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Two fast screening methods (GC-MS and TLC-ChEI assay) for rapid evaluation of potential anticholinesterasic indole alkaloids in complex mixtures

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ABSTRACT

The pharmacotherapy for Alzheimer's disease (AD) includes the use of acetylcholinesterase inhibitors (AChEI). Recent investigations for novel AD therapeutic agents from plants suggested that *Tabernaemontana* genus is a promising source of novel anticholinesterasic indole alkaloids. In this work two fast screening techniques were combined in order to easily identify novel cholinesterase inhibitors (ChEI). Gas chromatography-mass spectrometry (GC-MS) of the less polar alkaloidic fractions obtained from the acid-base extraction of the stalk of *T. laeta* revealed thirteen monoindole alkaloids, four of them confirmed by co-injection with previously isolated alkaloids. The others were tentatively identified by mass fragmentation analysis. By gas chromatography with flame ionization detection (GC-FID) and using isatin as internal standard, affinisine and voachalotine were determined as major compounds. These fractions and fourteen previously isolated alkaloids, obtained from root bark of *T. laeta* and *T. hystrix* were investigated for acetyl (AChE) and butyrylcholinesterase (BuChE) inhibitory activities by the modified Ellman's method in thin layer chromatography (TLC-ChEI). Results showed selective inhibition of the alkaloids heyneanine and N_b-methylvoachalotine for BuChE, and 19-*epi*-isovoacristine for AChE, whereas olivacine, affinisine, ibogamine, affinine, conodurine and hystrixnine inhibited both enzymes. In addition to confirming that monoterpenoid indole alkaloids can be novel therapeutic agents for AD, this is the first report of the ChEI activity of olivacine, a pyridocarbazole alkaloid.

Key words: *Tabernaemontana*, indole alkaloids, cholinesterase inhibitor, thin-layer chromatography assay, Ellman´s method, GC-MS.

INTRODUCTION

The year 2006 marked a century since the first description of Alzheimer's disease (AD) by Alois Alzheimer. AD, considered the most common neurodegenerative disease that affects the elderly, is characterized by progressive memory loss, decline in language skills and other serious cognitive impairments (Goedert and Spillantini 2006).

The etiology of AD is not very clear and multiple factors, such as amyloid-β peptide (Aβ) and tau protein

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aggregation, excessive transition metals, oxidative stress and reduced acetylcholine (ACh) level, have been implicated (Ji and Zhang 2006). However, it is a cholinergic deficiency rather than other neurochemical deficits that is thought to play a pivotal role in the development of AD symptoms (Farlow 2002). For this reason, inhibition of brain acetylcholinesterase (AChE) has been the major therapeutic target of AD treatment strategies.

Whereas several new natural and synthetic compounds are reported in the scientific literature as cholinesterase inhibitors (ChEI) candidates, this research is continuously improved as better drugs for AD is still required. Within the structure diversity of the ChEI, plant alkaloids are the most studied already leading to the development of new drugs (*e.g.* galanthamine, huperzine and phenserines) (Houghton et al. 2006, Mukherjee et al. 2007).

Monoterpenic indole alkaloids have been extensively investigated for exhibiting numerous biological activities (such as anti-tumor, anti-microbial, anti-hypertensive or as a central nervous system stimulant) (Andrade et al. 2005, Saxton 1997). They can be found in plants of the Apocynaceae, Rubiaceae, and Loganiaceae families and a few have recently been reported as ChEI by different research groups (Hostettmann et al. 2006). Among the AChEI found are some indole glucoalkaloids from *Chimarrhis turbinate* (Rubiaceae) (Cardoso et al. 2004); sarpagan type alkaloids from the *Haplophyton crooksii* (Apocynaceae) (Mroue et al. 1996); iboga alkaloids from *Tabernaemontana australis* (Apocynaceae) (Andrade et al. 2005) and vobasinyl-iboga *bis*-indole alkaloids from *T. divaricata* (Ingkaninan et al. 2006). In fact, a recent article reported the inhibition of cortical AChE activity and the enhancement of neuronal activity of *T. divaricata* extracts in rats, suggesting that it is a reversible AChE inhibitor and could be beneficial as a novel therapeutic agent for AD (Chattipakorn et al. 2007).

The genus *Tabernaemontana* (Apocynaceae) has been used in traditional rejuvenation remedies, believed to improve memory, or central nervous system stimulant (Chattipakorn et al. 2007). It is especially rich in complex mixtures of monoterpenic indole alkaloids and several Brazilian native species have been chemically investigated being good candidates to furnish cholinesterase inhibitory compounds (Batina et al. 2000, Batista et al. 1996, Bolzani et al. 1984, Cardoso and Vilegas 1999, Cardoso et al. 1997, 1998, Henriques et al. 1996, Kato et al. 2002, Lemos et al. 1996, Marques et al. 1996, Medeiros et al. 1999, 2001, Monnerat et al. 2005, Pereira et al. 2004, Rattmann et al. 2005, Zocoler et al. 2005).

In this work we associated two fast screening techniques, a chemical screening by GC-MS and an enzymatic activity screening by Ellman's TLC assay (TLC-ChEI), supported by a collection of previously isolated monoindole alkaloids, in order to suggest a rapid evaluation of potential cholinesterase inhibitors alkaloids from Apocynaceae family.

MATERIALS AND METHODS

General Experimental Procedures

Acetylcholinesterase (AChE) from electric eel (EC 3117); acetylthiocoline iodide (ATCI); butyrylcholinesterase (BuChE) from horse serum (EC 3118); butyrylthiocoline chloride (BTCC); 5,5'-dithiobis-(2-nitrobenzoic acid) (DTNB); Trisma hydrochloride (Tris-HCl) buffer solution, pH 8.0; the reference compound physostigmine and thin layer chromatography (TLC) polyethylene sheets silica gel 60 F254, 0.2 mm thickness were purchased from Sigma (St. Louis, MO, USA).

Gas Chromatography Analyses

Gas chromatography (GC) analyses were carried out on an Agilent GC 6890 gas chromatography (Palo Alto, USA) equipped with a fused silica DB1 (J&W, 30 m $\times$ 250μm $\times$ 0.30μm) capillary column directly coupled to a quadrupole mass spectrometer Agilent 5973. EI-mass spectra were recorded at 70eV. Conditions: injector (splitless mode) at 250°C; oven temperature: 150°C (6 min) to 300°C (20 min) at 4°C min^{-1} and He as carrier gas at 1 mL min^{-1}.Samples were analyzed by 1μL injection of 5 mg mL^{-1} solutions in CH_2Cl_2. Isatin (0.5 mg mL^{-1}) was used as internal standard.

Plant Material

The stalk of *Tabernaemontana laeta* Mart. was collected in July 2004 at the Botanical Garden of Rio de Janeiro, Rio de Janeiro State, Brazil. A voucher specimen is deposited at the herbarium of the same Institution under the number RB 342087.

Extraction and Isolation of Alkaloids

Stalk of *T. laeta* (150 g) was dried at 25°C for 15 days, ground and extracted on a Soxhlet apparatus with EtOH for 72 h. After reduced pressure concentration, the average yield of samples extracted in triplicate was 1.46 g of a brown residue. Dried ethanolic extracts were suspended in 100 mL of 1% HCl and extracted with EtOAc and $CHCl_3$ (3 × 30 mL each). The pH of the aqueous acidic fraction was adjusted to 3, 5, 7, 9 and 11 with aqueous NH_4OH, extracted with $CHCl_3$ (3 × 30 mL) and dried over anhydrous Na_2SO_4. Triplicates of each pH extract were analyzed by silica gel TLC using CH_2Cl_2: MeOH (9:1) as eluent and staining with 50% H_2SO_4/heat or Dragendorff´s reagent. Under these conditions, fractions related to pH 7 and pH 9 were combined. After solvents evaporation under reduced pressure, concentration triplicate average yielded 14.5 mg (pH 3), 8.4 mg (pH 5), 17.5 mg (pH 7 and pH 9) and 3.9 mg (pH 11).

The alkaloids **1-17** (Table I) were previously isolated by the authors and the experimental procedures are described elsewhere (Andrade et al. 2005, Medeiros et al. 1999, 2001, Monnerat et al. 2005).

TABLE I
ChE inhibitory activity for indole alkaloids from *Tabernaemontana* spp.

Substance	ChEI activity	
	AChE	BuChE
Affinine (**1**)[a]	+[e]	+[f]
Affinisine (**2**)[a]	+[fg]	+[f]
N_b-Methyl affinisine (**3**)[a]	–	–
Conodurine (**4**)[b]	+[f]	+[f]
Coronaridine (**5**)[b]	+[g]	n.t.
Heyneanine (**6**)[b]	–	+[f]
Hystrixnine (**7**)[a]	+[e]	+[f]
Ibogamine (**8**)[a]	+[e]	+[f]
Iboxygaine (**9**)[b]	–	–
Isovoacangine (**10**)[b]	–	–
19-*epi*-Isovoacristine (**11**)[b]	+[f]	–
Olivacine (**12**)[a]	+[f]	+[f]
Voacamine (**13**)[b]	–	–
Voacangine (**14**)[c]	+[g]	n.t.
Voacangine hydroxyindolenine (**15**)[c]	+[g]	n.t.
N_b-Methyl voachalotine (**16**)[b]	–	+[f]
Tabernamine (**17**)[b]	–	–

[a]isolated from *T. histrix*; [b]isolated from *T. laeta*; [c]previously isolated from *T. australis* (Andrade et al. 2005); [e]0.051μmol; [f]0.005μmol; [g]previously assayed for AChEI activity (Andrade et al. 2005); n.t. = not tested in this work.

Cholinesterase Inhibitory Activity Determination (TLC-ChEI)

Acetylcholinesterase and butyrylcholinesterase inhibitory activity was determined using TLC assay method and staining with Ellman's reagent (DTNB), which follows the technique described by Rhee et al. (Rhee et al. 2001). Pure compounds (0.051 and 0.005 mol L^{-1}), fractions (5 mg mL^{-1}) or extracts (10 mg mL^{-1}) were diluted in appropriate solvent, then 1.0μL of each sample was spotted on the silica gel TLC plates and developed with CH_2Cl_2:MeOH; (95:5); 1.0μL of 0.36 mmol L^{-1} physostigmine solution in methanol was also spotted as reference. After developing the TLC plate, enzyme inhibitory activities were detected by spraying the substrate, dye and enzyme. 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. In these experiments the minimum amount (detection limit) of physostigmine that could be recognized as a positive result was 0.07μg.

False-positive reactions were eliminated according to the method described by Rhee et al (Rhee et al. 2003). Briefly, 3 U mL^{-1} AChE was incubated for 15 min at 37°C with 1 mmol L^{-1} of ATCI in buffer solution and then used as the thiocoline spray reagent. The developed TLC sample spots together with the known false-positive *p*-anisaldehyde were analyzed by spraying the dye followed by the thiocholine solution. White spots, indicating false-positive reactions, on a yellow background were recorded, and then compared with the results of the enzymatic assays. Four TLC plates were processed as above for, respectively, AChE, BuChE, and false positive assays, or revealed under UV and with H_2SO_4 50% or Dragendorff´s solutions before heating.

RESULTS AND DISCUSSION

GC-FID and GC-MS Analyses

The stalk of *Tabernaemontana laeta* Mart. collected in July 2004 at the Botanical Garden of Rio de Janeiro, Rio

de Janeiro State, Brazil, was extracted with ethanol and fractionated at five different pH (3, 5, 7, 9 and 11). Silica gel TLC of the fractions showed the presence of alkaloids after Dragendorff´s reagent stain. The less polar fractions observed in TLC (pH 3 and 5) were chosen for GC-MS analysis. By standard co-injection with previously isolated alkaloids from *Tabernaemontana* spp. and tentative identification by mass fragmentation pattern, 13 monoterpenic indole alkaloids were identified (Table II and Fig. 1). GC-MS and GC-FID using isatin as internal standard showed affinisine (**2**) and voachalotine (**23**) as major compounds in both fractions. As shown in Table II, other minor alkaloids with iboga, voacanga and sarpagan skeletons were also identified.

All the structures showed in Figure 1 were based on the more recent literature data and CAS registries.

ChE Inhibition Activity by Modified Ellman's Method in TLC

Some of these alkaloids were previously isolated from *T. australis* crude extracts and exhibited AChE inhibitory activity [affinisine (**2**), coronaridine (**5**), voacangine (**14**) and voacangine hydroxyindolenine (**15**)] (Andrade et al. 2005), suggesting that a major investigation of this class of natural compounds would reveal interesting results. So, the fractions obtained from the stalk of *T. laeta* and a small collection of previously isolated alkaloids such as: conodurine (**4**), heyneanine (**6**), iboxygaine (**9**), isovoacangine (**10**), 19-*epi*-isovoacristine (**11**), voacamine (**13**), N_b-methyl voachalotine (**16**) and tabernamine (**17**) from root bark of *T. laeta* (Medeiros et al. 2001); affinine (**1**), affinisine (**2**), N_b-methyl affinisine (**3**), hystrixnine (**7**), ibogamine (**8**) and olivacine (**12**) from *T. hystrix* (Monnerat et al. 2005) were assayed by Ellman´s TLC method for butyryl and acetylcholinesterase inhibitory activities (BuChEI and AChEI).

From the fractions of pH 3 and 5, four spots showed ChEI activity, three of them were associated to affinisine (**2**), ibogamine (**8**) and olivacine (**12**) and inhibited both enzymes. The last spot was associated with N_b-methyl voachalotine (**16**) and appears to be BuChE selective. Beside this, affinine (**1**), conodurine (**4**) and hystrixnine (**7**), also showed AChE and BuChE inhibitory activities, heyneanine (**6**) and Nb-methyl voachalotine (**16**) inhibited only BuChE and 19-*epi*-isovoacristine (**11**) appears to be AChE selective. From the alkaloids studied, only N_b-methyl affinisine (**3**), iboxygaine (**9**), isovoacangine (**10**), the *bis*-alkaloids voacamine (**13**) and tabernamine (**17**) did not inhibited either of the two enzymes in Elmann's TLC test.

The preliminary activity of the 17 alkaloids assayed in this work indicated that the structure-activity relationship of this class of compounds is complex and should be investigated in detail. Ingkaninan et al. suggested that substitutions at carbons 11', 12' and 16' affect the *bis*-indole alkaloids AChEI activity in Ellman´s assay (Ingkaninan et al. 2006). Although we have to be careful with the preliminary results obtained by TLC assays, this property was not observed in the present work.

Besides the iboga, voacanga and tetrahydro β-carboline sarpagan alkaloids, olivacine (**12**) was the only pyridocarbazole assayed and showed to be an active substance at the smallest quantity used ($0,051\mu$mol). The indication of dual or selective inhibition of BuChE and AChE of some alkaloids also suggested that these compounds can be interesting models to study the biochemical interactions with cholinesterases as well as the pharmacological benefits of such selectivity.

CONCLUSIONS

GC-MS is a valuable tool to screen indole alkaloids such as those with iboga and sarpagan skeletons. These compounds frequently show intense molecular and characteristic diagnostic ions. Present investigation of the stalk of *T. laeta* revealed thirteen alkaloids by GC-MS, whereas only five have been previously described.

The combination of two techniques such as GC-MS and TLC-ChEI assay has led to a rapid chemical and biological screening of already known indole alkaloids. These results also confirm the AChE and BuChE inhibitory activities of monoterpenic indole alkaloids, and corroborate that TLC assay based on Ellman´s method is simpler, fast and inexpensive method to search AChE and BuChE inhibitors from natural sources.

In addition, this is the first report of the ChEI activity of olivacine (**12**), a pyridocarbazole alkaloid.

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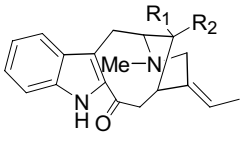
TABLE II

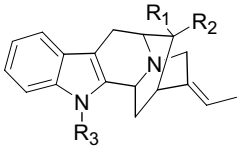
Gas Chromatography-Mass Spectrometry (GC-MS) and Gas Chromatography (GC-FID) analyses of pH3 and pH5 fractions obtained from *T. laeta* stalk extracts

Substance	MW	Main fragments (m/z)	Rt (min)	Rel. area (%)[f]	
				pH 3	pH 5
Ibogamine (**8**)[b]	280	280(40), 195(30), 154(10), 136(100), 135(65), 122(40)	32.26	0.56	–
Olivacine (**12**)[c]	246	246(100), 245(40), 229(5), 204(7), 123(8), 122(7)	35.06	–	2.03
Voacangine hydroxyindolenine (**15**)[c]	384	384(100), 367(60), 225(10), 207 (50), 122 (45)	35.21	0.50	–
n.i. EI[a]	320	320(100), 319(95), 279(10), 212(50), 197(25), 168(8)	36.25	7.16	–
Pericyclivine (**18**)[b]	322	322(65), 321(50), 263(30), 249(15), 169(100), 168(90)	37.12	1.11	–
N_b-Methyl voachalotine (**16**)[c]	381	336(100), 335(90), 321(30), 277(35), 182(70), 168(30)	37.33	3.93	6.27
Normacusine B (**19**)[b]	294	294(85), 279(10), 263(40), 249(10), 169(100), 168(70)	37.33	1.62	–
Affinisine (**2**)[c]	308	308(90), 307(60), 277(30), 249(10), 183(100), 182(80)	37,79	20.48	44.09
Vobasine (**20**)[b]	352	352(8), 336(5), 321(5), 292(5), 194 (8), 180(100)	38.42	–	1.59
n.i.[a]	322	322(100), 249(30), 210(30), 184(20), 168(20), 150(40)	38.89	–	6.18
Coronaridine (**5**)[c]	338	338(100), 323(30), 149(30), 136(95), 135(65), 122(40)	39.17		2.42
n.i.[a]	380	380(100), 321(30), 242(25), 236(20), 212(15), 159(25)	40.78	4.21	–
Dehydrovoachalotine (**21**)[b]	364	364 (26), 333(10), 305(10), 196(30), 182(100), 168(8)	41.12	–	tr
Conopharyngine (**22**)[b]	398	398(100), 274(15), 208(15), 136(90), 135(30), 122 (40)	41.51	4.05	7.95
Voachalotine (**23**)[b]	366	366(100), 365(15), 307 (70), 263 (5), 183(20), 182(10)	41.69	19.15	10.26
Voachalotine oxindole (**24**)[b]	382	382(20), 365(15), 323(10), 279(100), 172(25), 144(20)	42.20	9.69	tr
Amyrin acetate (isomers α/β)[c]	468	468 (5; 5), 218(100; 100); 203 (15; 30); 189(10; 10)	47.58	8.94	–
Total				**81.39**	**90.79**

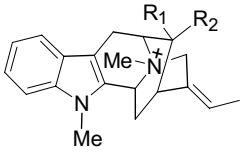
tr – trace; [a]n.i. – not identified with fragment ions measured in EI mode in decreasing order of relative abundance; [b]tentative identification by mass fragmentation pattern; [c]co-injected with isolated substance; [f]relative area corrected by the use of isatin as internal standard.



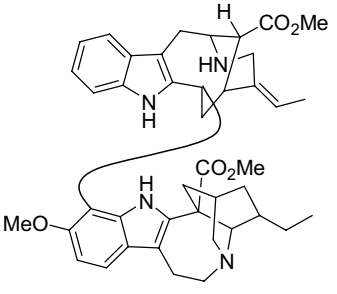
	R_1	R_2
(1) Affinine	CH_2OH	H
(7) Hystrixnine	H	CH_2OMe
(20) Vobasine	CO_2Me	H



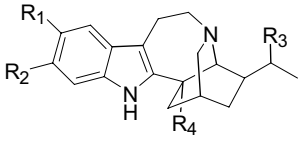
	R_1	R_2	R_3
(2) Affinisine	H	CH_2OH	Me
(18) Peryclyclivine	CO_2Me	H	H
(23) Voachalotine	CH_2OH	CO_2Me	Me



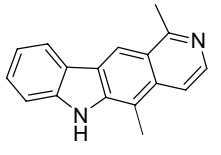
	R_1	R_2
(3) N_b-Methyl affinisine	H	CH_2OH
(16) N_b-Methyl voachalotine	CH_2OH	CO_2Me
(19) Normacusine B	H	CH_2OH



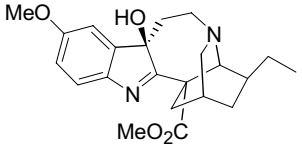
(4) Conodurine



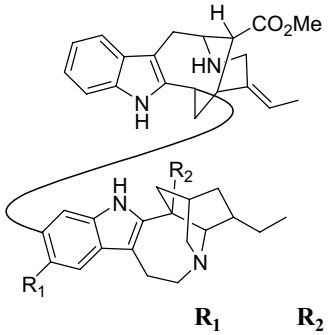
	R_1	R_2	R_3	R_4
(5) Coronaridine	H	H	H	CO_2Me
(6) Heyneanine	H	H	β-OH	CO_2Me
(8) Ibogamine	H	H	H	H
(9) Iboxygaine	OMe	H	β-OH	H
(10) Isovoacangine	H	OMe	H	CO_2Me
(11) 19-*epi*-Isovoacristine	H	OMe	α-OH	CO_2Me
(14) Voacangine	OMe	H	H	CO_2Me
(22) Conopharyngine	OMe	OMe	H	CO_2Me



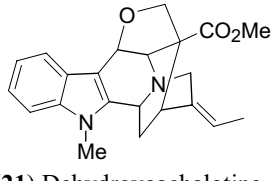
(**12**) Olivacine



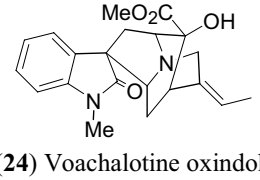
(**15**) Voacangine hydroxyindolenine



	R_1	R_2
(13) Voacamine	OMe	CO_2Me
(17) Tabernamine	H	H



(**21**) Dehydrovoachalotine



(**24**) Voachalotine oxindole

Fig. 1 – Alkaloids from *Tabernaemontana* spp.

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RESUMO

Dentre os tratamentos da doença de Alzheimer (DA) está o uso de inibidores da enzima acetilcolinesterase. Pesquisas recentes visando a descoberta de novos agentes terapêuticos naturais para esta doença sugerem que o gênero *Tabernaemontana* é uma fonte promissora de alcalóides indólicos anticolinesterásicos. Neste trabalho, duas técnicas de análise em mistura foram associadas de modo a identificar facilmente novos inibidores colinesterásicos. A cromatografia em fase gasosa acoplada a espectrometria de massas (CG-EM) das frações alcaloídicas apolares, obtidas da extração ácido-base do caule de *T. laeta*, revelou a presença de treze alcalóides monoindólicos, quatro deles confirmados por co-injeção com padrões previamente isolados. Os outros alcalóides foram tentativamente identificados pelo padrão de fragmentação de massas. Por cromatografia em fase gasosa com detecção por ionização de chama (CG-DIC) e utilizando isatina como padrão interno, affinisina e voachalotina foram identificadas como substâncias majoritárias. As frações alcaloídicas obtidas e os quatorze alcalóides previamente isolados das raízes de *T. laeta* e *T. hystrix* foram analisados quanto à atividade inibitória das enzimas acetil (AChE) e butirilcolinesterase (BuChE) pelo método de Ellman em cromatografia em camada delgada (CCD-ChEI). Os resultados revelaram uma inibição seletiva dos alcalóides heyneanina e N_b-metilvoachalotina para BuChE e de 19-*epi*-isovoacristina para AChE, enquanto que olivacina, affinisina, ibogamina, affinina, conodurina e hystrixnina inibiram ambas as enzimas. Além de confirmar que alcalóides indólicos monoterpênicos são agentes terapêuticos promissores para o tratamento da DA, este é o primeiro relato da atividade anticolinesterásica de olivacina, um alcalóide piridocarbazólico.

Palavras-chave: *Tabernaemontana*, alcalóides indólicos, inibidor colinesterásico, cromatografia em camada delgada, método de Ellman, CG-EM.

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