A New Imidazole Alkaloid and Other Constituents from *Pilocarpus grandiflorus* and their Antifungal Activity

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The stems of *Pilocarpus grandiflorus* have afforded the new imidazole alkaloid 4,6-dehydro-1,2,4,5-tetrahydro-2,5-dioxopilocarpine in addition to the 17 known compounds germanicol, β-amiryn, ocotillone, stigmast-4-en-3-one, 3β-hydroxy-stigmast-5-en-7-one, 6β-hydroxy-stigmast-4-en-3-one, β-sitosterol, scopoletin, 3-(1',1'-dimethylallyl)-scopoletin, elisin, dictamine, 4-methoxy-2-quinolone, platydesmine, syringaresinol, syringaldehyde, syringic acid and vanillic acid. Their structures were elucidated on the basis of chemical and spectroscopic evidence. The phenolic compounds vanillic acid and syringaldehyde and the furoquinoline alkaloid platydesmine exhibited antifungal activity against *Leucoagaricus gongylophorus*, the symbiotic fungus of leaf-cutting ants (*Atta sexdens rubropilosa*).

Key words: Pilocarpus grandiflorus, Jaborandi, Imidazole Alkaloid, *Leucoagaricus gongylophorus*, *Atta sexdens rubropilosa*

Introduction

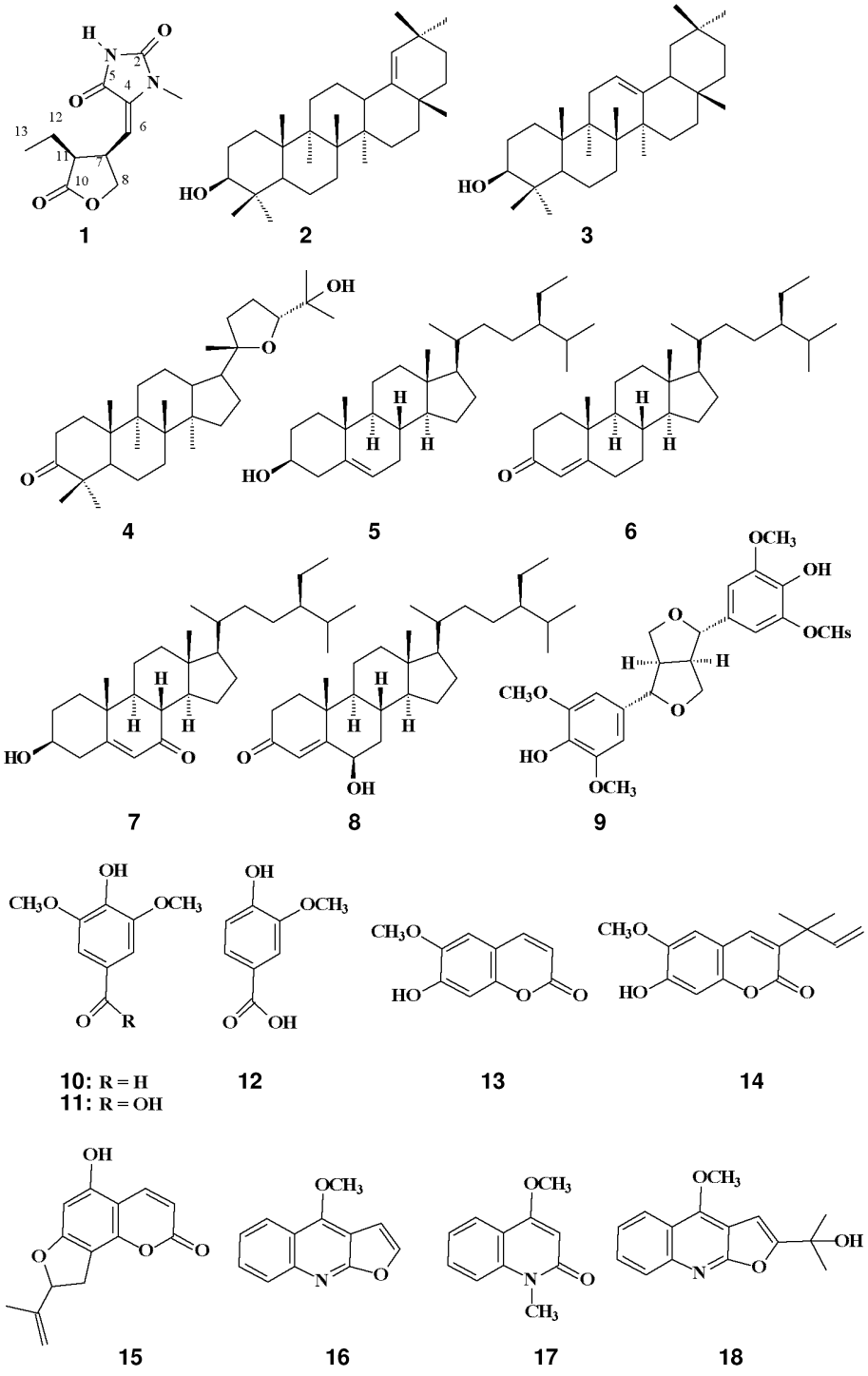
Pilocarpus commonly known as "Jaborandi" is a shrub belonging to the Rutaceae and comprises 16 neotropical species spread over tropical and subtropical America. In Brazil, the occurrence of thirteen species [1] has been reported. This genus has long been known as the most important source of the imidazole alkaloid pilocarpine. Imidazole alkaloids are histamine derivatives found only in the genus *Casimiroa* and *Pilocarpus* [2]. The alkaloid content in some *Pilocarpus* (0.5 – 1.0%) consists mainly of the imidazole alkaloid pilocarpine, together with small amounts of pilosine and related structures. Pilocarpine salts are valuable in ophthalmic practice and used in eyedrops as miotics and for the treatment of glaucoma [3].

Leaf-cutting ants of the genera *Atta* and *Acromyrmex* are among the most polyphagous agricultural pests of the South American countries. These ants cut vegetables for substrate of their symbiotic food fungus and produce consistent economic damage to the harvesting of the cultivated plants. The agricultural pest control practices generally involve toxic bait applications damaging the environment [4]. An alternative control for these insects can be achieved by inhibiting the growth of their symbiotic fungus through more selective and less toxic natural products than the baits existent in the market.

In this paper, we report the isolation of 18 compounds, the structural elucidation of a new imidazole alkaloid and the evaluation of their activities as inhibitors of the growth of the symbiotic fungus *Leucoagaricus gongylophorus* of leaf-cutting ants, *Atta sexdens rubropilosa*.

Results and Discussion

In continuation of our search for compounds to be used in the control of leaf-cutting ants [5 – 15], we have investigated the dichloromethane extract of the stems of *P. grandiflorus* leading to the isolation of a new imidazole alkaloid (**1**) in addition to the known compounds germanicol (**2**) [16], β-amiryn (**3**) [17], ocotillone (**4**) [18], β-sitosterol (**5**) [19], stigmast-



4-en-3-one (**6**) [20], 3β-hydroxy-stigmast-5-en-7-one (**7**) [21], 6β-hydroxy-stigmast-4-en-3-one (**8**) [22], syringaresinol (**9**) [23], syringaldehyde (**10**) [24], syringic acid (**11**) [25], vanillic acid (**12**) [26], scopoletin

Table 1. ^{1}H (400 MHz) and ^{13}C (100 MHz) NMR data of **1** ($CDCl_3$, δ).

No.	^{1}H	^{13}C
2	–	152.4
4	–	132.2
5	–	162.4
6	5.23 *d* (11)	112.3
7	4.76 *dddd* (11.5; 7; 5.4; 2)	35.7
8b	4.08 *dd* (9; 2)	71.7
8a	4.42 *dd* (9; 5.4)	
10	–	177.7
11	2.67 *ddd* (8; 7; 5)	45.4
12b	1.38 *m*	19.5
12a	1.84 *m*	
13	1.01 *t* (7.4)	12.2
N-CH_3	3.07 *s*	25.9

(**13**) [27], 3-(1',1'-dimethylallyl)-scopoletin (**14**) [28], elisin (**15**) [29], dictamine (**16**) [30], 1-methyl-4-methoxy-2-quinolone (**17**) [31] and platydesmine (**18**) [32].

The known compounds were identified by comparison of their physical and spectral data with those already reported in the literature. The new imidazole alkaloid had its structure determined on the basis of chemical and spectral evidence. The new alkaloid **1** had spectroscopic characteristics of the known imidazole alkaloid pilocarpine. The HREIMS spectrum displayed a molecular ion at m/z 238.0935 in accordance with the molecular formula $C_{11}H_{14}O_4N_2$. The ^{1}H NMR spectrum (Table 1) showed signals at $\delta =$ 1.01 (3H, *t*, $J = 7.4$ Hz), $\delta = 1.84$ (1H, *m*) and $\delta =$ 1.38 (1H, *m*) indicating an ethyl group; two double-doublets at $\delta = 4.42$ (1H, *dd*, $J = 9.0$, 5.4 Hz) and $\delta = 4.08$ (1H, *dd*, $J = 9.0$, 2.0 Hz) for protons of oxygenated carbons at $\delta = 71.7$ and other lactone carbon at $\delta = 177.8$ and the signals at $\delta = 2.67$ (1H, *ddd*, $J = 8.0$, 7.0, 5.0 Hz), $\delta = 4.76$ (1H, *dddd*, $J = 11.5$, 7.0, 5.4, 2.0 Hz) indicating a pattern similar to pilocarpine. The ^{13}C NMR spectrum for **1** (Table 1) showed eleven carbons in agreement with the imidazole alkaloid structure. The signals at $\delta = 177.7$, 162.4, 152.4, 132.2 and 112.3 suggesting a change in the structure of pilocarpine with two additional carbonyl groups and a double bond. The absence of a signal in the aromatic region and the signal at $\delta =$ 5.23 (1H, *d*, 11.0 Hz) in addition to the two carbonyl observed in the ^{13}C NMR spectrum suggests a modification of the imidazole ring. This is supported by HMBC spectrum (Table 2), which showed a correlation of the N-methyl group to the carbonyl C-2 and C-9 at $\delta = 152.4$ (^3J) and 162.4 (^3J), and from H-6 to the C-4 and C-5 at $\delta = 132.2$ (^3J) and 162.4 (^3J). In the HSQC spectrum, the carbon at $\delta = 35.7$ was correlated with the signal at $\delta = 4.76$. This value can be explained by anisotropic effects of the carbonyl group and the double bond. The G-NOESY experiments (Table 3) showed correlations of H-6 with the N-3-methyl group and H-8, requiring a *cis* configuration for the double bond.

Table 2. Correlations in the HMBC and G-NOESY two-dimensional observed for compound **1**.

H	— HMBC —		G-NOESY
	$^2J_{C-H}$	$^3J_{C-H}$	H
6	4	5; 8; 11	8b; N-CH_3
7	6; 11	4; 10	8a; 8b; 11
8a		6	7; 8b; 11
8b	7	6; 10; 11	6; 7; 8a
11	7; 12	6; 13	7; 8a; 12a
12a	11; 13	7; 10	
12b	11; 13	7; 10	
13	12	11	
N-CH_3		2; 4	6

Table 3. Evaluation of the growth inhibitory activity of dichloromethane extract and compounds of *P. grandiflorus*.

Extract/compounds	Amount (μg ml^{-1})	Growth inhibition (%)
Dichloromethane extract	1000	100
Germanicol (**2**)	100	0
β-Amyrin (**3**)	100	0
β-Sitosterol (**5**)	100	0
Stigmast-4-en-3-one (**6**)	100	0
3β-Hydroxystigmast-5-en-7-one (**7**)	60	20
Syringaresinol (**9**)	50	0
Syringaldehyde (**10**)	50	80
Vanillic acid (**12**)	50	80
Dictamine (**16**)	40	40
1-Methyl-4-methoxy-2-quinolone (**17**)	60	0
Platydesmine (**18**)	50	80

The dichloromethane extract of the stems as well as fractions of *P. grandiflorus* showed strong inhibitory activity on the growth of *L. gongylophorus*. Among the 18 isolated compounds, the phenolic compounds syringaldehyde (**10**) and vanillic acid (**12**) and the furoquinoline alkaloid platydesmine (**18**) exhibited higher inhibition of the fungal growth at 50 μg ml^{-1}.

Antifungal activities have been described for phenolic compounds against *Cladosporium herbarum* [33], *Aspergillus flavus and A. parasiticus* [34], *Geotrichum candidum*, *Coriolus versicolor*, *Phanerochaete chrysosporium* and *Mycelia sterilia* [35].

Grayer and Harbone [36] suggested that furoquinoline alkaloids might also play a role in the defense of plants against potentially pathogenic fungi. The result

of our search confirmed the antifungal activity for this compound against *L. gongylophorus*.

Experimental Section

General experimental procedures

^{1}H NMR (400 MHz) and ^{13}C NMR (100 MHz): Bruker ARX-400 spectrometer, in $CDCl_3$ containing TMS as int. standard. EIMS (70 eV): VG Platform II instrument and Shimadzu QP5000. HRMS: Autospec-Micromass EBE. CC were performed on silica gel 60 H (0.04 – 0.005 mm) and sílica gel (0.063 – 0.2 mm), respectively. Analytical TLC were performed on precoated Merck F_{254} sílica gel plates and visualized on UV (254 – 360 nm) and by spraying with vaniline-H_2SO_4.

Plant material

The stems of *P. grandiflorus* was collected in Poços D'antas, município de Murici, Alagoas state, Brazil in 1993. Identification of the plant was done by Professor Dr. José R. Pirani, and a voucher is deposited in the Herbarium of São Paulo University, Biosciences Institute.

Extraction and isolation

Stems of *P. grandiflorus* were dried and powdered (5.2 kg) and extracted with hexane, dichloromethane and methanol respectively. The dichloromethane extract (11.7 g) was submitted to vacuum column chromatography over silica gel using hexane, dichloromethane, ethyl acetate and methanol as eluents.

The dichloromethane fr. (3.8 g) was subjected to Column Chromatography over silica gel using dichloromethane, ethyl acetate and methanol at different rations of increasing polarity, furnishing 23 fr. Fr. 4-6 were purified by column Lobar Si60 using Hex-EtOAc-MeOH as eluents to yield **2** – **3** (16.3 mg), **5** (107.9 mg), **6** (16.0 mg) and **7** (7.0 mg). Fr. 22 was subjected to CC using gradient elution with Hex-AcOEt to yield the compounds **10** (4.5 mg), **13** (5.0 mg) and **14** (3.0 mg).

The ethyl acetate fr. (11 g) was subjected to chromatography on a silica gel column, with dichloromethane, ethyl acetate and methanol as eluents, furnishing 17 fr. Fr. 2 was subjected to CC using gradient elution with Hex-AcOEt to yield the compounds **4** (1 mg), **8** (0.7 mg), **15** (1.3 mg), **16** (20.5 mg) and **17** (5.6 mg). The Fr. 3 was purified by CC using gradient elution with Hex-AcOEt to give the compounds **16** (5.0 mg) and **18** (8.3 mg). Fr. 13 was subjected to CC using gradient elution with CH_2Cl_2-AcOEt-MeOH furnishing 5 fr. These fractions were further chromatographed by prep. TLC (silica gel, Hex-EtOAc, 8:2) to give **16** (6.0 mg), **9** (42.5 mg), **12** (7.7 mg), **10** (5.5 mg), **11** (6.0 mg) and **1** (6.0 mg).

Fungicidal assay

The fungus *L. gongylophorus* Singer was isolated from *Atta sexdens rubropilosa* Forel nest, kept in culture media and the fungicidal activity performed according to established protocols [37].

*4,6-Dehydro-1,2,4,5-tetrahydro-2,5-dioxopilocarpin (**1**)*

Yellow oil. $[\alpha]_D^{25} + 19.6$ (c 0.005, $CHCl_3$). – UV (MeOH): $\lambda_{max}(\lg\varepsilon) = 284$ nm (3.98), 242 (3.46). – IR (film): $\tilde{\nu} =$ 3444, 2967, 2932, 1767, 1731, 1604, 1442, 1382, 1175, 1121, 1065, 1019, 704, 641 cm^{-1}. – ^{1}H NMR and ^{13}C NMR: Table 1. – HRMS (EI, 70 eV): found 238.0938 [M^+], $C_{11}H_{14}N_2O_4$ requires 238.0954. – MS (EI, 70 eV): *m/z* (%) = 238 (47) [M^+], 209 (20), 192 (56), 180 (93), 165 (53), 151 (67), 139 (35), 126 (60), 94 (100), 81 (62), 68 (83).

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