Pharmacogn. Mag.
A multifaceted peer reviewed journal in the field of Pharmacognosy and Natural Products
www.phcog.com | www.phcog.net

ORIGINAL ARTICLE

In vitro Anticholinesterase and Neurotoxicity Activities of *Ocotea aciphylla* Fractions

Monique Marylin A. de A. Carneiro[1,2], Rodrigo Souza Conceição[1], Isabella Mary Alves Reis[3], Alessandra Bispo Da Silva[2], Joana Da Luz Oliveira[2], Alexsandro Branco[3], Silvia Lima Costa[2], Mariana Borges Botura[1]

[1]Department of Health, Laboratory of Toxicology, [3]Department of Health, Laboratory of Phytochemistry, State University of Feira de Santana, Feira de Santana, [2]Department of Biochemistry and Biophysics, Institute of Health Sciences, Laboratory of Neurochemistry and Cell Biology, Federal University of Bahia, Salvador, Bahia, Brazil

Submitted: 18-08-2017 Revised: 19-10-2017 Published: 10-09-2018

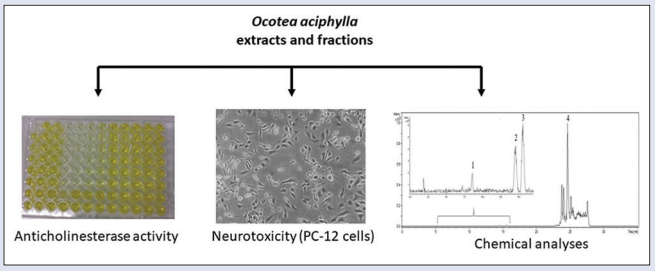
ABSTRACT

Background: *Ocotea* species are known to produce secondary metabolites with a range of biological activities. This study aimed to evaluate the *in vitro* acetylcholinesterase (AChE) inhibition and neurotoxicity activities of the *Ocotea aciphylla* leaves. **Materials and Methods:** The *in vitro* anticholinesterase effect of crude extracts of *O. aciphylla* was investigated by means of spectrophotometric microplate assay. The most active extract, aqueous extract (AQE), was fractionated using column chromatography with silica gel as stationary phase to furnish several fractions that were also evaluated for the anticholinesterase effect. The neurotoxicity activity of AQE and active fraction (F9) was investigated in rat adrenal medulla pheochromocytoma strain cultures by 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test. The chemical characterization of the most active fraction was performed through high-performance liquid chromatography coupled with mass spectrometry multistage (HPLC-MS/MS). **Results:** Ethanolic extract (EE) and AQE exhibited significant inhibitory effects of the activity of AChE, with inhibitory concentration (IC_{50}) of 443.7 and 412.8 μg/mL, respectively. Among the fractions, F9 was more effective AChE inhibition with IC_{50} of 286.2 μg/mL. In the neurocytotoxicity assays, only the F9, at the highest concentration (500 μg/mL), induced a significant reduction in cell viability. HPLC-MS/MS analysis of the active fraction enabled the characterization of the natural compounds, i.e., procyanidin B-type dimer, propelargonidin dimer, catechin, and methoxy-luteolin-deoxyhexose-hexose. **Conclusion:** The leaves of *O. aciphylla* showed *in vitro* anticholinesterase activity and low neurotoxicity, and these effects might be related to the presence of phenolic compounds.

Key words: Acetylcholinesterase, cytotoxicity, high-performance liquid chromatography-mass spectrometry, *Ocotea aciphylla*, phenolic compounds

SUMMARY

- Ocotea aciphylla extracts and fractions showed inhibitory effect on acetylcholinesterase activity
- The aqueous extract and active fraction exhibited low neurotoxicity on rat adrenal medulla pheochromocytoma stain cultures
- The most active fraction contains phenolic compounds (procyanidin B-type dimer, propelargonidin dimer, catechin, and methoxy-luteolin-deoxyhexose-hexose).



Abbreviation used: AChE: acetylcholinesterase; ACTI: Acetylthiocholine iodide; AD: Alzheimer's disease; AQE: Aqueous extract; BSA: Bovine serum albumin; DMSO: Dimethyl sulfoxide; DTNB: 5,5'-dithiobis (2-nitrobenzoic acid); EAE: Ethyl acetate extract; EE: Ethanolic extract; EtOAc: Ethyl acetate; HE: Hexane extract; Hex: Hexane; HPLC-MS/MS: High-performance liquid chromatography coupled with mass spectrometry multistage; MeOH: Methanol; MTT: 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; PC-12: Rat adrenal medulla pheochromocytoma strain; RPMI: Roswell park memorial institute medium.

Correspondence:

Prof. Mariana Borges Botura,
Departamento de Saúde, Laboratório de Toxicologia,
Universidade Estadual de Feira de Santana,
44.036-900 Feira de Santana, Bahia, Brazil.
E-mail: mbbotura@uefs.br

DOI: 10.4103/pm.pm_366_17

Access this article online
Website: www.phcog.com
Quick Response Code:

INTRODUCTION

Cholinesterase inhibitors have several important applications, such as in the treatment of neurodegenerative diseases, especially Alzheimer's disease (AD). The inhibition of acetylcholinesterase (AChE, EC 3.1.1.7), an enzyme responsible for the hydrolysis of acetylcholine, increases the availability of acetylcholine in the cholinergic synapses, thus improving the cognitive function and behavioral symptoms in AD patients.[1]

The AChE-inhibitor drugs used for the treatment of AD are donepezil, rivastigmine, and galantamine. The alkaloid galantamine is commonly found in species of the Amaryllidaceae family, which is currently commercialized in its synthetic form. However, these agents have high cost and are often associated with undesirable side effects, such as hepatotoxicity and gastrointestinal disorder. These drawbacks have stimulated the research for new safer AChE inhibitors from natural sources.[2]

Medicinal plants represent an important source of new drugs against various pharmacological targets.[3] Brazil holds almost one-third of the world's flora represented in the various national biomes.[4] Among the plant species found in the Brazilian northeastern semi-arid region, there is the genus *Ocotea*, which is characterized by a wide range of

Cite this article as: de A. Carneiro MM, Conceição RS, Alves Reis IM, Da Silva AB, Da Luz Oliveira J, Branco A, *et al*. *In vitro* anticholinesterase and neurotoxicity activities of *Ocotea aciphylla* fractions. Phcog Mag 2018;14:S448-52.

biological activities such as antifungal,[5] antinociceptive,[6] cytotoxic,[7] and anticholinesterase.[8] There are different classes of secondary metabolites described, such as alkaloids,[6] flavonoids,[9] lignans,[10] and terpenes.[11]

The *Ocotea aciphylla* occurs throughout tropical and subtropical America, including the semi-arid region of Brazil. This plant is characterized by having wood resistance against insects and also by being popularly used as antirheumatic and depurative. A chemical study on *O. aciphylla* enabled the identification of neolignans and phenylpropanoids.[10,12]

The scientific validation of the therapeutic activity of medicinal plants is necessary, since these products must have the same criteria of efficacy and safety as do the synthetic drugs.[13] Among the toxicological assays, cytotoxicity studies in glial and neuronal cell cultures have been shown to be an excellent model for testing new drugs, including plant extracts.[14,15]

Considering the limited number of AChE-inhibitor drugs and the few scientific information about *O. aciphylla*, the present study aimed to evaluate the *in vitro* AChE activity and toxicity for the neural cells of the extracts and fractions obtained from leaves of *O. aciphylla*. In addition, chemical analyses were performed to characterize the main constituents present in the most active fraction.

MATERIALS AND METHODS

Materials

All solvents used were of analytical grade. For column chromatography (CC), silica gel 60 (70–230 mesh, VETEC) was used. High-performance liquid chromatography (HPLC)-grade solvent (Merck) and ultrapure water (Milli-Q system) were used in the HPLC coupled with mass spectrometry (HPLC-MS) analysis.

For biological assays, enzyme AChE from *Electrophorus electricus* Type VI (EC: 3.1.1.7), 5,5'-dithiobis (2-nitrobenzoic acid) (DTNB or Ellman's reagent), acetylthiocholine iodide, bovine serum albumin (BSA), eserine, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT), and dimethyl sulfoxide (DMSO) were purchased from Sigma-Aldrich® (St. Louis, MO, USA). Roswell Park Memorial Institute medium (RPMI), gentamycin, and fetal equine serum (FES) were obtained from Cultilab (Campinas, São Paulo, Brazil). The cells were cultured in polystyrene plates and polystyrene culture dishes (TPP, Trasadingen, Switzerland).

Preparation of *Ocotea aciphylla* extracts

The leaves of *O. aciphylla* (Nees and Mart.) Mez were collected (1.25 kg) in the Rio de Contas Municipality (Bahia) in December 2013. The botanical identification was carried in the Herbarium of the State University of Feira de Santana with a voucher number (205865). The leaves of *O. aciphylla* were dried in a ventilated oven at 40°C and pulverized by a mechanical grinder. The powdered material was subjected to successive solvent extraction by maceration for 72 h using solvents of increasing polarity (hexane [Hex], ethyl acetate [EtOAc], ethanol, and water). After filtration, the solvents were evaporated under reduced pressure to obtain the hexane (HE), ethyl acetate (EAE), ethanolic (EE), and aqueous (AQE) extracts.

Fractionation of the active extract

The AQE was subjected to fractionation through column chromatography (CC) eluted with the following eluent systems using hexane (Hex), ethyl acetate (EtOAc), methanol (MeOH) and water (H_2O): Hex (100%), Hex/EtOAc (70:30), Hex/EtOAc (50:50), EtOAc (100%) EtOAc/MeOH (70:30), EtOAc/MeOH (50:50) MeOH (100%) MeOH/H_2O (70:30), MeOH/H_2O (50:50), H_2O (100%). After fractionation, 17 fractions were obtained.

In vitro anticholinesterase activity assay of extracts and fractions

The AChE activity was measured according to the method of Ellman *et al*.[16] modified by Atta-ur-Rahman *et al*.[17] The extracts and fractions were evaluated at concentration ranges of 125–1000 μg/mL.

For this assay, in each well of a 96-well microplate, 140 μL of phosphate buffer (100 mM) was added, containing 0.1% BSA, 20 μL of AChE enzyme (0.22 U/mL), and 20 μL of extracts/fractions. Negative controls (DMSO 0.1% and phosphate buffer) and positive control (eserine, 0.06–13.8 μg/mL) were also included. Plates were mixed and incubated at 37°C for 30 min. After incubation, 10 μL of acetylcholine iodide (75 mM) and 10 μL of DTNB at 10 mM were added. The absorbance was measured at 405 nm on a microplate reader at times 0 and 30 min. AChE percentage inhibition was calculated according to the following equation: Inhibition (%) = (1− [reaction rate sample/reaction rate control − phosphate buffer]) ×100.

In vitro neurotoxicity activity of aqueous extract and active fraction (F9)

Cell culture and treatments

Rat adrenal medulla pheochromocytoma strain (PC-12) was cultured until confluence in 10 mm polystyrene plates, trypsinized, and replated on 10-mm polystyrene culture dishes (1.5×10^5 cells/plate). These cells were grown in RPMI supplemented with 10% FES and 5% fetal bovine serum, a nutrient mixture (7 mM glucose, 2 mM glutamine, and 0.011 g/l pyruvate), and gentamycin (6.25 mg/mL) for 8 days in a humidified atmosphere with 5% CO_2 at 37°C.

PC-12 cells were seeded in 96-well plates at a density of 5×10^3 cells/cm^2 for 24 h before treatments. Thereafter, cells were treated with AQE and fraction 9, at concentrations between 31.25 and 500 μg/mL. The negative control corresponded to DMSO (0.1%). After 24 h of treatment, the viability cell was evaluated by MTT test and phase-contrast microscopy.

Cell viability assay

The cytotoxicity of AQE and fraction 9 on PC-12 cells was determined by MTT assay as described previously.[18] The cells were incubated with MTT (1 mg/mL) for 2 h. Thereafter, cells were lysed with 20% (w/v) sodium dodecyl sulfate, 50% (v/v) acetic acid, and 2.5% (v/v) 1 M HCl, and the plates were kept overnight at 37°C to dissolve formazan crystals. The cell viability was quantified by measuring the conversion of yellow MTT to purple MTT formazan by mitochondrial dehydrogenases of living cells. The optical density of each sample was measured at 595 nm using a spectrophotometer (Thermo Scientific® Flash Varioskan). Results from the MTT test were expressed as percentages of the viability of the treated groups related to the untreated groups.

Morphological changes and cellularity evaluation

All cultures were examined through microscopy by phase contrast with green filter (Labomed® TCM400 Microscope), which were performed evaluating cellularity (cell density in the culture) and cell morphology.

High-performance liquid chromatography with electrospray ion source coupled with mass spectrometry multistage analysis of active fraction

The analysis through HPLC-MS was held at USP (University of São Paulo) analytical center in mass spectrometer Bruker Daltonics® (Esquire Plus 3000 model) with electrospray ion source (ESI) and ion trap analyzer and chromatograph Shimadzu with Phenomenex Luna C-18 (250 × 4.6 mm – 5 μm), Pumps LC-20AD, CBM-20A Controller, Detector SPD-20A, and autosampler SIL-20AC. The solvent system

consisted of a gradient H_2O – 0.1% $C_2H_4O_2$ (A) and MeOH (B) in the following conditions: 0–20 min (75% A and 25% B), 20–25 min (100% B), and 25–35 min (75% A and 25% B). The column oven temperature and the flow of the mobile phase were 40°C and 1 mL/min, respectively.

Statistical analysis

The biological assays were conducted with eight replicates and values expressed as mean ± standard deviation. The data were analyzed by ANOVA followed by Tukey test (0.001%). The inhibitory concentration (IC_{50}) of the extracts and active fractions was calculated through nonlinear regression analysis. All statistical analysis was performed using the GraphPad Prism statistical program (version 5.0, GraphPad Software Inc., La Jolla, California, USA).

RESULTS

In vitro anticholinesterase activity of extracts and fractions

All extracts promoted *in vitro* inhibition of AChE activity, in a concentration-dependent manner, although differences in the degree of activity were observed. The mean inhibition percentage at the highest concentration tested (1000 µg/mL) corresponded to 62.6% HE, 80.9% EAE, 94.4% EE, and 99.0% AQE. The anticholinesterase effect of eserine (positive control) ranged from 32.6 to 92.9%. There was no statistically significant difference ($P > 0.001$) between the inhibition percentage of eserine (13.8 µg/mL) compared to the EE at 1000 µg/mL and the AQE at concentrations of 1000 and 750 µg/mL [Figure 1]. The IC_{50} values were 0.237, 443.7, and 412.8 µg/mL for eserine, EE, and AQE, respectively.

The fractions obtained from the AQE were tested in a preliminary assay at concentrations of 500 and 1000 µg/mL. All fractions showed AChE inhibitory effect, except for fraction 17. The fractions 2, 3, 8, 9, and 13 exhibited inhibition percentages greater than 50%, and the fractions 8 and 9 were more effective, with the maximum inhibition equal to 94.65 and 99.21%, respectively [Table 1].

The most active fractions (F8 and F9) were evaluated with five different concentrations to determine the IC_{50} value. All concentrations of F8 and F9 differed from the negative control ($P < 0.001$). There was no statistically significant difference between the positive control and F8/F9 at concentrations of 1000 and 750 µg/mL. F9 showed greater potency of inhibition of the enzymatic activity ($IC_{50} = 256.2$ µg/mL) compared to fraction 8 ($IC_{50} = 516.8$ µg/mL) and the AQE ($IC_{50} = 412.8$ µg/mL).

In vitro neurotoxicity effect of aqueous extract and active fraction (F9)

For the investigation of neurotoxicity, the MTT assay was performed in cultures of neural PC-12 cells, which measure the activity of mitochondrial dehydrogenases. We observed that after 24 h, the exposure to the AQE has no effect on the viability of PC-12 cells [Figure 2]. However, the fraction 9 reduced cell viability significantly ($P < 0.001$) at the highest concentration tested (500 µg/mL). The negative control did not display any significant effect on the parameters analyzed when compared to cultures that were not exposed to this solvent.

The induction of change of morphology and cellularity was also investigated with phase-contrast microscopy. The decrease in cytoplasmic shrinkage and cellularity was observed in PC-12 at a concentration of 500 µg/mL of the AQE and 250 and 500 µg/mL of F9.

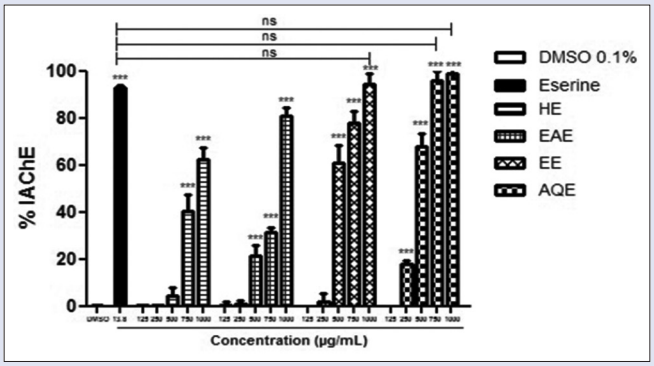


Figure 1: *In vitro* acetylcholinesterase inhibitory activity of *Ocotea aciphylla* extracts (***). Significant differences from negative control (0.1% dimethyl sulfoxide) values ($P < 0.001$) (ns). Values not significantly different as compared to positive control with eserine ($P > 0.001$)

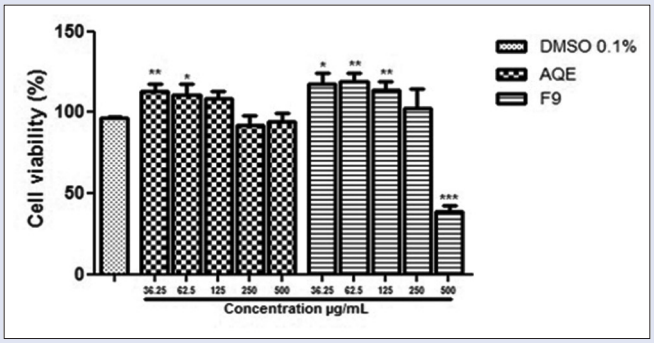


Figure 2: Percentage of Rat adrenal medulla pheochromocytoma strain cell viability after 24 h of exposure to the aqueous extract and F9 from *Ocotea aciphylla*. Statistical values significantly different as compared to negative control (dimethyl sulfoxide) ***$P < 0.001$; **$P < 0.01$; *$P < 0.05$

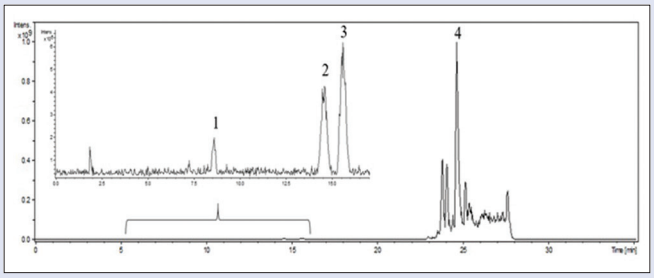


Figure 3: Chromatogram of total ions obtained through high-performance liquid chromatography-mass spectrometry of F9 of *Ocotea aciphylla*

Chemical analyses of active fraction

The most active fraction (F9) of *O. aciphylla* was analyzed through HPLC-ESI-MS/multistage (MS). Figure 3 shows the respective chromatogram of total ions. The retention times (t_R), molecular ions $[M + H]^+$, and MS^2 fragmentation of the main peaks are described [Table 2].

DISCUSSION

AChE inhibition is considered as an important strategy for the treatment of neurodegenerative disorders and bioactive components derived from

Table 1: Mean and standard deviation of the inhibition percentage of acetylcholinesterase activity after treatment with the fractions obtained from aqueous extract of *Ocotea aciphylla*

Fraction	Percentage AChE inhibition	
	500 µg/mL	1000 µg/mL
1	1.62±2.8	6.83±5.0
2	37.8±7.9	51.6±3.5
3	4.3±2.0	51.4±8.8
4	1.4±2.4	11.5±3.1
5	4.2±2.5	25.7±2.4
6	0.0	5.2±2.3
7	0.7±1.8	12.2±2.1
8	55.0±4.9	94.7±5.6
9	82.1±9.0	99.2±2.8
10	12.0±4.9	23.6±1.9
11	10.2±5.4	34.0±2.9
12	18.8±8.8	45.0±4.8
13	0.0	60.6±5.5
14	20.0±6.6	47.0±1.9
15	14.9±1.4	45.5±1.7
16	12.0±5.3	27.8±5.1
17	0.0	0.0

AChE: Acetylcholinesterase; AQE: Aqueous extract

Table 2: Peak assignments of the F9 from aqueous extract of *Ocotea aciphylla*

Peak	Tr (min)	$M + Na^+$	$M + H^+$	MS^2	Compound
1	8.7	-	579	427, 409, 291	Procyanidin B-type dimer
2	14.5	-	563	437, 411, 291	Propelargonidin dimer
3	15.5	313	-	-	Catechin
4	24.6	-	579	301	Methoxy-luteolin-deoxyhexose -hexose

MS: Mass spectrometry

natural products as promising drug candidates for this target.[2] Vinutha *et al.*[19] proposed the following classification of plant extracts with anticholinesterase action: potent inhibitors (>50% inhibition), moderate inhibitors (30%–50% inhibition), and weak inhibitors (<30% inhibition). Considering this classification, all extracts from *O. aciphylla* tested were presented as potent inhibitors of AChE, and the best result was obtained with fraction 9 of the AQE.

HPLC-MS/MS chemical analysis led to the characterization of five phenolic compounds in the active (F9) through mass spectral analysis and comparison with literature data: two proanthocyanidins (procyanidin B-type dimer and propelargonidin dimer), a flavan-3-ol catechin and a flavone methoxy-luteolin-deoxyhexose-hexose.[20-22]

Previous studies have attributed AChE inhibition to the occurrence of phenolic constituents in extracts of plants. Amoo *et al.*[8] demonstrated that MeOH extract of the bark of *Ocotea bullata* (1 mg/mL) promoted high *in vitro* effect on AChE, and this activity was associated to the presence of tannins and phenolic compounds. *In vitro* anticholinesterase effect of the proanthocyanidin-rich extract of *Cymbopogon schoenanthus* was reported by Khadri *et al.*[23]

In the present study, we observed low cytotoxicity of the AQE and F9 against PC-12 cells. The fraction 9 did not affect cell viability at a concentration of 250 µg/mL, a concentration close to that promoted inhibition of enzyme activity of AChE by 50% (IC_{50} = 286.2 µg/mL). These results reaffirm the estimated potential of the *O. aciphylla* as AChE inhibitor.

The tumor source cells can be cultured as cell lines and can provide reliable models for the study of the initial screening drug candidates for therapeutic agents.[24,25] The PC-12 cell line is cells transformed from the adrenal gland of rat (*Rattus norvegicus*) and is widely used in studies of neurobiology. In this work, undifferentiated cells were used, and so it was not possible to characterize the formation of neurites. Even undifferentiated, they are still used as template function, development, and neuronal survival.[26]

Similar results were reported concerning the flavonoid fraction of *Ocotea notata* (100 and 200 µg/mL), which induced alteration in the morphology of Vero cells (epithelial cells of African green monkey kidney), although more than 50% of the cells remained viable at the highest concentration tested (50% cytotoxic concentration >200 µg/mL).[9] The major compounds identified in this fraction were A-type proanthocyanidin trimer, isoquercitrin, reynoutrin, miquelianin, quercitrin, and afzelin. Ballabeni *et al.* (2010)[27] described that the essential oil of *Ocotea quixos* did not cause cytotoxic effect on J774 macrophage cell lineage at a concentration of up to 10 µg/mL, while the trans-cinnamaldehyde (main component of the essential oil) exhibited high cytotoxicity, with only 10% viable cells.

In the cytotoxic evaluation, we observed slight morphological changes in the cells treated with the AQE (500 µg/mL) and F9 (250 and 500 µg/mL). Cytomorphological alterations are evidence of toxicity of a substance, since cell damage does not always lead to cell death. Structural or constitution of the plasma membrane and cytoskeletal changes may be associated with the loss of important functions. Cell death, either by apoptosis or necrosis, also brings with it changes in shape, size, and cell structures or intracytoplasmic changes as granular inclusions or vacuoles.[28]

CONCLUSION

All crude extracts of *O. aciphylla* leaves have pronounced *in vitro* inhibitory effect on the activity of AChE. However, the AQE was the most effective that furnished an active fraction containing four phenolic compounds. The active fraction also showed slight cytotoxicity against neural cells (PC-12). Thus, *O. aciphylla* is a promising source of active compounds that can be used for the development of new anticholinesterase drugs.

Acknowledgement

The authors would like to thank the Foundation for Research Support of the State of Bahia (FAPESB, JCB0032/2013), National Counsel of Technological and Scientific Development (CNPq), Federal University of Bahia and State University of Feira de Santana) the financial support.

Financial support and sponsorship

Nil.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Massoud F, Léger GC. Pharmacological treatment of Alzheimer disease. Can J Psychiatry 2011;56:579-88.
2. Ahmed F, Ghalib RM, Sasikala P, Ahmed KK. Cholinesterase inhibitors from botanicals. Pharmacogn Rev 2013;7:121-30.
3. Atanasov AG, Waltenberger B, Pferschy-Wenzig EM, Linder T, Wawrosch C, Uhrin P, *et al.* Discovery and resupply of pharmacologically active plant-derived natural products: A review. Biotechnol Adv 2015;33:1582-614.
4. Dutra RC, Campos MM, Santos AR, Calixto JB. Medicinal plants in Brazil: Pharmacological studies, drug discovery, challenges and perspectives. Pharmacol Res 2016;112:4-29.
5. Cárdenas C, Pozo W, Rojas M, Roque A, Mihai R. Antifungal activity of two botanical

extracts on Rose crop (*Rosa L.* Sp.), against *Sphaerotheca Pannosa* var. *Rosae*. Agric Agric Sci Procedia 2016;10:465-74.

6. Montrucchio DP, Miguel OG, Zanin SM, da Silva GA, Cardozo AM, Santos AR, *et al.* Antinociceptive effects of a chloroform extract and the alkaloid dicentrine isolated from fruits of *Ocotea puberula*. Planta Med 2012;78:1543-8.
7. Cuca LE, Coy ED, Alarcón MA, Fernández A, Aristizábal FA. Cytotoxic effect of some natural compounds isolated from *Lauraceae* plants and synthetic derivatives. Biomedica 2011;31:335-43.
8. Amoo SO, Aremu AO, Moyo M, Van Staden J. Antioxidant and acetylcholinesterase-inhibitory properties of long-term stored medicinal plants. BMC Complement Altern Med 2012;12:87.
9. Garrett R, Romanos MT, Borges RM, Santos MG, Rocha L, Silva AJ. Antiherpetic activity of a flavonoid fraction from *Ocotea notata* leaves. Braz J Pharmacogn 2012;22:306-13.
10. Felicio JD, Motidome M, Yoshida M, Gottlieb OR. Further neolignans from *Ocotea aciphylla*. Phytochemistry 1986;25:1707-10.
11. Ballabeni V, Tognolini M, Giorgio C, Bertoni S, Bruni R, Barocelli E, *et al. Ocotea quixos* lam. essential oil: *In vitro* and *in vivo* investigation on its anti-inflammatory properties. Fitoterapia 2010;81:289-95.
12. Romoff P, Yoshida M, Gottlieb OR. Neolignans from *Ocotea aciphylla*. Phytochemistry 1984;23:2101-4.
13. Rates SM. Plants as source of drugs. Toxicon 2001;39:603-13.
14. Silva-Neto JP, Barreto RA, Pitanga BP, Souza CS, Silva VD, Silva AR, *et al.* Genotoxicity and morphological changes induced by the alkaloid monocrotaline, extracted from *Crotalaria retusa*, in a model of glial cells. Toxicon 2010;55:105-17.
15. Banerjee S, Pandey S, Mukherjee P, Sayeed A, Pandurangi A, George SK, *et al.* Investigation of cytotoxicity induced by *Nigella sativa* and *Azadirachta indica* using MDA-MB-231, HCT 116 and SHSY5Y cell lines. Pharmacogn J 2017;9:192-5.
16. Ellman GL, Courtney KD, Andres V Jr., Feather-Stone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.
17. Atta-ur-Rahman, Parveen S, Khalid A, Farooq A, Choudhary MI. Acetyl and butyrylcholinesterase-inhibiting triterpenoid alkaloids from *Buxus papillosa*. Phytochemistry 2001;58:963-8.
18. Hansen MB, Nielsen SE, Berg K. Re-examination and further development of a precise and rapid dye method for measuring cell growth/cell kill. J Immunol Methods 1989;119:203-10.
19. Vinutha B, Prashanth D, Salma K, Sreeja SL, Pratiti D, Padmaja R, *et al.* Screening of selected indian medicinal plants for acetylcholinesterase inhibitory activity. J Ethnopharmacol 2007;109:359-63.
20. de Souza LM, Cipriani TR, Iacomini M, Gorin PA, Sassaki GL. HPLC/ESI-MS and NMR analysis of flavonoids and tannins in bioactive extract from leaves of *Maytenus ilicifolia*. J Pharm Biomed Anal 2008;47:59-67.
21. Huang Y, Chen L, Feng L, Guo F, Li Y. Characterization of total phenolic constituents from the stems of *Spatholobus suberectus* using LC-DAD-MSn and their inhibitory effect on human neutrophil elastase activity. Molecules 2013;18:7549-56.
22. Beelders T, de Beer D, Stander MA, Joubert E. Comprehensive phenolic profiling of *Cyclopia genistoides* (L.) vent. By LC-DAD-MS and -MS/MS reveals novel xanthone and benzophenone constituents. Molecules 2014;19:11760-90.
23. Khadri A, Neffati M, Smiti S, Falé P, Lino AR, Serralheiro ML, *et al.* Antioxidant, antiacetylcholinesterase and antimicrobial activities of *Cymbopogon schoenanthus* L. Spreng (lemon grass) from Tunisia. LWT. Food Sci Technol 2010;43:331-6.
24. Lal PG, Ghirnikar RS, Eng LF. Astrocyte-astrocytoma cell line interactions in culture. J Neurosci Res 1996;44:216-22.
25. Pédeboscq S, L'Azou B, Liguoro D, Pometan JP, Cambar J. Interindividual differences in anticancer drug cytotoxicity in primary human glioblastoma cells. Exp Toxicol Pathol 2007;58:247-53.
26. Teng KK, Angelastro JM, Cunningham ME, Greene L. Cultured for neuronal PC12 cells: A model function, differentiation, and survival. In: Celis J, Carter N, Simons K, Small J, Hunter T, Shotton D, editors. Cell Biology: A Laboratory Handbook. 3nd ed. USA: Elsevier; 2006. p. 171-6.
27. Ballabeni V, Tognolini M, Bertoni S, Bruni R, Guerrini A, Rueda GM, *et al.* Antiplatelet and antithrombotic activities of essential oil from wild *Ocotea quixos* (Lam.) Kosterm. (*Lauraceae*) calices from Amazonian Ecuador. Pharmacol Res 2007;55:23-30.
28. Willingham MC. Cytochemical methods for the detection of apoptosis. J Histochem Cytochem 1999;47:1101-10.