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Indole alkaloids from *Tabernaemontana australis* (Müell. Arg) Miers that inhibit acetylcholinesterase enzyme

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Abstract—Ten indole alkaloids from the chloroform extract of stalk of *Tabernaemontana australis* (Müell. Arg) Miers were tentatively identified by GC–MS, *viz.*, coronaridine (**1**), voacangine (**2**), voacangine hydroxyindolenine (**3**), rupicoline (**4**), ibogamine (**5**), ibogaine (**6**), ibogaline (**7**), desethyl-voacangine (**8**), voachalotine (**9**), and affinisine (**10**). Of these, the first four were isolated by silica gel open column chromatography, identified by uni- and bidimensional NMR, IR, MS and showed anti-cholinesterasic activity at the same concentration as the reference compounds physostigmine and galanthamine (detection limit of 0.01 mM) by TLC assay using the modified Ellman's method.

1. Introduction

Alzheimer's disease (AD) is the most common neurodegenerative disorder of this century and the most prevalent cause of dementia with aging. Symptomatic pharmacological treatment of AD is mainly based on the use of acetylcholinesterase inhibitors (AChEI) (e.g., donezepil, rivastigmine, and galanthamine), which have beneficial effects on cognitive, functional, and behavioral symptoms of the disease as well as undesired side effects.[1] The need of novel treatments and the fact that the role of cholinesterase inhibitors in AD are still not completely unveiled led to the investigation of new natural AChEI. Several alkaloids from terrestrial plants have been tested as AChEI as well as other natural products.[2]

Monoterpenoid indole alkaloids have been extensively investigated for a wide variety of pharmacological effects, such as contraceptive, anti-tumor, anti-inflammatory, anti-malarial, anti-HIV, bactericide, and leishmanicide activities, as well as a stimulatory action on the central nervous system.[3] Up to date, more than 2000 different compounds of this class have been isolated and besides the anti-cancer Vinca alkaloids, coronaridine (**1**) and ibogaine (**6**) are ones of the most studied Apocynaceae alkaloids due their activity in the CNS and their potential use as anti-addiction agents.[4]

The species *Tabernaemontana australis* (Müell. Arg) Miers (sin. *Peschiera australis*), which flourishes in Brazil, Argentina, Uruguay, and Paraguay, has been poorly investigated with regard to its chemical composition and specific pharmacological activities. Recent studies reported the anti-leishmanial activity of the ethanolic extracts and of the isolated monoterpenoid indole alkaloid coronaridine **1**.[5]

Although tryptophan derived alkaloids can inhibit acetylcholinesterase enzyme [e.g., physostigmine (eserine)],[6] up to date, only monoterpenoid indole alkaloids from the Apocynaceae *Haplophyton crooksii* have been assayed as cholinesterase inhibitors,[7] which led the authors to investigate this pharmacological behavior in iboga alkaloids.

Keywords: Indole alkaloids; *Tabernaemontana australis* (Müell. Arg) Miers; Acetylcholinesterase inhibitors; Thin-layer chromatography assay.
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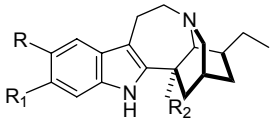
2. Results and discussion

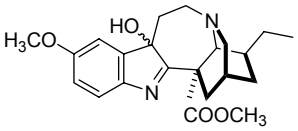
T. australis (Müell, Arg) Miers (Apocynaceae) was collected at Botanical Garden of Rio de Janeiro. TLC chromatography of the crude stalk chloroform extract indicated the presence of alkaloids revealed by Dragendorff's reagent. TLC assay using the modified Ellman's method[8] and comparison with Dragendorff's positive spots revealed the presence of acetylcholinesterase inhibitors among the alkaloids.

GC–MS analysis of the crude indicated the presence of alkaloids with molecular weight ranging from 280 to 384. Analysis of the fragmentation pattern of the alkaloids in the mass spectrometry, associated with the Wiley 275 MS library, comparison with the literature data[9] and standards co-injection suggested the presence of coronaridine (**1**), voacangine (**2**), voacangine hydroxyindoleine (**3**), rupiculine (**4**), ibogamine (**5**), ibogaline (**7**), desethyl-voacangine (**8**), voachalotine (**9**), and affinisine (**10**) (Fig. 1).

Silica gel open column chromatography of the crude chloroform extract afforded nine fractions. Fractions 1–4 were eluted in silica gel open column chromatography with a gradient of cyclohexane and ethyl acetate to give compounds **1**, **2**, **3**, and **4**. Spectroscopic analyses by 1D and 2D NMR, IR, and MS confirmed the presence of coronaridine (**1**), voacangine (**2**), voacangine hydroxyindoleine (**3**), and rupicoline (**4**).[10] Alkaloids **5**–**10** were obtained as mixtures after column chromatography.

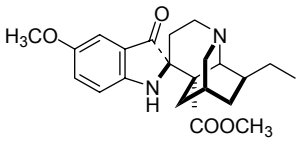
TLC assay of the crude extract in silica gel, based on Ellman's method, showed significant acetylcholinesterase inhibition in three large regions but not in all of the revealed by Dragendorff's reagent spots. After column chromatography, these spots were associated with compounds **1**, **2**, **3**, **4**, and **10**, although the last one was obtained as a mixture as revealed by HRGC. Analyses of four pure alkaloids **1**, **2**, **3**, and **4** in the same TLC assay showed a significant AChE inhibition in the same concentration of the reference compounds physostigmine and galanthamine detection limits (0.01 mM).



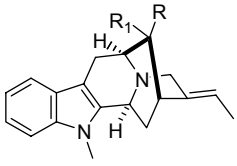


Voacangine hydroxyindolenine (**3**)

Coronaridine (**1**): R=R$_1$=H; R$_2$=COOCH$_3$
Voacangine (**2**): R=H; R$_1$=OCH$_3$; R$_2$=COOCH$_3$
Ibogamine (**5**): R=R$_1$= R$_2$= H
Ibogaine (**6**): R= OCH$_3$; R$_1$= R$_2$= H
Ibogaline (**7**): R=R$_1$= OCH$_3$; R$_2$= H
Desethyl-voacangine (**8**): R=H; R$_1$=OCH$_3$; R$_2$=COOCH$_3$



Rupicoline (**4**)



Voachalotine (**9**): R=COOOH$_3$; R$_1$=CH$_2$OH
Affinisine (**10**):R=CH$_2$OH; R$_1$=H

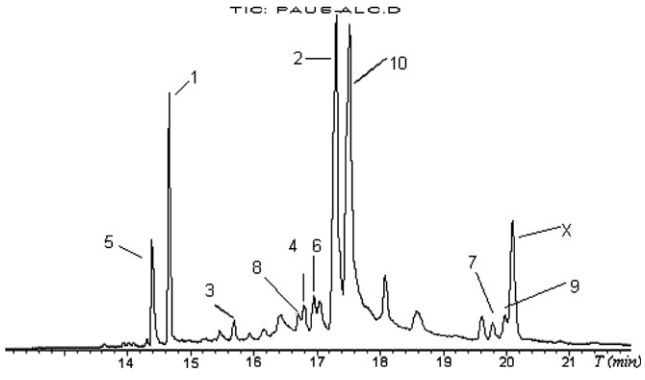


Figure 1. Total ion chromatogram of *T. australis* crude stalk chloroform extract (coronaridine **1**, voacangine **2**, voacangine hydroxyindoleine **3**, rupicoline **4**, ibogamine **5**, ibogaine **6**, ibogaline **7**, desethyl-voacangine **8**, voachalotine (**9**), and affinisine (**10**) (X = unidentified)).

Indole alkaloids from *H. crooksii* showed different anticholinesterasic activities by colorimetric in vitro acetylcholinesterase assay. No indole alkaloids with iboga nucleus were described from *H. crooksii* and some of the most significant active ones are from sarpagan type as 10-methoxy-*N*1-methylpericycline and akuammidine,[7] as are voachalotine (**9**) and affinisine (**10**) obtained from *T. australis*.

The activity of ibogaine and derivatives, such as 18-methoxycoronaridine, as anti-addictive agents seems to be related to their antagonist effect in subtype α3β4 nicotinic receptors (nChRs), binding with low affinity to other types of receptors, including α4β2 nChRs.[1,11]

On the other hand, α4β2 nChRs agonists, as well as acetylcholinesterase inhibitors, are important targets for AD treatment. In fact, a number of nicotinic receptor

agonists are in preclinical or clinical testing, even though they are difficult to dose and can cause desensitization rather than increase activation of nicotinic receptors.[11]

As far as we known, no other iboga or sarpagan natural alkaloids has been investigated in relation to its action in nicotinic receptors nor to anti-cholinesterasic activity.[7]

The presence of such compounds as major metabolites on the active anti-cholinesterase alkaloidic fraction of *T. australis* suggests iboga and sarpagan alkaloids from Apocynaceae family as interesting probes for further studies on CNS biochemical processes, including its action in nicotinic receptors and cholinesterase enzyme inhibition.

This is the first report of the anti-cholinesterasic activity of iboga alkaloids as compounds **1**–**4**. These results also corroborate that TLC assay based on Ellman's method is the simplest, faster, and cheapest method to screen new anti-cholinesterasic inhibitors from plants.

3. Experimental

3.1. General

GC analyses were carried out on an Agilent GC 6890 gas chromatography equipped with a fused silica DB1 (J&W, 25 m × 0.25 mm × 0.25 μm) capillary column directly coupled to a quadrupole mass spectrometer Agilent 5973. EI-mass spectra were recorded at 70 eV. Conditions: injector (split mode, 1:30) at 250 °C; oven temperature: 150–290 °C (5 min) at 4 °C min^{-1}, He as carrier gas at 1 mL min^{-1}. The NMR spectra (δ ppm and J in Hz) were recorded on a Bruker DRX-300 spectrometer in $CDCl_3$ as solvent and as internal reference. FTIR spectrum with a KBr disc was recorded on a Nicolet AVATAR-FTIR spectrometer.

Acetylcholinesterase (AChE) from electric eel (EC 3.1.1.7); acetylthiocoline iodide (ATCI; product no. A5751); 5,5′-dithiobis-(2-nitrobenzoic acid) (DTNB); trisma hydrochloride (Tris–HCl) buffer solution, pH 8.0; and the reference compound physostigmine were obtained from Sigma (St. Louis, MO, USA). TLC aluminum sheets silica gel 60 F_{254}, 0.2-mm thickness were purchased from Merck (Darmstadt, Germany).

3.2. Plant material

T. australis was collected at Botanical Garden of Rio de Janeiro where a voucher specimen is deposited under the number ICN-68457.

3.3. Extraction and isolation of alkaloids

Stalk (631 g) was dried at 80 °C for seven days, ground and extracted on a Soxhlet apparatus with EtOH to give 53 g of a brown residue after reduced pressure concentration. The dried ethanolic extract was suspended in 300 mL of 5% HCl and extracted four times with $CHCl_3$ (80 mL). The pH of the aqueous acidic fraction was adjusted to 9 with NH_4OH, extracted four times with $CHCl_3$ (100 mL), and dried over anhydrous Na_2SO_4. Reduced pressure concentration yielded 2.6 g of a crude chloroform extract. 1.5 g of this extract was submitted to silica gel (60–220 mesh) open column chromatography in a gradient elution of cyclohexane/ethyl acetate/methanol to afford nine fractions. Silica gel column chromatography (cyclohexane/ethyl acetate in gradient mode) of fractions 1–4 gave three pure compounds: coronaridine **1** (14 mg), voacangine **2** (29 mg), and voacangine hydroxyindoleine **3** (25 mg) identified on the basis of its spectral data and comparison of the literature and also rupicoline **4** (90% pure by GC, 5 mg). Silica gel column chromatography was applied to the other fractions where alkaloids were obtained as mixtures.

3.4. Screening for AChE activity

Acetylcholinesterase inhibitory activity was determined using TLC assay method and staining with Ellman's reagent (DTNB). Briefly, crude extract or pure compounds were diluted in CH_2Cl_2 at a concentration of 10 mg mL^{-1} or 0.01 and 0.1 mM, respectively. A volume of 2.5 μL of each sample was spotted on the silica gel TLC plate and developed with the solvent hexane/ethyl acetate (1:1); 2.5 μL of 0.1 and 0.01 mM physostigmine and galanthamine solutions in methanol were also spotted as reference compounds. After developing the TLC plate, enzyme inhibitory activities of the developed spots were detected by spraying the substrate, dye and enzyme. TLC analysis without solvent development was also used to test the enzyme inhibition in different pure compounds concentrations.

The presence of cholinesterase inhibitory activity was determined by the formation of well-defined white spots made visible by spraying with DTNB, which gives a yellow background.

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