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Anticholinesterase activity evaluation of alkaloids and coumarin from stems of *Conchocarpus fontanesianus*

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Abstract: *Conchocarpus fontanesianus* (A. St.-Hill.) Kallunki & Pirani, Rutaceae, popularly known as *pitaguará*, is a native and endemic tree from São Paulo and Rio de Janeiro States, Brazil. Based in the information that anticholinesterasic derivatives could act as new prototypes to treatment of Alzheimer disease, this work describes the fractionation guided by evaluation of the anticholinesterase activity of the ethanolic stems extract from *C. fontanesianus.* This procedure afforded the alkaloids dictamnine (**1**), γ-fagarine (**2**), skimianine (**3**), and 2-phenyl-1-methyl-4-quinolone (**4**), as well as the coumarin marmesin (**5**).

Introduction

The genus *Conchocarpus* (Rutaceae) comprises 45 species distributed in Central and South America (Kallunki & Pirani, 1998). In Brazil, the genus can be found mainly in Amazonian, Atlantic Forrest and Cerrado regions (Pirani, 2010). Phytochemically, several constituents have been found in this genus, mainly alkaloids and flavonoids. Cortez et al. (2009) identified from the stems of *C. gaudichaudianus* four alkaloids, three indoloquinazolonic type and one quinoline type, along with two amides, *N-trans*-cumaroyltyramine, and *N-trans*-feruloyltyramine. Ambrozin et al. (2008) isolated from leaves of *C. heterophyllus* simple and hydroxylated flavones, and a mixture of new piranoflavones, while from the stems were isolated steroids, flavones, and alkaloids (haplotusine and 2-phenyl-1-methyl-4-quinolone). Additionally, from the leaves of *C. paniculatus*, Vieira et al. (1992) isolated two acridone alkaloids (cuspanine and cusculine).

Conchocarpus fontanesianus (A. St.-Hill.) Kallunki & Pirani, Rutaceae, popularly known as pitaguará, is Brazilian native and endemic species, distributed in Atlantic Rain Forest, mainly in the states of São Paulo and Rio de Janeiro (Pirani, 2002). Despite of the antifungal, antitumoral, and antimicrobial activities from leaves extract has been already described (Agripino et al., 2004), there is no information about the phytochemicals isolated from this plant.

The Alzheimer disease (AD), a neurodegenerative disease of a great socio-economic impact, is associated with deficits in brain neurotransmitters (Guela & Mesulam, 1994). The symptomatic treatment of AD primarily involves restoration of cholinergic function (Francis et al., 1999; Trevisan, 2003). The inhibition of acetylcholinesterase (AChE) is the most effective therapeutic approach to optimize the cholinergic system in patients with AD. Several plant-derived drugs (rivastigmine and galanthamine) that inhibit AChE can be used to treat early stages of AD, since these compounds increase the endogenous levels of acetylcholine to boost cholinergic neurotransmission (Scott & Goa, 2000). In a recent review, 260 chemically defined natural molecules were evaluated for AChE inhibition. The compounds tested were classified in alkaloids (139), monoterpenes

(27), coumarins (18), triterpenes (17), flavonoids (14), benzenoids (13), diterpenes (8), oxygen heterocycles (5), sesquiterpenes (5), stilbenes (3), lignans (2), sulfur compounds (2), proteids (2), polycyclic (1), quinoid (1), benzoxazinone (1), carotenoid (1) and alycyclic (1) (Howes et al., 2003; Barbosa-Filho et al., 2006; Houghton et al., 2006). In this sense, the discovery of new compounds from natural sources with anticholinesterase activity is of great interest.

In continuation with trials conducted with native species of Cerrado and Atlantic Rain Forest, the crude ethanol (EtOH) extract from the stems of *C. fontanesianus* showed anticholinesterase potential. Thus, the aim of this work was perform the chemical study of the stems of *C. fontanesianus*, guided by inhibitory AChE activity assays.

Material and Methods

General experimental procedures

High performance liquid chromatography (HPLC) was performed using Varian Pro Star 310 with loop of 20 µL, detection in UV detector at 242 nm, using mobile phase acetonitrile (ACN), methanol (MeOH) and deionized water (H_2O, milli Q) in the proportion of 10:45:45 v/v (isocratic mode) and a flow at 1 mL/min, during 30 min. LC-MS (Liquid Chromatography/Mass Spectormetry) and LC-DAD (Liquid Chromatography/ Photodiode Array Detector) analyzes were recorded in a Shimadzu equipment, using a pump LC-10AD vp, UV-Vis detector SPD-M10A vp, SIL-10AF autosampler, and a Phenomenex Luna C-18 reverse phase column (250 x 4.6mm - 5µ). Mass spectra obtained by HRESIMS (positive mode) were recorded in a Micromass QTof MS spectrometer (Manchester, UK), with the electrospray ionization source and TOF analyzer. The general conditions used were: source temperature 100 °C, capillary voltage of 2.1 kV and cone voltage of 40 V. All data were obtained in positive mode (+) and were treated using the software Mass Lynx 3.5 (Waters, Manchester, UK). The samples were dissolved in MeOH (100+0.1% HCO_2H) and analyzed by direct insertion. Analyses by GC-LREIMS (Gas Chromatography/Mass Spectrometry) were performed using a gas chromatography Agilent 6890 Series with an HP-5MS apolar column (30 m x 0.25 mm - 0.25 µm film thickness) coupled with mass spectrometer Agilent 5937 (electron impact ionization at 70 eV), ion source temperature of 230 °C, with a quadrupole analyzer. Helium has used as carrier gas and general conditions were: initial temperature 100 °C (1 min), increasing 15 °C/min to 300 °C, holding at 300 °C (10 min). Preparative TLC were performed on glass plates of 20 x 20 cm (0.5 mm thickness) of silica gel 60 PF_{254} (Merck).

Plant material and extraction

Stems of *Conchocarpus fontanesianus* (A. St.-Hil.) Kallunki & Pirani, Rutaceae, were collected at Ecological Station Jureia-Itatins (license SMA process n° 260.108-007.532 2009), Nucleus Arpoador/Peruíbe, São Paulo State, Brazil (24° 22'58.5" S, 47° 01'07.5" W, 38 m above sea level) and identified by Dra. Inês Cordeiro. A voucher specimen (RSCabral 09) has been deposited at the Herbário do Estado 'Maria Eneyda P. Kaufmann Fidalgo (SP)`, São Paulo, SP, Brazil.

The powder of the stems of *C. fontanesianus* (534.23 g) was submitted to extraction with EtOH under pressure (1500-1700 psi) at 60 °C using an ASE300 extractor (DIONEX). The EtOH solution was concentrated in vacuum, to give crude EtOH extract (EE).

Compounds isolation procedures

EtOH extract (14.53 g) was partially dissolved in aqueous acid solution (0.1 M HCl), filtered and the soluble acid solution was partitioned with hexane, yielding the *n*-hexane fraction (0.0963 g). The acid aqueous fraction was treated with NH_4OH (pH 10) and partitioned with $CHCl_3$ obtaining the alkaloid fraction (0.3134 g). All fractions were evaluated for their anticholinesterase potential.

The purification of alkaloid fraction was performed by prep TLC, eluted with $CHCl_3$:MeOH:NH_4OH (90:7.5:2.5 v/v). This procedure afforded a fraction composed by **1** + **2** (26.5 mg), and compounds **3** (31.0 mg), **4** (27.7 mg), and **5** (35.2 mg).

Dicatmine (**1**). Yellow amorphous solid. HPLC R_t = 16.6 min. UV λ_{max} (log ε): 240 (3.93), 311 (3.22). HRESIMS: $[M+H]^+$ *m/z* 200.0688, calc. to $C_{12}H_{10}O_2N$ 200.0712. LREIMS *m/z* (int. rel.): 199 (M^+, 100), 184 (72), 156 (36), 130 (19), 128 (36), 101 (26), 76 (25), 75 (20), 50 (17).

γ-*Fagarine* (**2**). Yellow amorphous solid. HPLC R_t = 14.7 min. UV λ_{max} (log ε): 242 (4.78), 308 (3.81). HRESIMS: $[M+H]^+$ *m/z* 230.0814, calc. to $C_{13}H_{12}O_3N$ 230.0818. LREIMS *m/z* (int. rel.): 229 (M^+, 100), 228 (82), 214 (34), 200 (83), 199 (36), 185 (26), 184 (31), 156 (41).

Skimiamine (**3**). Yellow amorphous solid. HPLC R_t = 12.5 min. UV λ_{max} (log ε): 251 (3.77), 328 (3.04). HRESIMS: $[M+H]^+$ *m/z* 260.0862, calc. to $C_{14}H_{13}O_4N$ 260.0923. LREIMS *m/z* (int. rel.): 259 (M^+, 61), 258 (25), 244 (100), 230 (56), 229 (27), 228 (23), 216 (24), 213 (27), 201 (26), 199 (16).

2-Phenyl-1-methyl-4-quinolone (**4**). Yellow amorphous

solid. HPLC R_t = 7.88 min. HRESIMS: $[M+H]^+$ *m/z* 236.1011, calc. to $C_{16}H_{14}ON$ 236.1076. LREIMS *m/z* (int. rel.): 235 (M^+, 76), 207 (100), 165 (19), 130 (11), 102 (18), 89 (17), 77 (37), 51 (25).

Marmesin (**5**). Yellow amorphous solid. HPLC R_t = 6.57 min. UV λ_{max} (log ε): 212 (3.89), 335 (4.09). HRESIMS: $[M+H]^+$ *m/z* 247.0993, calc. to $C_{14}H_{15}O_4$ 247.0971. LREIMS *m/z* (int. rel.): 246 (M^+, 28), 213 (21), 188 (58), 187 (100), 160 (31), 131 (26), 77 (17), 59 (74), 51 (15), 43 (28).

Inhibitory activity of acetilcholinesterase (IAChEs)

Qualitative evaluation procedure

The AChE inhibitory activity of EtOH crude extract, alkaloid fraction, hexane fraction and purified compounds were detected using a TLC autographic assay as previously described (Marston et al., 2002). The TLC layers were spotted with 200, 100 and 50 µg for crude extract, fractions and purified compounds, respectively, developed with $CHCl_3$:MeOH:NH_4OH (90:7.5:2.5 v/v) and subsequently dried. The plates were then sprayed with the enzyme solution (6.66 U/mL), thoroughly dried, and incubated at 37 °C for 20 min (moist atmosphere). Enzyme activity was detected by spraying with a solution consisting of 0.25% of 1-naphthyl acetate in EtOH plus 0.25% aqueous solution of Fast Blue B salt. Potential acetylcholinesterase inhibitors appeared as clear zones on a purple colored background. Electric eel AChE type V (product no. C 2888, 1000 U) and the other reagents were purchased from Sigma-Aldrich.

Quantitative evaluation

A Biotek ELISA reader, model Power Wave, "KC4 software" was used to establish each reation rate. In each well of a 96-well microplate was added 25 mL acetylthiocholine iodide (15 µM), 125 µL of 5,5'-ditiobis-[2-nitrobenzoic] in solution C (3 µM DTNB or Ellman's reagent), 50 µL of solution B, 25 µL of samples (EtOH crude extract, alkaloid fraction, *n*-hexane fraction and purified compounds) dissolved in MeOH and diluted in solution A at concentrations of 0.78-200 µg/mL (Rhee et al., 2001; Trevisan et al., 2003). The absorbance was measured at 405 nm for 30 s. Then, 25 µL of the enzyme AChE (0.22 U/mL) was added and absorbance was measured again after 5, 10, 15 and 20 min of incubation of the enzyme. The percentage of AChE inhibition was calculated by comparing the reaction rates (hydrolysis of the substrate) samples as compared to negative control (10% MeOH in the solution A, considered the total activity of AChE, 100%). The standard used as positive control was physostigmine at concentrations of 0.097-200 µg/mL. Solutions: A. Tris/HCl 50 mM, pH 8; B. Tris/HCl 50 mM, pH 8, with 0.1% bovine albumin fraction V; C. Tris/HCl 50 mM, pH 8, with NaCl (0.1 M) and $MgCl_2.6H_2O$ (0.02 M) (Ellman et al., 1961 modified by Rhee et al., 2001).

Statistical analysis

Data were presented as means±SEM of experiments realized at least in triplicate. The IC50 values were calculated by means of regression analysis.

Results and Discussion

Partition of EtOH crude extract from stems of *Conchocarpus fontanesianus* (A. St.-Hil.) Kallunki & Pirani, Rutaceae, gave *n*-hexane and alkaloid fractions (0.63 and 2.15% yield, respectively). The results of the TLC qualitative evaluation of the EtOH extract (EE), *n*-hexane (HEX), and alkaloid (ALK) fractions indicated that all presented AChE inhibitory potential, being more intense in hexane and alkaloid fractions (Figure 1). Similarly, in quantitative analysis, the EE extract, the HEX and ALK fractions inhibited AChE activity (Table 1 and Figure 2), being the greatest potential observed to ALK fraction (IC50 31.73±0.45 µg/mL).

Figure 1. TLC qualitative acetylcholisterase assay. 1. EtOH crude extract (200 µg); 2. Alkaloid fraction (100 µg); 3. Hexane fraction (100 µg), and 4. Physostigmine (0.05 µg). TLC elution system: $CHCl_3$:MeOH:NH_4OH, 90:7.5:2.5 v/v.

The purification of the alkaloid fraction by preparative TLC afforded compounds **1-5**. In the case of compounds **1-3**, the absorption bands at λ_{max} 240-

251 and 308-328 nm, were indicative of furoquinoline alkaloids (Grundon & McCorkindale, 1957; Cortez et al., 2006). HRESIMS showed ion peaks $[M+H]^+$ at *m/z* 200.0688, 230.0814 and 260.0862 corresponding, respectively, to molecular formulas $C_{12}H_9NO_2$, $C_{13}H_{11}NO_3$, and $C_{14}H_{13}NO_4$. This information, associated with fragmentation patterns observed in GC-LREIMS and comparison with those described in the literature (Paulini et al., 1989; Inada et al., 2008; Li et al., 2011), allowed the identification of dictamnine (**1**), γ-fagarine (**2**) and skimianine (**3**). Compound **4** showed a ion peak $[M+H]^+$ at *m/z* 236.1011 in the HRESIMS spectrum, corresponding to molecular formula $C_{16}H_{13}NO$ and a fragmentation pattern characteristic of quinolone alkaloid (Biavatti et al., 2002). Comparison of MS data with those recorded in the literature, allowed its identification as 2-phenyl-1-methyl-4-quinolone (Ambrozin et al., 2008). UV spectrum of 5 showed absorptions at λ_{max} at 212 and 335 nm, characteristic of furanocoumarins (Abu-Mustafa & Fayez, 1961; Joo et al., 2004). The ion peak $[M+H]^+$ at m/z 247.0993, corresponding to molecular formula $C_{14}H_{14}O_4$, associated with LREIMS fragmentation pattern and comparison with literature data (Trumble & Milla, 1996), allowed the identification of the compound **5** as marmesin.

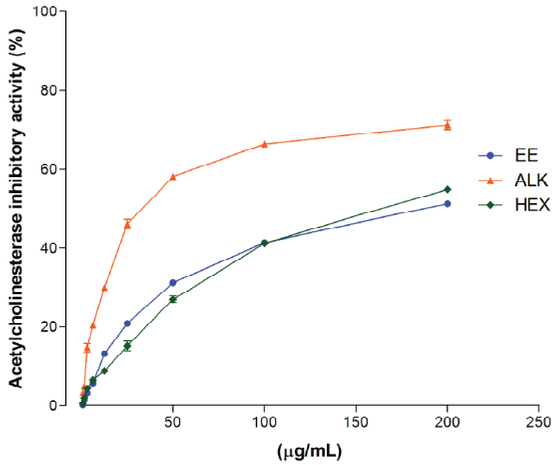
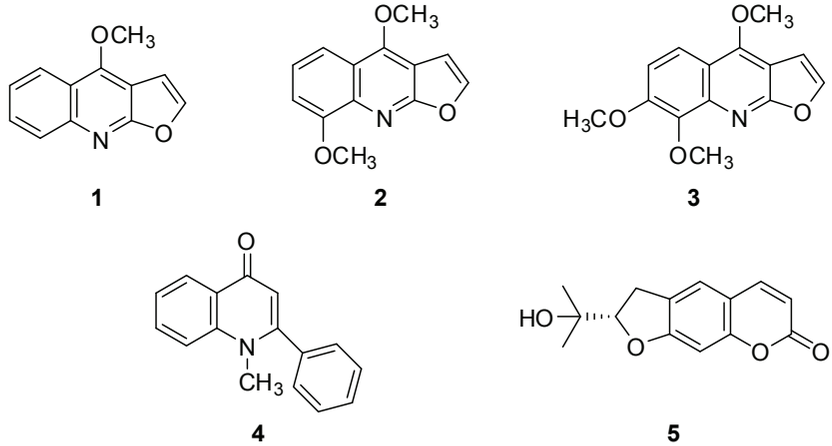


Figure 2. Acetylcholinesterase inhibitory potential of the EtOH crude extract (EE), alkaloid fraction (ALK) and hexane fraction (HEX) (0.78 to 200 µg/mL).

Compounds **1-5** showed AChE inhibitory potential in the TLC qualitative (Figure 3) as well as in the quantitative assay, as shown in Table 2. TLC qualitative assay indicated that more actives compounds

Table 1. Acetylcholinesterase inhibitory potential of the EtOH crude extract, alkaloid fraction and hexane fraction (0.78 to 200 µg/mL).

Concentration (µg/ml)	Ethanolic crude extract % of inhibition±SEM	Alkaloid fraction % of inhibition±SEM	Hexane fracion % of inhibition±SEM
0,78	0,169±0,089	3.305±0,242	0,450±0,238
1,56	1.435±0,206	5.115±0,192	2.025±0,192
3,12	3.253±0,18	14.725±0,628	4.520±0,139
6,25	5.883±0,079	20.416±0,28	6.663±0,05
12,50	13.234±0,425	30.061±0,441	8.966±0,108
25,00	20.852±0,326	46.008±0,672	15.190±0,698
50,00	31.248±0,723	58.163±0,255	26.997±0,494
100,00	41.356±0,631	66.326±0,195	41.159±0,31
200,00	51.234±0,518	71.087±0,658	54.945±0,224



were furocoumarin (**5**) and quinolone alkaloid (**4**). In the quantitative assay the mixture of alkaloids dictamnine (**1**) and γ-fagarine (**2**) reduced the activity of AChE from 1.12±0.14 to 52.0±0.2% while alkaloid skimmianine (**3**) reduced the activity from 0.98±0.26 to 67.21±0.29%. Similarly, alkaloid 2-phenyl-1-methyl-4-quinolone (**4**) reduced the activity of AChE of 0.34±0.09 to 48.60±0.23%. Coumarin marmesin (**5**) reduced the activity from 0.61±0.13% to 52.87±0.25% (Figure 4).

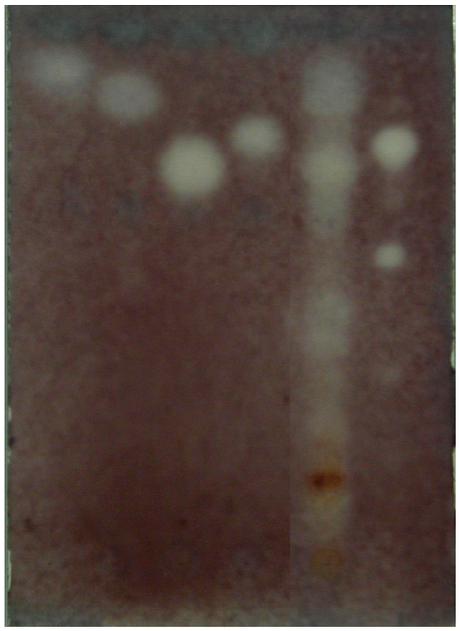


Figure 3. TLC qualitative acetylcholisterase assay. **1.** dictamin+γ-fagarine (50 µg), **2.** skimianine (50 µg), **3.** marmesin (50 µg), **4.** 2-phenyl-1-methyl-4-quinolone (50 µg), **5.** Alkaloid fraction (100 µg), and **6.** physostigmine (0.05 µg). TLC elution system: $CHCl_3$/MeOH/NH_4OH (90:7.5:2.5 v/v).

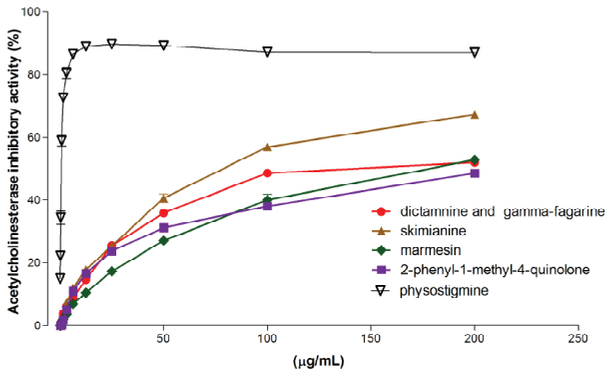


Figure 4. Quantitative evaluation of the anticholinesterase activity of the purified compounds (0.78 to 200 µg/mL), compared with the standard physostigmine.

Comparatively, alkaloid skimmianine (3) showed to be more active in the AChE quantitative assay with IC50 74.09±0.33 mg/mL. However, despite of the interesting potential detected to compound 3, this result was higher than that calculated to positive control physostigmine (IC50 0.61±0.037 µg/mL). Skimianine (3) was isolated from alkaloid fraction of *Esenbeckia leiocarpa*, and showed weaker inhibitory potential (IC50 1.4 mM or 362.6 µg/mL) when compared to reference compounds (Cardoso-Lopes et al., 2010). In this same work, the authors evaluated the AChE inhibitory activity of compounds obtained from alkaloid fraction from stems of *Esenbeckia leiocarpa* (Rutaceae), being the more active leptomerine (IC50 2.5 µM), with potential close to the reference compounds physostgmine (IC50 0.4 µM) and galantamine (IC50 1.7 µM). Comparatively, leptomerine showed a similar structure of compound 4, which among the isolated alkaloids, showed the lower potential. Thus, the obtained data suggested that the promising inhibitory potential of leptomerine could be

Table 2. Acetylcholinesterase inhibitory potential of the purified compounds (0.78 to 200 µg/mL).

Concentration (µg/mL)	Dictamnine plus γ-Fagarine	Skimianine	Marmesin	2-Phenyl-1-methyl-4-quinolone	Physostigmine
	% of inhibition±SEM	% of inhibition±SEM	% of inhibition±SEM	% of inhibition±SEM	% of inhibition±SEM
0,78	1.449±0,314	0,985±0,259	1.285±0,662	0,347±0,096	58.976±0,91
1,56	3.802±0,529	3.12±0,379	2.064±0,425	1.839±0,376	72.639±0,324
3,12	6.131±0,665	7.295±0,498	3.584±0,061	5.041±0,315	80.540±1,032
6,25	8.876±0,193	11.98±0,598	6.954±0,657	11.060±0,407	86.671±0,124
12,50	14.622±0,798	17.780±0,154	10.550±0,272	16.597±0,494	89.066±0,174
25,00	25.507±0,601	25.398±0,363	17.194±0,357	23.742±0,344	89.736±0,121
50,00	35.822±0,886	40.562±0,786	27.089±0,574	31.237±0,673	89.296±0,147
100,00	48.652±0,503	57.026±0,323	39.985±1,073	37.928±0,217	87.296±0,085
200,00	52.002±0,203	67.213±0,298	52.869±0,257	48.606±0,235	87.158±0,401

explained by structural difference, since the compound 4 contains a phenyl substituent in place of n-propyl.

In this paper, the IAChE property of extracts, fractions and purified compounds from the stems of *C. fontanesianus* as well as the identification of dictamnine, γ-fagarine, skimianine, 2-phenyl-1-methyl-4-quinolone and marmesin, already isolated from other species of the family Rutaceae, are herein reported for the first time. As previously mentioned, the search for new compounds which could act as inhibitor of AChE from Brazilian vegetal species is of great interest, since some of the substances already used as medicine for the treatment of Alzheimer's, present limitations such as low bioavailability and high hepatotoxicity.

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