In vitro Antioxidant and Enzymatic Approaches to Evaluate Neuroprotector Potential of *Blechnum* Extracts without Cytotoxicity to Human Stem Cells

Juliana Maria de Mello Andrade, Renata Biegelmeyer, Roger Remy Dresch, Natasha Maurmann[1,2], Patrícia Pranke[1,2], Amélia T. Henriques

Laboratory of Pharmacognosy, [1]Hematology and Stem Cell Laboratory, [2]Stem Cell Research Institute, School of Pharmacy, Federal University of Rio Grande do Sul, Porto Alegre, RS, Brazil

ABSTRACT

Background: Investigation of selected plant extracts on multi-targets related to neurodegeneration, such as monoamine oxidases (MAO), cholinesterase enzymes, and antioxidant activities (AOA) is a useful tool for identification of new scaffolds. **Objective:** This work investigated biological effects of three *Blechnum* methanol extracts from Brazil and chemical profile of the most active sample. **Materials and Methods:** AOA included scavenging of hydroxyl and nitric oxide radicals, also lipid peroxidation inhibition. Enzymatic modulation of *Blechnum binervatum*, *Blechnum brasiliense*, and *Blechnum occidentale* extracts on MAO and cholinesterases was conducted. Moreover, total phenol content was performed with all samples, and high-performance liquid chromatography-diode array detection mass spectrometry HPLC-DAD-MS analysis was carried out with *B. brasiliense*. Possible toxic effects were evaluated on Wistar rats polymorphonuclear cells (PMN) and human stem cells. **Results:** *B. brasiliense* extract presented the highest phenolic amount (9.25 g gallic acid equivalent/100 g extract) and lowest IC_{50} values (112.3 ± 2.61 and 176.1 ± 1.19 µg/mL) against hydroxyl radicals and on lipid peroxidation, respectively, showing strong AO effects. On nitric oxide assay and cholinesterase inhibition, all extracts were considered inactive. MAO-A selective action was evidenced, being *B. brasiliense* powerful against this enzyme (IC_{50}: 72.7 µg/mL), followed by *B. occidentale* and B. *binervatum* (IC_{50}: 130.85 and 165.2 µg/mL). No cytotoxic effects were observed on PMN and human stem cells treated with *Blechnum* extracts. HPLC-DAD-MS analysis of *B. brasiliense* allowed the identification of chlorogenic and rosmarinic acids. **Conclusion:** Our results especially highlight *B. brasiliense*, with pronounced phenols content and strong effects on selected targets related to neurodegeneration, being characterized as a natural safe source of bioactive hydroxycinnamic acids.

Key words: Antioxidants, *Blechnum*, high-performance liquid chromatography-diode array detection-mass spectrometry, monoamine oxidases inhibitors, phenolic content, stem cells

SUMMARY

- *Blechnum* crude extracts showed high phenolic amounts and valuable IC_{50} values on targets related with neurodegenerative disorders
- *Blechnum brasiliense* was the most active sample, with strong radical scavenging and lipid peroxidation inhibition, also with monoamine oxidases:

A selective modulation

- No cytotoxic effects were observed on polymorphonuclear cells rat cells and human stem cells treated with *Blechnum* extracts
- High-performance liquid chromatography-diode array detection-mass spectrometry analysis of *Blechnum brasiliense* allowed the identification of hydroxycinnamic derivatives: Chlorogenic and rosmarinic acids.
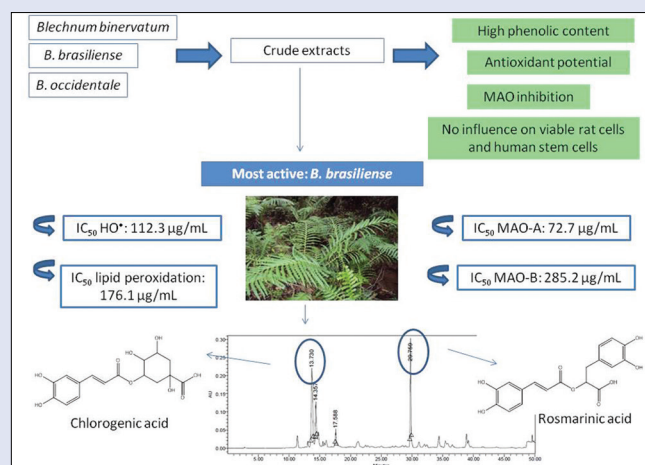
Abbreviations used: IC_{50}: half maximal inhibitory concentration; MAO: monoamine oxidase; MAO-A: monoamine oxidase isoform A; MAO-B: monoamine oxidase isoform B; HO•: hydroxyl radical.

Correspondence:

Dr. Juliana Maria de Mello Andrade,
Avenida Ipiranga, 2752/505H, 90610-000,
Porto Alegre, RS, Brazil.
E-mail: juandradep@gmail.com

Access this article online

Website: www.phcog.com

Quick Response Code:

INTRODUCTION

Ferns are vascular plants, globally distributed, covering approximately 13,600 species, found in several biomes. Additionally, ferns present a great chemical diversity and several biological activities, including anti-inflammatory, antioxidant and are capable to modulate enzymes related to neurodegeneration.[1] The genus *Blechnum* is chemically characterized by the presence of phenolic compounds such as flavonols, hydroxycinnamic acids, and lignans; compounds with recognized potential central nervous system activities. Among species studied in this work, *Blechnum occidentale* is used in folk medicine for treatment of inflammatory and pulmonary diseases, urinary

tract infections, and others.[2] Nonato *et al*.[3] showed that methanol extracts of *B. occidentale* leaves, when administered intraperitoneally

and orally, produced anti-inflammatory and antinociceptive effects in animals.

Our research group have investigated the multifunctional feature of extracts from selected plants and isolated compounds able to inhibit targets related to neurodegeneration, such as monoamine oxidases (MAO), cholinesterase enzymes and antioxidative activities, according to the proposal scheme by Novaroli *et al.*[4] The imbalance between reactive species formation and repairing overproduction thereof, results in oxidative damage to membranes, proteins, lipids, and nucleic acids. Evidence have supported its involvement in the etiology of several diseases, including neurodegeneration.[5]

Pharmacological activity associated with preclinical evaluation of toxicity from natural product extracts should be performed by appropriate bioassays. Determination of action mechanisms is important, also selection of the most active crude mixture devoid of toxicity, aiming to search novel potential natural sources of compounds, as by bioassay-guided fractionation.[6] Hence, the aim of this study was to perform *in vitro* antioxidant, enzymatic and toxicological studies of methanolic extracts of *Blechnum binervatum*, *Blechnum brasiliense*, and *B. occidentale* fronds, in addition to quantify the total phenol contents. Different biological systems were employed, including stabilization of hydroxyl and nitric oxide radicals, inhibition of lipid peroxidation, effects against MAO and cholinesterases, and possible toxic responses of rat cells and human stem cells after incubation with *Blechnum* extracts. In addition, high-performance liquid chromatography-diode array detection-mass spectrometry (HPLC-DAD-MS) analysis of most active sample was performed in order to search substances responsible for powerful properties.

MATERIALS AND METHODS

Materials

Human MAO-A and MAO-B supersomes were acquired from BD Gentest (Woburn, MA). Acetylcholinesterase from *Electrophorus electricus*, butyrylcholinesterase from equine serum, acetylthiocholine iodide (AChE), S-butyrylthiocholine iodide (BChE), 5,5'-dithiobis (2-nitrobenzoic acid) (Ellman's reagent), physostigmine, kynuramine, pargyline, clorgyline, 2-deoxy-D-ribose, sodium nitroprusside dihydrate, sulfanilamide, N-(1-naphthyl) ethylenediamine dihydrochloride, (+)-sodium L-ascorbate, Triton X-100, dimethyl sulfoxide (DMSO), tris (hydroxymethyl) aminomethane, methanol, acetonitrile and formic acid were purchased from Sigma-Aldrich Chemical (St. Louis, MO, USA).

Phosphate salts, potassium chloride, and sodium hydroxide came from Fluka (Buchs, Switzerland). Trichloroacetic acid, hydrogen peroxide, and ferrous sulfate were obtained from Synth (Diadema, SP, Brazil). Phosphoric and 2-thiobarbituric acids were obtained from Merck (Darmstadt, Germany). Lactate dehydrogenase (LDH) commercial kit was purchased from Doles (Goiás, Brazil). To the culture experiments, *in vitro* products used included inactivated fetal bovine serum (Cultilab, SP, Brazil), Rh-TGF-β-1 (ImmunoTools). Following reagents were purchased from Sigma-Aldrich: Dulbecco's Modified Eagle's Medium (DMEM), penicillin, streptomycin, trypsin-EDTA solution, dexamethasone, L-ascorbic acid 2-phosphate sesquimagnesium salt hydrate, insulin, transferrin, selenium (ITS) supplement, insulin from bovine pancreas, indomethacin, rosiglitazone, β-glycerophosphate hydrate, and 3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT). The antibodies for flow cytometry analysis were obtained from BD (Becton Dickinson, San Diego, CA, USA). LDH Liquiform Ref.: 86-1/100 was obtained from Labtest Diagnóstica SA.

Plant material

Blechnum species were harvested in Rio Grande do Sul state, with previous approval from Conselho de Gestão do Patrimônio Genético and identified by botanical Maria Angelica Kieling-Rubio. Exsiccates were deposited in the Herbarium of Instituto de Biociências (ICN) from Federal University of Rio Grande do Sul (UFRGS). *B. binervatum* (ICN 171553) and *B. brasiliense* (ICN 177668) were harvested in Morro Reuter (29°32'17"S, 51°04'51"W) city while *B. occidentale* (ICN 177667) was harvested in Campo Bom (29°40'39"S, 51°01'97"W).

Preparation of methanolic extracts

Fronds of *Blechnum* species were dried at room temperature in the shadow. Reduction was performed using mill of knives and plants were extracted exhaustively with methanol by maceration (3 × 5 days), using 1:20 proportion (drug: solvent). Extracts were combined and evaporated under reduced pressure at temperature below 40°C. Yields obtained were 13.6%, 11.6%, and 15.8% for *B. binervatum*, *B. brasiliense*, and *B. occidentale*, respectively.

Determination of total phenol content

Total phenol content was determined according to Folin-Ciocalteu method, using a spectrophotometer at 760 nm. Results were expressed as grams of gallic acid equivalent (GAE)/100 g of crude extract.[7,8]

Antioxidant assays

Stabilization of hydroxyl radical

Extracts (1-500 µg/mL) were diluted in phosphate buffer and added to the reaction system in 96-well plate, containing 2-deoxyribose (50 mM), ferrous sulfate (60 µM), hydrogen peroxide (29%) and phosphate buffer (20 mM, pH 7.2). After, the addition of phosphoric acid (4%) and thiobarbituric acid (TBA, 1%) were performed. The plates were incubated for 30 min and absorbance measured at 532 nm in SpectraMax® apparatus (Molecular Devices, CA, USA). As positive control, chlorogenic and caffeic acids were employed.[9] Analyzes were performed in triplicate, and half maximal inhibitory concentration values (IC_{50}) were estimated.

Nitric oxide radical scavenging activity

Samples (1-500 µg/mL) were prepared in phosphate saline buffer (20 mM, pH 7.2) and added to sodium nitroprusside solution (20 mM) in 96-well plates. Incubation was performed for 60 min, at room temperature. After, Griess reagent (sulphanilamide [2%] and naphthylethylenediamine dihydrochloride [0.1%]) was included and incubation of reactions was performed in the dark, for 7 min. Finally, formed nitrite levels were quantified at 546 nm using SpectraMax® apparatus.[10] The experiment was carried out in triplicate and IC_{50} values from extracts and standards (chlorogenic and caffeic acids) were calculated.

Thiobarbituric acid reactive substances assay

To perform thiobarbituric acid reactive substances (TBARS) experiment, approval by Ethics Committee on Animal Use from UFRGS was obtained (Protocol 23374). Cortex and hippocampus of adult male Wistar rats were employed, weighing between 180 and 220 g. Animals were sacrificed by decapitation and brain structures were immediately removed and washed with tris (hydroxymethyl)aminomethane (TRIS) buffer (20 mM, pH 7.4). Tissues were placed in Potter-Elvehjem homogenizer to obtain a cell homogenate which was centrifuged at 7500 rpm, for 5 min, removing the supernatant.

Samples were diluted in purified water (1–500 µg/mL) and incubated with homogenate brain tissue supernatant, $FeSO_4$ (10 mM) and ascorbic acid (0.1 mM), for 60 min at 37°C. After, addition of trichloroacetic acid (28%) and TBA (2%) was performed, a further incubation by 20 min. Supernatant obtained after centrifugation was measured at 532 nm in spectrophotometer (SpectraMax®).[11] Analyzes were performed in triplicate, and IC_{50} values were obtained.

Inhibition of monoamine oxidase enzyme

In black microplates were added in this order: Phosphate saline buffer (20 mM, pH 7.4), kynuramine (5 mM), and samples (1-500 µg/mL) in DMSO (final concentration of 1%). Mixtures were incubated at 37°C, for 20 min. Following, enzyme isoforms were added separately, MAO-A (0.09 mg/mL) and MAO-B (0.15 mg/mL), and microplates were incubated at 37°C, for 30 min. At the end of incubation, sodium hydroxide (2 M) was added to stop reactions. Fluorescence readings (excitation $\lambda = 320$ nm and emission $\lambda = 400$ nm) were measured in SpectraMax® plate reader.[12] Clorgyline and pargyline were used as control inhibitors of MAO-A and MAO-B, respectively. IC_{50} values of samples were calculated after triplicate analysis.

Inhibition of acetyl and butyrylcholinesterase enzymes

To evaluate inhibition of acetyl and butyrylcholinesterase, Ellman reagent (10 mM), substrate solution 14 mM (AChE and BChE), samples diluted in DMSO (1%) and respective enzyme (1 UI/mL) were added in 96-well plates. After enzyme addition, reactions were immediately measured in kinetic mode at 412 nm (SpectraMax®), during 6 min at intervals of 30 s.[13,14] Physostigmine was used as positive control and *Blechnum* extracts (100-500 µg/mL) were evaluated in triplicate to estimate IC_{50} values.

Evaluation of cytotoxicity and cell viability

Polymorphonuclear animal cells assay

Polymorphonuclear cells (PMN) obtained from plasma of Wistar rats (final concentration of 1.5×10^7 cells/mL) were employed to evaluate cytotoxicity of *Blechnum* extracts. Animals were previously treated to obtain a PMN cells pellet, as described by Andrade *et al.*[1] PMN cells were preincubated with samples at 37°C, for 30 min. After centrifugation, enzyme substrate and ferric alum were added to supernatant and mixture was incubated for 3 min. Nicotinamide adenine dinucleotide was added, and incubation for 5 min was performed. At the end, stabilization solution was added to reaction medium and analysis was performed at 492 nm using a commercial kit of LDH enzyme (Doles Reagents, Goiás, Brazil). Extracts were assessed in triplicate (1 mg/mL), and Triton X-100 (1%) was used as positive control.

Human mesenchymal stem cells assay

Stem cells from human exfoliated deciduous teeth (SHED) were isolated as previously described by Bernardi *et al.*,[15] in partnership with Graduate Program in Dentistry at UFRGS, approved by the Ethics Committee of UFRGS (Protocol 36403514.6.0000.5347). Cell cultures were seeded and maintained in DMEM supplemented medium (pH 7.4) in an atmosphere containing 5% CO_2 at 37°C. After culture, SHED characterization was performed by flow cytometry (FACSAria III BD®, CA, USA) incubated with specific monoclonal antibodies (CD14, CD34, CD45, CD73, CD90, CD105, and human leukocyte antigen - antigen D related [HLA]-DR). Additionally, cells were analyzed for their ability to differentiate into osteoblasts, chondrocytes, and adipocytes through induction media.[15]

SHED were plated (7×10^3 cells/well) and after reached confluence, were treated with crude extracts at concentrations of 100, 250 and 500 µg/mL, dissolved in DMSO (2%). As negative control, DMSO 2% was used and positive control consisted of Triton X-100 (1%). After 24 h incubation, cell viability was determined by reduction of MTT.[16] In this assay, cells were incubated with MTT (0.25 µg/mL) during 4 h; after, supernatant was removed, and DMSO (200 µL) was added. The measure of absorbance at 570 and 630 nm was performed in SpectraMax® and results were obtained by two independent experiments, each one in triplicate.

Extension of cell membrane integrity was determined in supernatant of cultures, using LDH assay, through commercial test kit (Labtest Diagnostica SA, Minas Gerais, Brazil) as standardized by Pranke *et al.*[17] SHED culture was treated with extracts, as described above. Readings were performed in LabMax 560 equipment (Labtest Diagnostica SA), and cytotoxicity was calculated according to the damage of cell membrane, expressed as a percentage of intracellular LDH release compared to negative control (100%). Independent experiments were performed in triplicate.

Chromatographic and mass spectrometric analysis

Based on biological results, we performed liquid chromatography coupled with photodiode array (PDA) detector (HPLC-PDA) analysis of the most active sample, in order to verify chemical composition and detect active compounds. Separation Module Waters 2695 and ultraviolet (UV) visible Waters 996 PDA detector were employed, data acquisition and integration were managed with Waters Empower software (Waters, Milford, MA, USA). A C18 reversed-phase column (Kromasil, 150 mm × 4.6 mm × 5 µm) was used, operating at a temperature of 24 ± 2°C.

Dried methanol crude extract from *B. brasiliense* was dissolved in methanol (LC grade) to obtain final concentration of 10 mg/mL. Sample was filtered through a 0.45 µm pore size membrane (Millipore, Bedford, USA) before LC system analysis. A linear gradient system was used with mobile phases consisting of a ultrapure water: formic acid (100:0.2; v/v) mixture (A) and acetonitrile (100; v) (B). Gradient profile was: 0–45 min from 5 to 35% of B, 45–46 min from 35 to 50% of B, 46–47 min from 50 to 100% of B, 47–50 min 100% of B. The flow rate was 0.8 mL/min, and injection volume was 10 µL. Compound detection was performed at 320 nm, and identity was confirmed by comparison with reference substances, injected under the same conditions.

Additional chemical structure information was obtained by mass spectrometry with electrospray ionization (ESI-MS). A mass spectrometer Waters microTOF-Q Micromass (Waters Corp., Milford, MA, USA) was employed, high purity nitrogen was used as nebulizer and auxiliary gas argon as the collision gas. MS/MS spectra was obtained in negative ion mode, with 25 eV collision energy, by direct injection. Data acquisition was performed using Waters MassLynx software (Waters, Milford, MA, USA).[18]

Statistical analysis

Statistical analysis from determination of total phenols, assessment of cell viability and cytotoxicity were evaluated by analysis of variance followed by Bonferroni's test, using Prism 5.0 software (GraphPad Software, Inc., CA, USA). Significant differences were considered when $P < 0.05$. Results obtained in antioxidant assays, and enzyme inhibition tests were interpreted using the same program, and IC_{50} values were obtained after adjustment of experimental data (% inhibition vs. inhibitor concentration) for nonlinear regression curves, through equation described below (Equation 1):

$$Y = Bottom + (top-bottom)/(1 + 10^{[(logIC_{50} - X)*HillSlope]})$$

Equation 1. Calculation of IC_{50} value from samples, using nonlinear regression curves obtained by Prism 5.0 software (GraphPad Software, Inc., CA, USA).

RESULTS

Total polyphenols content

Results of total polyphenols content in methanolic extracts of *Blechnum* species demonstrated *B. brasiliense* with higher content (9.25 ± 0.35 g GAE/100 g extract), followed by *B. occidentale* (7.22 ± 0.20 g GAE/100 g extract) and *B. binervatum* (5.17 ± 0.32 g GAE/100 g extract), with values significantly different between samples ($P < 0.05$).

Antioxidant assays

Hydroxyl radicals are reactive oxygen species mainly responsible for biological damage and lipid oxidation.[9] *Blechnum* extracts were effective in scavenger hydroxyl radicals, formed by deoxyribose degradation. *B. brasiliense* showed the highest activity in hydroxyl radical stabilization, with IC$_{50}$ of 112.3 ± 2.61 µg/mL [Table 1]. Against nitric oxide, *Blechnum* extracts showed low activity, with IC$_{50}$ values above 500 µg/mL. Hydroxycinnamic acids, used as standards, were less active compared with results for other used methods. In addition, antioxidant activity of extracts was assessed by TBARS assay, which is based on the formation of malondialdehyde, a byproduct of lipid peroxidation. Potential activity was observed for *B. brasiliense* extract with IC$_{50}$ value of 176.1 ± 1.19 µg/mL [Table 1]. Chlorogenic and caffeic acids presented IC$_{50}$ values equal to 37.5 ± 1.25 and 55.9 ± 1.22 µM, respectively.

Enzyme inhibition

According to results presented in Table 2, all samples were more active against isoform A of MAO, with pronounced activity for *B. brasiliense* extract (IC$_{50}$ 72.7 ± 1.09 µg/mL). In addition, *B. brasiliense* showed a good selectivity index, since its MAO-A inhibition was 3.92 times higher than MAO-B modulation (IC$_{50}$ 285.2 ± 1.03 µg/mL). The irreversible MAO-A inhibitor, clorgyline, showed IC$_{50}$ value of 0.009 ± 0.0002 µM and selective MAO-B inhibitor, pargyline, presented IC$_{50}$ of 0.207 ± 0.0112 µM.

All *Blechnum* extracts presented IC$_{50}$ values above of 500 µg/mL, in both AChE and BChE inhibition, being considered inactive against cholinesterases. Physostigmine, a positive control, exhibited strong inhibitory activity with IC$_{50}$ values of 0.0165 ± 0.0019 µM (AChE) and 0.0718 ± 0.0018 µM (BChE).

Toxicity evaluation in rodents cells

Cell toxicity was not observed for *Blechnum* samples, tested by measuring the release of LDH. At 1 mg/mL, results were similar to negative control (cells nontreated), indicating the feasibility of membrane from PMN [Figure 1]. On the other hand, Triton X-100 (1%) showed strong cell membrane damage.

Assessment of viability and cytotoxicity on stem cells

This study also evaluated effects of *Blechnum* extracts on stem cells viability, through MTT assay [Figure 2a]. Characterization of mesenchymal stem cells was performed according to established criteria by the International Society for Cellular Therapy, showing typical fibroblasts morphology. Cells presented plastic adherence and expressed positivity (≥95%) for CD73, CD105, and CD90. In addition, cells did not express (≤2%) surface markers: CD34, CD45, CD14, CD11b and HLA-DR and were differentiated into osteoblasts, adipocytes, and condroblastos *in vitro*.[19] Added of extracts, (100-500 µg/mL), cells maintained viability, with values comparable to nontreated group (*P* < 0.05). Triton X-100 (1%) showed extensive cell death [Figure 2a].

In agreement with MTT results, cells treated with *Blechnum* extracts by 24 h showed no cytotoxic effect by LDH release method [Figure 2b]. Triton X-100 (1%) showed significant increase in LDH leakage of treated cells. Samples demonstrated similar profiles and were comparable to negative control (*P* < 0.05). In summary, the results showed *Blechnum* extracts are capable of maintaining stem cells viability, and no cytotoxic effects were observed at concentrations of 100–500 µg/mL.

Table 1: Half maximal inhibitory concentration values of *Blechnum* extracts, chlorogenic and caffeic acid standards, in antioxidant experiments

Assay	IC$_{50}$* (µg/mL)			IC$_{50}$* (µM)	
	Blechnum binervatum	*Blechnum brasiliense*	*Blechnum occidentale*	CGA[b]	CA[c]
Hydroxyl radical	311.1±1.66	112.3±2.61	198.2±1.86	45.6±1.52	63.3±1.43
Nitric oxide radical	>500	>500	>500	159.8±1.44	180.9±1.87
TBARS[a]	489.5±2.98	176.1±1.19	455.7±2.56	37.5±1.25	55.9±1.22

[a]Thiobarbituric acid reactive substances assay; [b]Chlorogenic acid; [c]Caffeic acid; *Values expressed as mean±standard deviation. IC$_{50}$: Half maximal inhibitory concentration

Figure 1: Results of lactate dehydrogenase method to evaluate cytotoxic effect from *Blechnum* samples. Values of 100% indicate total cell viability (negative control data). Triton X-100 (1%) exhibited cytotoxicity. #Indicates significant differences compared to nontreated cells (*P* < 0.05)

Table 2: Enzyme inhibition of *Blechnum* extracts against monoamine oxidases and cholinesterase enzymes

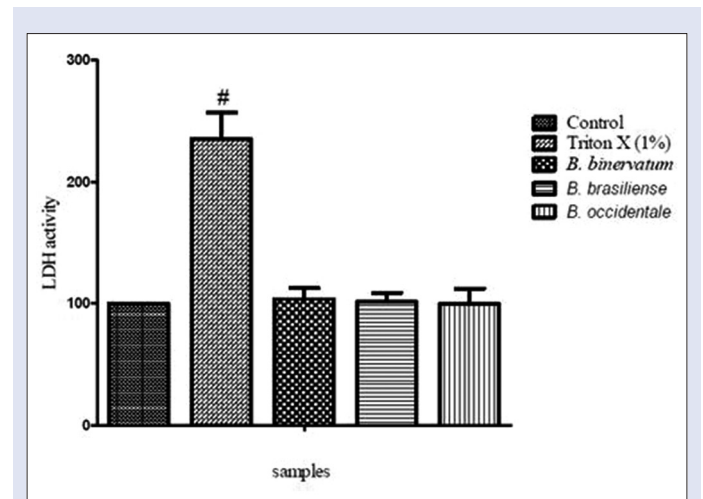| Sample	MAO-A	MAO-B	SI[a]	AChE	BChE
IC$_{50}$* (µg/mL)					
B. binervatum	165.2±1.10	499.7±2.24	3.02	>500	>500
B. brasiliense	72.7±1.09	285.2±1.03	3.92	>500	>500
B. occidentale	130.8±1.05	421.5±1.01	3.22	>500	>500
IC$_{50}$* (µM)					
Clorgyline	0.009±0.0002	NT**	-	NT**	NT**
Pargyline	NT**	0.207±0.0112	-	NT**	NT**
Physostigmine	NT**	NT**	-	0.0165±0.0019	0.0718±0.0018

[a]Selectivity index (IC$_{50}$ MAO-B/IC$_{50}$ MAO-A); *Values expressed as mean±standard deviation; **NT: Not-tested; IC$_{50}$: Half maximal inhibitory concentration; MAO: Monoamine oxidases; AChE: Acetylcholinesterase; BChE: Butyrylcholinesterase

Chromatographic and mass spectrometric analysis

Analysis by HPLC-PDA was conducted with *B. brasiliense* extract, most active sample in antioxidant assays and inhibition of MAO. This sample presented four detectable peaks, being two of them, the majorities, with retention times of 13.73 and 29.76 min [Figure 3]. Compounds showed maxima UV absorption characteristic of hydroxycinnamic acids.[20] Comparing with standard reference, compound in 13.73 min was characterized as chlorogenic acid.

In addition, compound with retention time of 29.76 min was analyzed by mass spectroscopy in negative mode. The substance presents $C_{18}H_{16}O_8$ molecular formula and molecular mass of 360 g/mol. Pseudomolecular ion (m/z 357 [359-H_2]$^-$), was fragmented by MS/MS [Figure 3d], generating characteristic product-ions: m/z 197, m/z 179 and base peak m/z 161.[21-23] Compiled data conducted to the identification of rosmarinic acid in *B. brasiliense* extract.

DISCUSSION

For some species of the *Blechnum* genus, Bohm[24] showed the presence of rosmarinic and chlorogenic acids in ethanol extracts, using thin layer chromatography analysis. Blechnic acid was also observed in *B. spicant*

extract. According to Farias *et al.*,[25] the crude extract of *B. brasiliense* presented 2.352 g GAE/100 g extract. Comparing with our results, we observed the higher content of total polyphenols in this species.

Antioxidant action mechanism of polyphenols, evaluated by hydroxyl scavenging assay, is usually attributed to the scavenging ability of these compounds. However, many studies have been shown that these phenols do not only block the degradation of deoxyribose by stabilization of hydroxyl radicals but also act as antioxidants via formation of complexes with iron. Thus, iron cannot participate in Fenton's reaction, which is responsible to triggering generation of free radicals through oxidation of organic substrates.[26,27] With regards to the NO• radical, literature reports as one of reactive nitrogen species that contributes to the development of several diseases, including inflammation and neurodegenerative disorders.[28] TBARS was performed since therapeutic strategies capable to modulate lipid peroxidation may be promising in the prevention of several disorders.[29] Thus, substances with free radical scavenging properties, also lipid peroxidation inhibition are valuable for new drugs development.

Farias *et al.*[25] previously demonstrated inhibition of DPPH formation by *B. brasiliense*, which IC_{50} values of crude extract and ethyl acetate fraction were 4.14 and 1.43 mg/mL, respectively. Significant differences observed
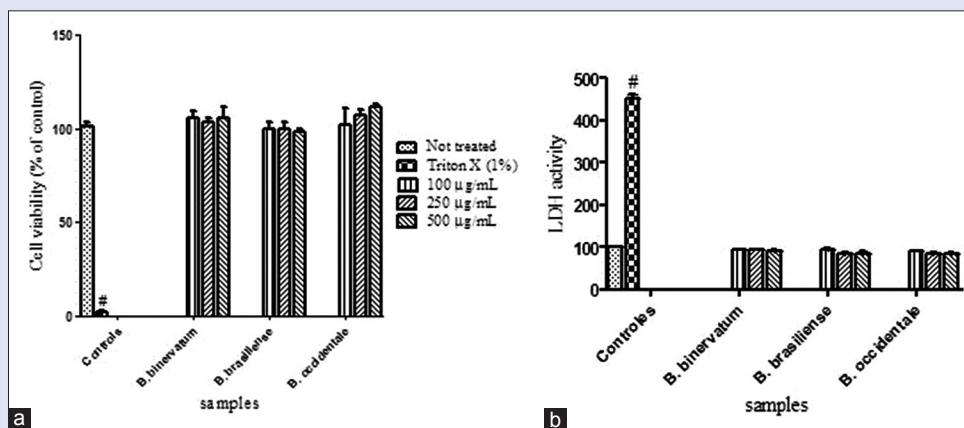
Figure 2: Effect of *Blechnum binervatum*, *Blechnum brasiliense*, and *Blechnum occidentale* extracts on stem cells viability (3-(4,5-dimethythiazol-2-yl)-2,5-diphenyltetrazolium bromide assay) (a) and cytotoxicity (lactate dehydrogenase leakage) (b). For these tests, cells were pre-cultivated in 96 well-microplates and were then incubated with samples, at 100–500 µg/mL, for 24 h, at 37°C. Data are expressed as mean ± standard deviation of two independent experiments. Percentage of cell viability and lactate dehydrogenase activity was treated as 100% in the control group. #Indicates no viability and cytotoxicity compared to not-treated group ($P < 0.05$)
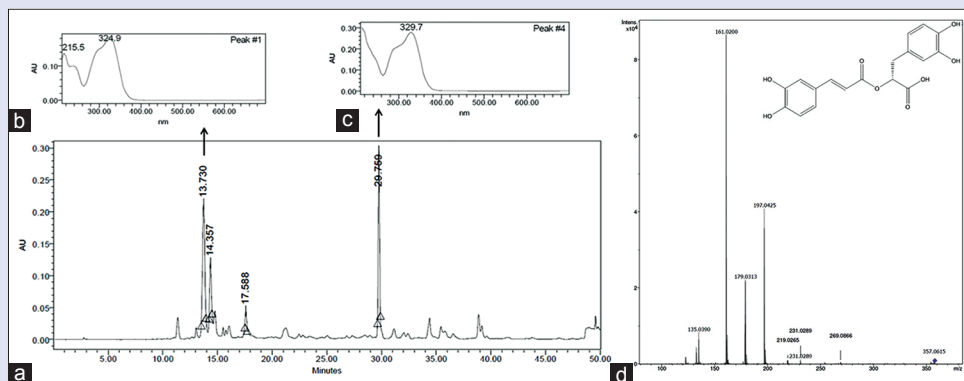
Figure 3: Chromatogram at 320 nm from *Blechnum brasiliense* extract (a); ultraviolet spectra of major compounds: peak 1 at 13.73 min (b), and peak 4 at 29.76 min (c). Mass spectrum obtained after MS/MS fragmentation of pseudomolecular ion m/z 357 (359-H_2)-, of rosmarinic acid (d)

can be explained by chemical nature of samples and also by employed assay. Distinct methodologies present limitations and applicability, mainly due to complex nature of phytocompounds and their interactions. Thus, multiple antioxidant methodologies become an indispensable approach to evaluate complex phenol mixtures, which are involved in scavenging free radicals and inhibiting lipid peroxidation in tissues.[30]

MAO activity was determined, in this study, by fluorometric assay proposed by Novaroli *et al.*[31] employing kynuramine as substrate. This substance, after oxidation, produces 4-hydroxyquinoline, a fluorescent compound, easily detected and quantified.[1,12] MAO inhibitors are considered key prototypes for drug development, applied for neurodegenerative diseases and also depression and anxiety treatment.[32] Mazzio *et al.*[33] ranked plant crude extracts regarding their MAO-B inhibition, according to levels: Very strong (IC_{50} <70 µg/mL), strong (IC_{50} <200 µg/mL), moderately strong (IC_{50} >200 <400 µg/mL), moderate (IC_{50} >400 <700 µg/mL) and weak (IC_{50} = 700 µg/mL). Considering this classification, *B. brasiliense* extract showed moderately strong inhibition (IC_{50}: 285.2 µg/mL), *B. binervatum* and *B. occidentale* extracts presented moderate inhibition of MAO-B (IC_{50}: 499.7 and 421.5 µg/mL, respectively).

Covering the proposal of multiple targets search, the inhibitory activity of AChE was assessed, which is characterized as the main strategy employed in Alzheimer's disease treatment.[34] This activity promotes cognition improvement in patients. Besides AChE, BChE is also responsible for inactivation of acetylcholine in brain tissue.[35] Our results showed no effects of *Blechnum* extracts against cholinesterases, highlighting the modulation of targets related to Parkinson disease.[4]

Some of the available MAO modulator drugs in therapeutics of depression and Parkinson diseases present many side effects and their physiological activity can persist for up to 2–3 weeks.[36] Thus, the search for novel inhibitors with few adverse effects is strongly encouraged. In this sense, natural compounds from different classes are being studied as MAO inhibitors, as well as, multifunctional substances on targets related to neurodegeneration. Flavonoids and coumarins have already demonstrated strong MAO inhibition, being apigenin the most active compound, among the tested (IC_{50}: 1 µmol).[37]

Generally, bicyclic compounds, including coumarin and conjugated hydroxycinammic acids, are known to present considerable inhibitory activity against both MAO-A and MAO-B enzymes.[38] Gallic acid and derivatives, isolated from *Liquidambar formosana* fruits, showed strong MAO inhibition, with IC_{50} values lower than 2 µg/mL.[39] Recent studies showed that infusions from green tea and citrus peels presented MAO inhibitory ability,[40] being linked to their phenolic content, due to its structural similarity to synthetic inhibitors.[41] Phenolic compounds are well known antioxidant and anti-inflammatory substances and these properties, added to their MAO inhibition, makes them interesting neuroprotective compounds designed to drug discovery.

Our previous research studies assessed 11 species of ferns from Brazil, regarding its antioxidant and anti-inflammatory potential (10 µg/mL) and also inhibitory capacity against MAO-A and MAO-B enzymes (100 µ/mL). *Asplenium serra, Lastreopsis amplissima*, and *Cyathea dichromatolepis* showed potential antioxidant activity by total reactive antioxidant potential method (TRAP). MAO-A inhibition was predominantly observed for *Dydymochlaena truncatula, Alsophila setosa, Cyathea phalerata,* and *Cyathea delgadii*, whose inhibition values ranged from 70.32% to 82.61% at the concentration tested. No toxic effects were observed to PMN cells, when incubated with fern species, at 100 µg/mL.[1]

In addition to the toxic assay using animal cells, our studies have evaluated effects of *Blechnum* extracts on human stem cells, considering their high rate of proliferation and capacity of differentiation in several cell types, including neurons. Thus, this cells can predict toxicity of plant extracts and isolated molecules in specific differentiated cells.[42-44] Furthermore,

human exfoliated deciduous teeth allows obtaining stem cells with minimal invasive damage to donor since teeth are discarded after natural replacement process.[45] Human cells usage enables more sensitivity design to predict toxicity and contribute to animals replacement.[46]

According with HPLC-DAD-MS analysis, the most active sample presented two hydroxycinnamic acids in extract composition, characterized as chlorogenic and rosmarinic acid. Studies have reported effects of chlorogenic acid on H_2O_2 induced damages[47] as well as its protective ability against neurotoxicity promoted by oxidative stress and reduction of lipid peroxidation in brain structures.[48] For rosmarinic acid, significant antioxidant activity has been reported,[49] being responsible for cytoprotective effects of this molecule.[50] Moreover, this substance presented protective ability on human dopaminergic cell line (SH-SY5Y).[51] Thus, our findings point to new safe sources of neuroactive compounds, capable of modulate targets related to neurodegenerative disorders, stimulating the development of new scaffolds to compose therapeutic strategies to combat these pathologies.

CONCLUSION

Our results highlight the importance of fern species studies, especially from *Blechnum* genus, which few reports are described in the literature. This is the first report assessing *Blechnum* species for antioxidant activity and enzymatic selected methods demonstrating safety of these plants in animal and human stem cells. Taken together, data indicate *B. brasiliense* extract with most relevant results, which can be related to higher levels of total phenols and also to the presence of chlorogenic and rosmarinic acids. These compounds contributed to the pronounced capacity of stabilize hydroxyl radical and inhibit lipid peroxidation. In addition, *B. brasiliense* extract displayed strong and selective inhibition of MAO-A enzyme, showing no toxic effects and maintaining viability of rodents and human stem cells. Compiled data point to these fern species as important sources of natural and safe compounds, capable to prevent damage caused by oxidants and lipid peroxidation, which is associated with numerous pathologies. The lack of AChE and BChE enzyme activities also shows selectivity, indicating these plant species as inhibitors of specific biochemical targets related degenerative disorders, such as Parkinson's disease.

Conflicts of interest

There are no conflicts of interest.

REFERENCES

1. Andrade JM, Passos Cdos S, Dresch RR, Kieling-Rubio MA, Moreno PR, Henriques AT. Chemical analysis, antioxidant, antichemotactic and monoamine oxidase inhibition effects of some pteridophytes from Brazil. Pharmacogn Mag 2014;10 Suppl 1:S100-9.

2. Barros IC, Andrade LH. Pteridófitas medicinais (samambaias, avencas e plantas afins). (in portuguese). Pernambuco, Brazil: Editora Universitária, Federal University of Recife; 1997. p. 214.

3. Nonato FR, Barros TA, Lucchese AM, Oliveira CE, dos Santos RR, Soares MB, *et al.* Antiinflammatory and antinociceptive activities of *Blechnum occidentale* L. extract. J Ethnopharmacol 2009;125:102-7.

4. Novaroli L, Daina A, Bertolini F, Di Giovanni S, Bravo J, Reist M, *et al.* Identification of novel multifunctional compounds for the treatment of some aging related neurodegenerative diseases. Chimia 2005;59:315-20.

5. de Moura MB, dos Santos LS, Van Houten B. Mitochondrial dysfunction in neurodegenerative diseases and cancer. Environ Mol Mutagen 2010;51:391-405.

6. van Breemen RB. Development of safe and effective botanical dietary supplements. J Med Chem 2015;58:8360-72.

7. Druckerei CH. European Pharmacopoeia. 4th ed. Nordlingen: Beck; 2002. p. 187.

8. Verza SG, Kreinecker MT, Reis V, Henriques AT, Ortega GG. Evaluation of analytical variables of the Folin-Ciocalteu method for the quantitation of the total tannins content using a *Psidium guajava* L. leaves aqueous extract as a model. Quím Nova 2007;30:815-20.

9. Lopes GK, Schulman HM, Hermes-Lima M. Polyphenol tannic acid inhibits hydroxyl radical formation from Fenton reaction by complexing ferrous ions. Biochim Biophys Acta 1999;1472:142-52.

10. Kumar RS, Sivakumar T, Sunderam RS, Gupta M, Mazumdar UK, Gomathi P, *et al.* Antioxidant and antimicrobial activities of *Bauhinia racemosa* L. stem bark. Braz J Med Biol Res 2005;38:1015-24.

11. Reis FS, Martins A, Barros L, Ferreira IC. Antioxidant properties and phenolic profile of the most widely appreciated cultivated mushrooms: A comparative study between *in vivo* and *in vitro* samples. Food Chem Toxicol 2012;50:1201-7.

12. Dos Santos Passos C, Soldi TC, Torres Abib R, Anders Apel M, Simões-Pires C, Marcourt L, *et al.* Monoamine oxidase inhibition by monoterpene indole alkaloids and fractions obtained from *Psychotria suterella* and *Psychotria laciniata*. J Enzyme Inhib Med Chem 2013;28:611-8.

13. Ellman GL, Courtney D, Andres V, Featherstone RM. A new and rapid colorimetric determination of acetylcholinesterase activity. Biochem Pharmacol 1961;7:88-95.

14. Passos CS, Simões-Pires CA, Nurisso A, Soldi TC, Kato L, de Oliveira CM, *et al.* Indole alkaloids of *Psychotria* as multifunctional cholinesterases and monoamine oxidases inhibitors. Phytochemistry 2013;86:8-20.

15. Bernardi L, Luisi SB, Fernandes R, Dalberto TP, Valentim L, Bogo Chies JA, *et al.* The isolation of stem cells from human deciduous teeth pulp is related to the physiological process of resorption. J Endod 2011;37:973-9.

16. Pardo Andreu GL, Maurmann N, Reolon GK, de Farias CB, Schwartsmann G, Delgado R, *et al.* Mangiferin, a naturally occurring glucoxilxanthone improves long-term object recognition memory in rats. Eur J Pharmacol 2010;635:124-8.

17. Pranke O, Kumar S, Raja B. Cytotoxic effect of cobaiba oil in stem cells. In: ISSCR 12th Annual Meeting of International Society for Stem Cell Research. Vancouver, Canada: Late Breaking Poster Abstracts; 2014. p. 13-4.

18. Dresch RR, Dresch MK, Guerreiro AF, Biegelmeyer R, Holzschuh MH, Rambo DF, *et al.* Phenolic compounds from the leaves of *Vitis labrusca* and *Vitis vinifera* L. as a source of waste byproducts: Development and validation of LC method and antichemotactic activity. Food Anal Methods 2014;7:527-39.

19. Dominici M, Le Blanc K, Mueller I, Slaper-Cortenbach I, Marini F, Krause D, *et al.* Minimal criteria for defining multipotent mesenchymal stromal cells. The International Society for Cellular Therapy position statement. Cytotherapy 2006;8:315-7.

20. Lin LZ, Harnly J, Zhang RW, Fan XE, Chen HJ. Quantitation of the hydroxycinnamic acid derivatives and the glycosides of flavonols and flavones by UV absorbance after identification by LC-MS. J Agric Food Chem 2012;60:544-53.

21. Liu AH, Guo H, Ye M, Lin YH, Sun JH, Xu M, *et al.* Detection, characterization and identification of phenolic acids in Danshen using high-performance liquid chromatography with diode array detection and electrospray ionization mass spectrometry. J Chromatogr A 2007;1161:170-82.

22. Hossain MB, Rai DK, Brunton NP, Martin-Diana AB, Barry-Ryan C. Characterization of phenolic composition in *Lamiaceae* spices by LC-ESI-MS/MS. J Agric Food Chem 2010;58:10576-81.

23. Barros L, Dueñas M, Dias MI, Sousa MJ, Santos-Buelga C, Ferreira IC. Phenolic profiles of cultivated, *in vitro* cultured and commercial samples of *Melissa officinalis* L. infusions. Food Chem 2013;136:1-8.

24. Bohm BA. Phenolic compounds in ferns – III. An examination of some ferns for caffeic acid derivatives. Phytochemistry 1968;7:1825-30.

25. Farias CC, Chagas MO, Bonani VF, Simionatto E, Hess SC, Peres MT. Avaliação da atividade antioxidante e determinação do teor de fenóis totais em extratos de quatro espécies de pteridófitas de MS. (in portuguese). In: 30a Annual Meeting of the Brazilian Chemical Society. Vol. 01. São Paulo, Brazil: SBQ; 2007.

26. Barbusiński K. Fenton reaction – Controversy concerning the chemistry. Ecol Chem Eng 2009;16:347-58.

27. Carocho M, Ferreira IC. A review on antioxidants, prooxidants and related controversy: Natural and synthetic compounds, screening and analysis methodologies and future perspectives. Food Chem Toxicol 2013;51:15-25.

28. Gupta SP, Patel S, Yadav S, Singh AK, Singh S, Singh MP. Involvement of nitric oxide in maneb- and paraquat-induced Parkinson's disease phenotype in mouse: Is there any link with lipid peroxidation? Neurochem Res 2010;35:1206-13.

29. Reed TT, Sellers ZP, Butterfield DA. Lipid peroxidation in age-related neurodegenerative disorders. In: Spickett CM, Forman HJ, editors. Lipid Oxidation in Health and Disease. Boca Raton, Florida: Taylor and Francis Publishers; 2015. p. 329-61.

30. Mihailović V, Matić S, Mišić D, Solujić S, Stanić S, Katanić J, *et al.* Chemical composition, antioxidant and antigenotoxic activities of different fractions of *Gentiana asclepiadea* L. roots extract. EXCLI J 2013;12:807-23.

31. Novaroli L, Daina A, Favre E, Bravo J, Carotti A, Leonetti F, *et al.* Impact of species-dependent differences on screening, design, and development of MAO B inhibitors. J Med Chem 2006;49:6264-72.

32. Youdim MB, Bakhle YS. Monoamine oxidase: Isoforms and inhibitors in Parkinson's disease and depressive illness. Br J Pharmacol 2006;147 Suppl 1:S287-96.

33. Mazzio E, Deiab S, Park K, Soliman KF. High throughput screening to identify natural human monoamine oxidase B inhibitors. Phytother Res 2013;27:818-28.

34. Hansen RA, Gartlehner G, Webb AP, Morgan LC, Moore CG, Jonas DE. Efficacy and safety of donepezil, galantamine, and rivastigmine for the treatment of Alzheimer's disease: A systematic review and meta-analysis. Clin Interv Aging 2008;3:211-25.

35. Francis PT, Palmer AM, Snape M, Wilcock GK. The cholinergic hypothesis of Alzheimer's disease: A review of progress. J Neurol Neurosurg Psychiatry 1999;66:137-47.

36. Fiedorowicz JG, Swartz KL. The role of monoamine oxidase inhibitors in current psychiatric practice. J Psychiatr Pract 2004;10:239-48.

37. Lee SJ, Chung HY, Lee IK, Oh SU, Yoo ID. Phenolics with inhibitory activity on mouse brain monoamine oxidase (MAO) from whole parts of *Artemisia vulgaris* L (mugwort). Food Sci Biotechnol 2000;9:179-82.

38. Thull U, Testa B. Screening of unsubstituted cyclic compounds as inhibitors of monoamine oxidases. Biochem Pharmacol 1994;47:2307-10.

39. Yang ZD, Liang JB, Xue WW, Sheng J, Shi Y, Yao XJ, *et al.* Phenolic compounds from *Liquidambar formosana* fruits as monoamine oxidase inhibitors. Chem Nat Comput 2014;50:1118-9.

40. Ademosun AO, Oboh G. Comparison of the inhibition of monoamine oxidase and butyrylcholinesterase activities by infusions from green tea and some citrus peels. Int J Alzheimers Dis 2014;2014:586407.

41. Benamar H, Rached W, Derdour A, Marouf A. Screening of Algerian medicinal plants for acetylcholinesterase inhibitory activity. J Biol Sci 2010;10:1-9.

42. Yarak S, Okamoto OK. Human adipose-derived stem cells: current challenges and clinical perspectives. An Bras Dermatol 2010;85:647-56.

43. Giordano G, La Monaca G, Annibali S, Cicconetti A, Ottolenghi L. Stem cells from oral niches: A review. Ann Stomatol (Roma) 2011;2:3-8.

44. Kanafi MM, Pal R, Gupta PK. Phenotypic and functional comparison of optimum culture conditions for upscaling of dental pulp stem cells. Cell Biol Int 2013;37:126-36.

45. Miura M, Gronthos S, Zhao M, Lu B, Fisher LW, Robey PG, *et al.* SHED: Stem cells from human exfoliated deciduous teeth. Proc Natl Acad Sci U S A 2003;100:5807-12.

46. Aguiar AM, Zych J, Reus T, Kuligovski C, Moraes E, Abud AP, et al. Células-tronco adultas humanas para ensaios de citotoxicidade: Uma alternativa aos ensaios animais. (in portuguese). In: Seminário Anual Científico e Tecnológico. Rio de Janeiro, Brazil: Fundação Osvaldo Cruz; 2013.

47. Kim J, Lee S, Shim J, Kim HW, Kim J, Jang YJ, *et al.* Caffeinated coffee, decaffeinated coffee, and the phenolic phytochemical chlorogenic acid up-regulate NQO1 expression and prevent H2O2-induced apoptosis in primary cortical neurons. Neurochem Int 2012;60:466-74.

48. Nakajima Y, Shimazawa M, Mishima S, Hara H. Water extract of propolis and its main constituents, caffeoylquinic acid derivatives, exert neuroprotective effects via antioxidant actions. Life Sci 2007;80:370-7.

49. Gu L, Wu T, Wang Z. TLC bioautography-guided isolation of antioxidants from fruit of *Perilla frutescens* var. *acuta*. LWT Food Sci Technol 2009;42:131-6.

50. Chkhikvishvili I, Sanikidze T, Gogia N, Mchedlishvili T, Enukidze M, Machavariani M, *et al.* Rosmarinic acid-rich extracts of summer savory (*Satureja hortensis* L.) protect Jurkat T cells against oxidative stress. Oxid Med Cell Longev 2013;2013:456253.

51. Lee HJ, Cho HS, Park E, Kim S, Lee SY, Kim CS, *et al.* Rosmarinic acid protects human dopaminergic neuronal cells against hydrogen peroxide-induced apoptosis. Toxicology 2008;250:109-15.

ABOUT AUTHOR

Dr. Juliana Maria de Mello Andrade, is a post-doctoral student at the Federal University of Rio Grande do Sul, where she graduated in Pharmacy and obtained Master and Doctoral degrees in Pharmaceutical Sciences. Her doctoral research focused on the chemical investigation and influence on targets related with neurodegenerative disorders of *Blechnum* samples and isolated compounds, also cytotoxicity on rat cells and human stem cells. She has experience in the area of Pharmacognosy, Chemistry of Natural Products, and Pharmacology.

Juliana Maria de Mello Andrade