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Original Article

Maytenus distichophylla and *Salacia crassifolia*: source of products with potential acetylcholinesterase inhibition

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ABSTRACT

The phytochemical study of the extract leaves from *Maytenus distichophylla* Mart. and *Salacia crassifolia* (Mart. ex Schult.) G. Don, Celastraceae, resulted in the isolation of 3-oxofriedelane, 3β-hydroxyfriedelane, 3β,24-dihydroxyfriedelane, 3-oxo-28,29-dihydroxyfriedelane, two mixtures of pentacyclic triterpenes (α-amyrin with β-amyrin and 3β-stearyloxy-urs-12-ene with 3β-stearyloxy-olean-12-ene), 3β-palmityloxy-urs-12-ene, the steroid β-sitosterol and its glycosylated derivative β-glucosyl-β-sitosterol, tritriacontanoic acid and the natural polymer gutta percha. The chemical structures of these constituents were established by IR, ^{1}H and ^{13}C NMR spectral data. Crude extracts, the mixtures of triterpenes and the isolated constituents were subjected to *in vitro* acetylcholinesterase inhibitory evaluation. Acetylcholinesterase inhibitory effect was observed for crude chloroform extract leaves from *M. distichophylla* (100%) and *S. crassifolia* (97.93 ± 5.63%) and for the triterpenes 3β,24-dihydroxyfriedelane (99.05 ± 1.12%), 3-oxo-28,29-dihydroxyfriedelane (90.59 ± 3.76%) and 3β-palmityloxy-urs-12-ene (97.93 ± 1.47%). The percent inhibitions induced by these natural products were very similar to those produced by physostigmine (93.94 ± 2.10%) a standard acetylcholinesterase inhibitor. Therefore, these results open perspectives for the use of these species as source of compounds with similar physostigmine pharmacological effect.

Introduction

Neurodegenerative diseases result from chronic breakdown and progressive functional or structural loss of neurons, particularly those of the central nervous system (CNS). The accumulation of aggregated proteins at neurons has been correlated to these types of diseases (Park, 2010). The neurodegeneration process observed in Alzheimer's disease (AD) has been characterized by progressive dementia and memory loss. Elevated levels of the peptide β-amyloid (Aβ) are associated with alterations of the synaptic function and neural network activity that probably underlies the cognitive deficits that occur in AD. Furthermore, the accumulation of this toxic peptide leads to deposition of Aβ into plaques and is thought to drive a pathologic cascade, which culminates in neuronal death (Cramer et al., 2012). The loss of cholinergic cells is accompanied by a decrease in the concentration of the acetylcholine (ACh). This endogenous compound is hydrolyzed by acetylcholinesterase (AChE), a hydrolytic enzyme of the serine class that is of major importance to physiology of the cholinergic synapses of the somatic system, the autonomic nervous system and the central nervous system (CNS) (Triggle et al., 1998). Therefore, one of the current accepted strategies in pharmacotherapy of AD has been the use of AChE inhibitors (Yang et al., 2012). As example, physostigmine (eserine) exerts a stereoselective inhibition of cholinesterase enzymes, such as AChE and butyrylcholinesterase (BuChE) by acting as a competitor or pseudosubstrate and transferring a carbamate residue to the enzyme's active site. Spontaneous hydrolysis regenerates the native enzyme and its function (Triggle et al., 1998).

The current drugs that act inhibiting AChE produce limited therapeutic results against AD, however, primarily provide a short-term alleviation of the symptoms, without blocking the progression of disease (Park et al., 2012). Until this moment, the development of more efficient AChE inhibitors, which act mainly in brain, has been

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considered as an effective approach to be applied for treating AD (Liu et al., 2013).

The nature is a rich source of biological and chemical diversity. Complex chemical structures isolated from natural products cannot be easily obtained by synthesis or semi synthesis in laboratories (Filho et al., 2006). The natural compounds, represented by the class of pentacyclic triterpenes (PCTT), are secondary plant metabolites that have a potential inhibitory property of AChE (Gurovic et al., 2010). The PCTT with skeleton lupane and friedelane can be included amongst the compounds to be used to treat CNS disorders observed in AD (Rodrigues et al., 2014).

The Celastraceae family represents a good source of PCTT that are of great interest, due to their wide range of biological activities (Silva et al., 2013). Species of this family, like *Maytenus ilicifolia*, have been used in traditional medicine of different regions of Brazil, for the treatment of gastric ulcers (De Andrade et al., 2007), inflammations, and diarrhea (Santos et al., 2007). In addition, the pharmacological potential of some Celastraceae species have being evidenced through its traditional use in Northeast of Brazil, as CNS stimulant, and to treat insomnia and migraine (Omena, 2009).

In the present work extracts and constituents from two species of the Celastraceae family, *Maytenus distichophylla* and *Salacia crassifolia*, were investigates in relation to the *in vitro* AChE inhibitory activity.

Materials and methods

^{1}H (400 MHz) and ^{13}C (100 MHz) NMR experiments were carried out on a Bruker *Avance* DRX-400, operating at 300 K. The chemical shifts assignments (δ) were expressed in parts-per-million (ppm) and coupling constants (J) registered in Hertz (Hz). Tetramethylsilane (TMS) was used as internal standard ($\delta_H = \delta_C = 0$). The infrared spectra (IR) (1% KBr soln, 400–4000 cm^{-1}) were obtained on Shimadzu IR408 spectrometer. Melting points were determined on MQAPF-302 apparatus (Microquímica Equipamentos Ltda).

Column chromatography (CC) processes were performed using silica gel 60 [0.063–0.200 mm (70–230 mesh ASTM)], as stationary phase, and organic solvent pure, or in mixtures of crescent polarity, as mobile phase. Silica gel 60 (Merck) was used to prepare plates (0.25 mm) for analytic thin layer chromatography (TLC).

The leaves of *Maytenus distichophylla* Mart., Celastraceae, were collected in Jequié, Bahia, Brazil, and the species was identified by Dra. Guadalupe Licona de Macedo of Departamento de Botânica of Universidade Estadual do Sudoeste da Bahia (UESB), Brazil. A voucher specimen (No. HUESB 2093) was deposited in the *Herbarium* of Departamento de Botânica of UESB. The leaves of *Salacia crassifolia* (Mart. ex Schult.) G. Don, Celastraceae, were collected in Montes Claros, Minas Gerais, Brazil, and the species was identified by Dra. Maria Olívia Mercadante-Simões of Universidade Estadual de Montes Claros, Brazil. A voucher specimen (No. HBCB 144624) is preserved in the *Herbarium* of Instituto de Ciências Biológicas, UFMG. The plant materials were collected in accordance with authorization (Process: 010119/2014-0) to access to the genetic patrimony emitted by Conselho Nacional de Desenvolvimento Científico e Tecnológico (CNPq), Brazil.

The leaves of *M. distichophylla* and *S. crassifolia* were dried at room temperature and fragmented on a mill, separately. Each powdered material was sequentially extracted with hexane, chloroform and ethyl acetate.

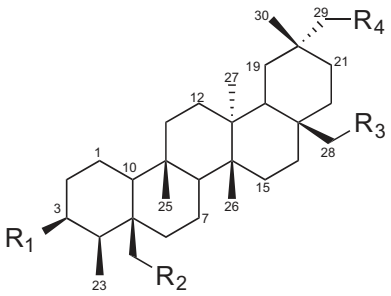
The chloroform extract (35.6 g) from *M. distichophylla* was subjected to silica gel CC, initially eluted with MeOH and then with $CHCl_3$, the last solvent yielded compound **13** (6 g). After removal the solvent, the fractions eluted with MeOH were submitted to successive silica gel CC, furnishing the constituents **7** and **8** as mixture (178 mg; hexane–$CHCl_3$ 70:30), **1** (55 mg; hexane–$CHCl_3$ 70:30), **2** (23 mg; hexane–$CHCl_3$ 60:40), **5** and **6** as mixture (35 mg; hexane–$CHCl_3$ 15:85), **10** (18 mg; hexane–EtOAc 95:05), **3** (272 mg; hexane–EtOAc 70:30), **12** (35 mg; $CHCl_3$–EtOAc 95:05), and **11** (87.3 mg; hexane–EtOAc 30:70).

The chloroform extract (44 g) from *S. crassifolia* was subjected to silica gel CC eluted with MeOH followed by $CHCl_3$ allowing the isolation of compound **13** (30 g). The fractions obtained with methanol, after the removal the solvent, were submitted to successive silica gel CC, furnishing the constituents, **9** (11.2 mg; hexane–$CHCl_3$ 80:20), **1** (10.2 mg; hexane–$CHCl_3$ 25:75), **2** (9.7 mg; hexane–$CHCl_3$ 15:85), **5** and **6** as mixture (3.7 mg; hexane–$CHCl_3$ 10:90), **10** (11.8 mg; hexane–$CHCl_3$ 5:95), **4** (24.4 mg; $CHCl_3$–EtOAc 27:75) and **12** (167.3 mg; $CHCl_3$–EtOAc 10:90).

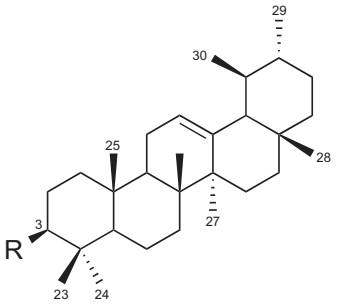
The *in vitro* AChE inhibitory activity of extracts and constituents was evaluated using a 96-well microtiter plate following the Ellman's method (Ellman et al., 1961). The buffer A (50 mM Tris–HCl, pH 8, containing 0.1 M NaCl and 0.02 M $MgCl_2 \cdot 6H_2O$), B (50 mM Tris–HCl, pH 8, containing 0.1% bovine serum albumin), and C (50 mM Tris–HCl, pH 8) were prepared and used in this assay. The volumes of 25 μl of ACh iodide (15 mM in water), 125 μl of 5,5-dithiobis-(2-nitrobenzoic acid) (3 mM in buffer A), 50 μl of buffer B, and 25 μl of sample (10 mg/ml in MeOH diluted by 10 times with buffer C, providing a final concentration of 1 mg/ml) were added into each well of a 96-well microtiter plate. Instead of the addition of sample solution, 25 μl of buffer C was used to prepare the blank sample. The positive control physostigmine was prepared using similar procedure. Each assay was carried out in quintuplicate. The absorbance was measured at 405 nm every 60 s by eight times using an Elisa Thermoplate microplate reader. After addition of 25 μl of AChE solution (0.226 U/ml in buffer B), the absorbance was again measured every 60 s, for 10 times. The increase in absorbance relative to spontaneous hydrolysis of substrate was corrected by reaction rate variation before and after addition of the enzyme. The inhibition percentage was calculated by comparing the results produced by the samples and physostigmine, in relation to blank.

Results and discussion

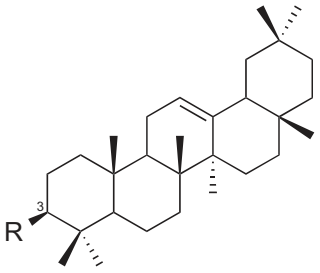
Using phytochemical methods the following known compounds were isolated and identified of the leaves from *M. distichophylla* and *S. crassifolia*: 3-oxofriedelane (**1**) (Mahato and Kundu, 1994), 3β-hydroxyfriedelane (**2**) (Mahato and Kundu, 1994), 3β,24-dihydroxyfriedelane (**3**) (Costa and Carvalho, 2003) and 3-oxo-28,29-dihydroxyfriedelane (**4**) (Weeratunga et al., 1982), mixture of α-amyrin (**5**) and β-amyrin (**8**) (Mahato and Kundu, 1994), mixture of derivates of PCTT 3β-stearyloxy-urs-12-ene (**6**) (Miranda et al., 2006) and 3β-stearyloxy-olean-12-ene (**9**) (Vieira-Filho et al., 2003), 3β-palmityloxy-urs-12-ene (**7**) (Vieira-Filho et al., 2003), the steroid β-sitosterol (**10**) (Lendl et al., 2005) and its glycosylated derivative β-glucosyl-β-sitosterol (**11**) (Lendl et al., 2005), tritriacontanoic acid (**12**) (Hamdan et al., 2014), and the natural polymer gutta percha (**13**) (Oliveira et al., 2006). Even though occurring in distinct biomes, located about 1100 km far from one another, both species, *M. distichophylla* and *S. crassifolia* presents the compounds **1**, **2**, **5**, **10**, **12** and **13**. This fact contributes to confirm that species of the Celastraceae family uses similar biosynthetic routes to produce its secondary metabolites. The main chemical shift assignments observed in the NMR spectra of compounds **1**–**13** are presented below.



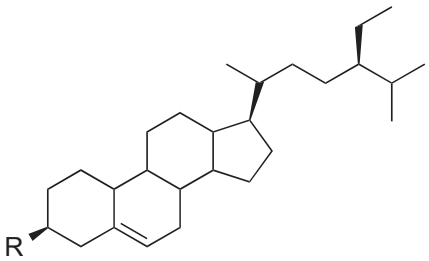
1 R_1=O; R_2=R_3=R_4=H
2 R_1=OH; R_2=R_3=R_4=H
3 R_1=R_2=OH; R_3=R_4=H
4 R_1=O; R_2=H; R_3=R_4=OH

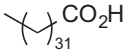


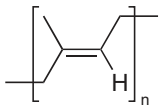
5 R=OH
6 R=OC=O$(CH_2)_{16}CH_3$
7 R=OC=O$(CH_2)_{14}CH_3$



8 R=OH
9 R=OC=O$(CH_2)_{16}CH_3$



10 R=OH
11 R=*O*-D-glucose
12 

13 

In the ^{1}H NMR spectra of PCTT **1**–**4**, a doublet signal at δ_H 0.87 (J = 6.80 Hz) was observed and it is in agreement to hydrogen of methyl C-23 of the friedelane skeleton. The ^{1}H and ^{13}C NMR spectra of constituent **5** present signals at δ_H 5.13 (J = 3.60 Hz) and at δ_C 124.45 and δ_C 139.61, which are characteristic of ursane skeleton. The signal of a multiplet at 5.30, in the ^{1}H NMR, associated to the signals of carbinolic (80.60) and carbonyl (173.69) carbons, observed in the ^{13}C NMR spectra of compounds **6** and **7**, indicate an ester chain attached to C-3 of the PCTT. The presence of signals at δ_C 124.30 and δ_C 139.50 corroborates to identify these esters as being derivatives of **5**. The signals at δ_C 121.60 and δ_C 145.20, correspondent to olefin carbon atoms, indicate compound **8** and **9** as oleanane derivatives. The signals at δ_C 80.61, correspondent to carbinolic carbon, and at δ_C 173.69, of carbonyl carbon, suggest compound **9** as an oleanane esther. In the ^{1}H NMR spectrum of compound **10**, the signal at δ_H 5.35 was attributed to olefin hydrogen and a multiplet at δ_H 3.56 to hydrogen of hydroxylated carbon. The ^{13}C NMR spectra of triterpenes **10** and **11** present similar profiles and the observed signal at δ_C 101.6 typical of glycoside, indicated **11** as a glycosylated steroid. The signals at δ_C 176.06 and at δ_C 14.12, observed in the ^{13}C NMR spectrum of **12,** were associated to carbonyl of carboxylic acid and the methyl group-end chain, respectively. Based on the signals of a triplet at δ_H 5.12 (J = 6.40 Hz), δ_C 134.90 and at δ_C 124.20, that are characteristic of olefin hydrogen, observed in the NMR spectra, together its appearance of a semi-solid compound **13** was identified as gutta percha. The constituents **1**–**13** isolated from leaves of *M. distichophylla* and *S. crassifolia* are in agreement with chemical profile of other species of the *Celastraceae* family (Silva et al., 2013). The compounds **3**, **10**, **11**, and **12**, and mixtures of **5** and **6**, **7** and **8** from *M. distichophylla*, and all constituents from *S. crassifolia* have been isolated for the first time in these species.

Extracts and compounds from Celastraceae species have been showed potential *in vitro* AChE inhibitory activity (Alarcón et al., 2008, 2015; Yang et al., 2012; Rodrigues et al., 2014; Sousa et al., 2016). For example, sesquiterpene of β-agarofuran (epoxyeudesmane) skeleton, isolated from *Maytenus disticha* and *Euonymus japonicas* (Alarcón et al., 2008, 2015), pentacyclic triterpenes, from *Maytenus* sp. (Rodrigues et al., 2014) and flavonol triglycosides of leaves from *Maytenus robusta*, showed high AChE activity. Alarcón et al. (2008) showed that in polyhydroxy dihydroagarofuran sesquiterpenoid with a nucleus of the alatol-type are most active than others sesquiterpenes. On these compounds the presence of a free hydroxy group at C-15, and of the OAc (C-2) group next to OBz (C-1) could be responsible for the activity. These terpenoids possess mixed or uncompetitive mechanisms of inhibition of AChE, and were considered as models for the development of new naturally occurring drugs for management strategies for neurodegenerative diseases (Alarcón et al., 2015). Sousa et al. (2012) and Rodrigues et al. (2014) reported the phytochemical and the biological studies of *Maytenus gonoclada* Mart. and *M. imbricata* Mart. ex Reissek.

Table 1
AChE inhibitory activity to chloroform extracts and compounds of the leaves from *Maytenus distichophylla* and *Salacia crassifolia.*

Samples	AChE activity		
	Inhibition (%)	Standard deviation	Coefficient of variation
Chloroform extract from *M. distichophylla*	100	Nd	Nd
Chloroform extract from *S. crassifolia*	97.93	5.63	0.06
3β,24-Dihydroxyfriedelane (**3**)	99.05	1.12	0.01
3-Oxo-28,29-dihydroxyfriedelane (**4**)	90.59	3.76	0.04
3β-Palmityloxy-urs-12-ene (**7**)	94.90	1.47	0.02
Physostigmine (Eserine)[a]	93.94	2.10	0.02

Nd, not detected.
[a] Positive control.

In this works were observed high AChE inhibition induced by the pentacyclic triterpenes 3-oxo-11α-hydroxylup-20(29)-ene, 3-oxo-29-hydroxyfriedelane and 3,7-dioxofriedelane (Rodrigues et al., 2014) and for 3β-hydroxy-21β-H-hop-22(29)-ene, and 3β,11β-dihydroxyfriedelane (Sousa et al., 2012). Therefore, sesquiterpenes and the triterpenes showed AChE inhibitory activity, opening possibilities to the employment of these compounds as drug leads to be used in the treatment of Alzheimer's disease.

Herein, the chloroform extracts from leaves of *M. distichophylla* and *S. crassifolia*, and constituents **1**–**11** were assayed to *in vitro* AChE inhibition. For both chloroform extracts, and for 3β,24-dihydroxyfriedelane (**3**), 3-oxo-28,29-dihydroxyfriedelane (**4**) and 3β-palmityloxy-urs-12-ene (**7**) were observed AChE inhibitory activity (Table 1). The other constituents did not present AChE inhibition. The extract from *M. distichophylla* furnished the active compound **3** and the *S. crassifolia* extract the compounds **4** and **9**. The extracts and compounds 3β,24-dihydroxyfriedelane (**3**) and 3β-palmityloxy-urs-12-ene (**7**) showed the same *in vitro* AChE inhibitory activity than physostigmine, used as positive control (Table 1). The results found in the present work confirmed that the PCTT have AChE inhibition property and open perspectives to the employment of these compounds in researches involving pharmacological activities similar to those presented by physostigmine.

Ethical disclosures

Protection of human and animal subjects. The authors declare that no experiments were performed on humans or animals for this study.

Confidentiality of data. The authors declare that they have followed the protocols of their work center on the publication of patient data.

Right to privacy and informed consent. The authors declare that no patient data appear in this article.

Authors' contributions

FLF, VGR, FCS, GDFS and LPD contributed to the experimental work, extraction and isolation of the metabolites from plants, the interpretation of all these data and wrote the manuscript. SAVF and DMO wrote and revised the manuscript. BLGM and JAT conducted the test *in vitro* AChE inhibitory activity. All authors read and approved the final manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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