reported in *P. trachyllophus* (Andrade-Neto et al. 1996) and in *P. microphyllus* (Abreu et al. 2007). In the present study, it was detected in the leaves of six species (*P. trachyllophus*, *P. microphyllus*, *P. carajaensis, P spicatus, P. racemosus* and *P. pennatifolius*), indicating that it is frequently found in this genus, and could possibly participate in different biosynthetic routes.

Pilocarpidine was observed in *P. jaborandi* (Santos and Moreno 2004) and its structure and synthesis have been described (Cordell 1981). In the present study, alkaloid 3 (m/z 195) with high resolution mass and fragmentation compatible with pilocarpidine, was only observed in the *P. jaborandi* sample.

Alkaloid **1** (m/z 179, Fig. 2) has high resolution mass and fragmentation compatible with dehydropilosinine, reported in the synthesis of pilocarpine proposed by Link and Bernauer (1972). This alkaloid was found in six of the species: *P. carajaensis, P. spicatus, P. trachyllophus, P. pennatifolius, P. jaborandi* and *P. racemosus*, indicating that it is also quite common in this genus.

Alkaloids **16** and **20** have never been reported before. They have the same high resolution mass, close retention times, and similar fragmentation patterns, suggesting that they are isomers. They were found in *P. pennatifolius* and both *P. microphyllus* samples, from São Paulo and Maranhão states; although more intense in the São Paulo sample. As the *P. pennatifolius* sample is also from São Paulo, local climatic conditions may be responsible for an increase in the production of these metabolites.

Alkaloids **18** and **19** (m/z 257) have never been reported before, and were only observed in the chromatograms of *P. pennatifolius*, from São Paulo state. They have the same high resolution mass, close

retention times and similar fragmentation patterns, indicating that they are isomers. Alkaloids **5**, **8** and **11** (m/z 273) are also isomers, with the same high resolution mass and similar fragmentation patterns. They were observed only in the samples grown in São Paulo (*P. pennatifolius* and *P. microphyllus*). These alkaloids have not been reported before and their molecular formulae were determined by high resolution mass.

Conclusions

In the present study, the leaf samples of most *jaborandi* species produced pilocarpine, as well as a series of other imidazole alkaloids. The alkaloid profiles and contents of most of these species are presented for the first time, containing approximately 1% of alkaloids in relation to the dry weight of the leaves, with the pilocarpine content varying drastically between 2 and 70% (w/w) of the total alkaloid composition. Some of these alkaloids have been reported previously, but nine others were observed for the first time. Their molecular formulae were determined by high resolution mass spectrometry and their structures have been tentatively characterized based on their mass spectra. Further studies will be necessary to isolate and determine the full structure of previously unidentified alkaloids and to screen for their biological activities. *P. microphyllus* grown in Maranhão had the highest yield of total alkaloids, but three other species were found to contain higher concentrations of pilocarpine (*P. jaborandi*, *P. racemosus* and *P. trachyllophus*) in relation to total alkaloids; which could make them candidates for pilocarpine extraction. On the other hand *P. jaborandi* did not contain pilosine or its isomers (as previously reported) and *P. spicatus* had only traces of pilocarpine. These species may be used as tools to study the biosynthetic pathway of pilocarpine and other imidazole alkaloids, since species with different alkaloids may be crossed and the F1 plants analyzed for their alkaloid constitution. In this study it became clear that *Pilocarpus* species show quite varied imidazole alkaloid profiles and show potential for the prospection of novel bioactive alkaloids.

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