Rev Bras Farmacogn 23(2013): 789-795

Revista Brasileira de Farmacognosia
BRAZILIAN JOURNAL OF PHARMACOGNOSY
www.sbfgnosia.org.br/revista

Original Article

Neuroprotection of *Persea major* extract against oxygen and glucose deprivation in hippocampal slices involves increased glutamate uptake and modulation of A1 and A_{2A} adenosine receptors

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ARTICLE INFO

Article history:
Received 21 June 2013
Accepted 1 September 2013

Keywords:
Persea major
Glutamate
Neuroprotection
Adenosine

ABSTRACT

Ischemic stroke is characterised by a lack of oxygen and glucose in the brain, leading to excessive glutamate release and neuronal cell death. Adenosine is produced in response to ATP depletion and acts as an endogenous neuromodulator that reduces excitotoxicity. *Persea major* (Meins.) L.E. Kopp (Lauraceae) is a medical plant that is indigenous to South Brazil, and the rural population has used it medicinally due to its anti-inflammatory properties. The aim of this study was to evaluate the neuroprotective effect of *Persea major* methanolic extract against oxygen and glucose deprivation and re-oxygenation as well as to determine its underlying mechanism of action in hippocampal brain slices. *Persea major* methanolic extract (0.5 mg/ml) has a neuroprotective effect on hippocampal slices when added before or during 15 min of oxygen and glucose deprivation or 2 h of re-oxygenation. Hippocampal slices subjected to oxygen and glucose deprivation and re-oxygenation showed significantly reduced glutamate uptake, and the addition of *Persea major* methanolic extract in the re-oxygenation period counteracted the reduction of glutamate uptake. The presence of A1 or A_{2A}, but not A_{2B} or A3 receptor antagonists, abolished the neuroprotective effect of *Persea major* methanolic extract. In conclusion, the neuroprotective effect of *Persea major* methanolic extract involves augmentation of glutamate uptake and modulation of A1 and A_{2B} adenosine receptors.

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Introduction

Persea major (Meins.) L.E. Kopp, Lauraceae, is a medical plant that is grown mainly in South Brazil. It is popularly known as "pau-andrade", "abacate-do-mato" or "canela-rosa", and the rural population has used it to treat skin wounds, inflammation and gastric disorders. Previous studies demonstrated that the methanolic extract obtained from the bark of *Persea major* displays antibacterial (Schlemper et al., 2001) and antispasmodic (Cechinel-Filho et al., 2007) effects. Gastroprotective and intestinal motility inhibitory

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effects were also observed with the hydroalcoholic extract of *Persea major* (Cosmo et al., 2007).

Ischemic stroke is caused by a transient or permanent reduction of blood flow to the brain, which leads to a complex cascade of events that culminate in neuronal death (Hofmeijer and van Putten, 2012). Ischemic stroke represents the main cause of long-lasting disabilities, and it is the third leading cause of death in major industrialised countries (Savitz and Mattle, 2013). Oxygen and glucose deprivation (OGD) in brain tissue preparations is often used as an *in vitro* model of cerebral ischemia (Arias et al., 1999; Brongholi et al., 2006; Matsuda et al., 1992; Oliveira et al., 2002) that allows for the evaluation of neural degeneration. Additionally, this model system allows for the study of the effects of putative protective compounds in a cerebral structure that is especially susceptible to ischemic insult in a tissue preparation that maintains the cellular architecture observed in situ (Dal-Cim et al., 2011).

During OGD, the lack of glucose and oxygen reduces ATP levels, which leads to the collapse of the Na^+/K^+ electrochemical gradient. After OGD, when oxygen levels are normalised, termed the re-oxygenation period, ischemic insult can be aggravated by the additional generation of reactive oxygen species, mostly in the mitochondrial electron transport chain, inflammation and the abnormal release of neurotransmitters (Kostandy, 2012). As a result, glutamate transporters operate in a reverse direction, releasing excessive glutamate (Danbolt, 2001), resulting in the overstimulation of the NMDA receptor (excitotoxicity) (Lai et al., 2010).

Adenosine is an endogenous compound that modulates neurotransmission. The intracellular concentration of adenosine is tightly linked to the energy charge of cells, as small decreases in the energy charge result in large increases in the intracellular levels of adenosine (Cunha, 2001), which can be released to the extracellular space by adenosine transporters. Extracellular adenosine then acts on metabotropic adenosine receptors located in the cell membrane, A1, A_{2A}, A_{2B} and A3 receptors. In the nervous system, adenosine exerts a wide range of effects (Fredholm, 2007; Fredholm et al., 2005), but it generally inhibits the pre-synaptic activity of glutamatergic transmission (Dolphin and Archer, 1983); thus, adenosine plays an important protective role against excitotoxic/ischemic damage in the brain (Lopes et al., 2011).

Despite extensive and continued research on the mechanisms of ischemia-induced cell death, there are currently no pharmacological interventions that provide significant neuroprotection against brain ischemia or injury in the clinical setting. Because inflammation is a key event in ischemic brain injury (Xia et al., 2010) and *Persea major* has anti-inflammatory activities (Cosmo et al., 2007), we evaluated the neuroprotective effect of the *Persea major* methanolic extract (PMME) against OGD-induced cell damage and sought to unravel its underlying mechanism of action in hippocampal brain slices.

Materials and Methods

Plant material

Persea major (Meins.) L.E. Kopp, Lauraceae, bark was collected in Canoinhas, Santa Catarina, Brazil, in 2010. The plant material was identified by M. L. Brotto, and a specimen voucher (number 290857) of the plant was deposited in the Municipal Botanical Museum of Curitiba, Brazil.

Extract preparation

Methanolic extract was obtained by successive percolation of dried and powdered *Persea major* bark (3 kg) with methanol for 21 days. The solvent was changed every seven days, in the dark, at room temperature. The extract was evaporated *in vacuo*, and the residue was powdered and maintained at -20 °C until use.

Drugs

[^{3}H]Glutamate was purchase from Amersham Life Science. All other reagents were obtained from Sigma (St. Louis, MO, USA). The selective adenosine A1 receptor selective antagonist 8-cyclopentyl-1,3-dipropylxanthine (DPCPX) and the selective adenosine A_{2A} receptor antagonist 4-(-2-[7-amino-2-{2-furyl}{1,2,4}triazolo{2,3-a}{1,3,5}triazin-5-yl-amino]ethyl)phenol (ZM241385) were dissolved and added to the hippocampal brain slices at a final concentration of 0,01% dimethylsulfoxide (DMSO). The selective A3 adenosine receptor antagonist *N*-(2-methoxyphenyl)-*N*'-[2-(3-pyridinyl)-4-quinazolinyl]-urea (VUF5574, 1 µM) was also prepared in DMSO and added to slices at a final concentration of 1% DMSO. The antagonists were diluted in physiological buffer to the indicated final concentrations: 100 nM DPCPX (Molz et al., 2009); 50 nM ZM241385 (Molz et al., 2009); 0,1 µM alloxazine (Brown and Dale, 2000) and 1 µM VUF5574 (Stella et al., 2003).

Animals

Male Swiss mice (60-90 days old, weighing 20-30 g) were obtained from our local breeding colony and maintained on a 12-h light-12 h dark schedule at 25° C with food and water *ad libitum*. All experiments followed the "Principles of laboratory animal care" (NIH publication No. 85-23, revised 1985) and were approved by the local Ethical Committee of Animal Research (CEP/UnC 087/2010).

Preparation and incubation of hippocampal slices

Mice were sacrificed by decapitation, and the hippocampi were rapidly removed and placed in ice-cold Krebs-Ringer bicarbonate buffer (KRB; 122 mM NaCl, 3 mM KCl, 1.2 mM $MgSO_4$, 1.3 mM $CaCl_2$, 0.4 mM KH_2PO_4, 25 mM $NaHCO_3$ and 10 mM D-glucose). The buffer was bubbled with 95% O_2-5% CO_2 at pH 7.4. Slices (0.4 mm thick) were rapidly prepared using a McIlwain Tissue Chopper, separated in KRB at 4 °C and allowed to recover for 30 min in KRB at 37 °C for stabilisation (Oliveira et al., 2002).

In the OGD model, hippocampal slices were incubated in a modified KRB buffer, where D-glucose was replaced with 10 mM 2-deoxyglucose (Pocock and Nicholls, 1998), and bubbled with nitrogen throughout the incubation period (Strasser and Fischer, 1995). During the re-oxygenation period, the OGD

buffer was replaced by physiological KRB and bubbled with 95% O_2–5% CO_2.

Treatment of hippocampal slices

To determine whether PMME was toxic to hippocampal slices, PMME was dissolved in aqueous buffers (KRB) and added to slices for 2 h and 15 min.

To verify the neuroprotective effect of PMME, the slices were treated as follows: (a) slices were pre-incubated with PMME for 15 min and then subjected to OGD and re-oxygenation; (b) PMME was added to slices only during the OGD period and then subjected to re-oxygenation; or (c) slices were subjected to OGD and then PMME was added during the re-oxygenation period (Oleskovicz et al., 2008). In all experiments, the slices were incubated for 2 h and 15 min in physiological buffer (KRB) alone as a control. In experiments where adenosine receptors antagonists were tested, hippocampal slices were treated with adenosine antagonists for 15 min before adding PMME, and the antagonists were present the during re-oxygenation period. The following antagonists were used: selective adenosine A1 receptor antagonist (DPCPX, 100 nM), adenosine A_{2A} receptor antagonist (ZM241385, 50 nM), adenosine receptor A_{2B} antagonist (alloxazine, 0,1 µM) and adenosine A3 receptor antagonist (VUF5574, 1 µM).

To assess the involvement of glutamate transport in the neuroprotective effect of PMME, the extract was dissolved in HBSS. Hippocampal brain slices were subjected to OGD, and PMME was added during the re-oxygenation period.

Cellular viability assay

Cell viability was determined by measuring the ability of cells to reduce MTT (3-(4,5-dimethylthiazol-2-yl-diphenyltetrazolium bromide; Sigma) (Mosmann, 1983). Hippocampal slices were incubated with MTT (0.5 mg/ml) in KRB for 30 min at 37 °C. The tetrazolium ring of MTT is cleaved by active dehydrogenases and produces precipitated formazan. Next, 200 µl DMSO was used to dissolve the formazan, generating a coloured compound. The optical density of each well was measured using a microplate reader (Labsystems Multiskan MS-550 nm).

Glutamate uptake assay

L-[^{3}H]-glutamate uptake was evaluated as previously described (Molz et al., 2009). After OGD and re-oxygenation, hippocampal slices were maintained for 15 min at 37 °C in HBSS containing the following (mM): 1.3 $CaCl_2$, 137 NaCl, 5 KCl, 0.65 $MgSO_4$, 0.3 Na_2HPO_4, 1.1 KH_2PO_4, 2 glucose and 5 HEPES. Uptake was assessed by adding 0.33 µCi/ml L-[^{3}H]-glutamate with 100 µM of unlabelled glutamate. After 7 min, incubation was stopped by discarding the incubation medium, and the slices were subjected to two ice-cold washes with 1 ml HBSS. The slices were solubilised by overnight incubation in 0.1% NaOH/0.01% SDS. Aliquots of the resulting slice lysates were collected to determine the intracellular L-[^{3}H]-glutamate content of using a scintillation counter. Sodium-independent uptake was determined using choline chloride instead of sodium chloride in the HBSS buffer. Non-specific sodium-independent uptake (approximately 30% of total glutamate uptake) was subtracted from the total uptake to obtain the specific sodium-dependent glutamate uptake. The results are expressed as nmol of L-[^{3}H]-glutamate taken up per mg of protein per min. Protein content was determined according to Peterson (1977), using bovine albumin as a standard.

Statistical analysis

The results are expressed as the means ± standard error of mean (SEM). Comparisons among groups were performed by one-way analysis of variance (ANOVA) followed by Tukey's test (Graphpad Prism), and $p \leq 0.05$ was considered to be statistically significant.

Results and discussion

The viability of hippocampal slices incubated in control conditions with increasing concentrations of PMME is shown in Fig. 1. No significant changes on cellular viability were observed at any concentration tested. The results are expressed as the percentage viability with respect to the control group (100%).

Considering that the extract was not toxic to hippocampal slices, they were pre-incubated in KRB buffer for 15 min with increasing concentrations of PMME (0.5 to 500 µg/ml) and then subjected to OGD and re-oxygenation (OGD+R). Ischemic conditions (OGD+R) significantly decreased cellular viability ($p \leq 0.001$) (Fig. 2). Pretreatment with PMME extract (5 µg/ml) significantly inhibited OGD+R induced cell death ($p \leq 0.001$) to a similar extent as MK-801 (50 µM), an NMDA receptor antagonist. Based on this result, this PMME concentration was used in all subsequent experiments.

Next, PMME extract was added to slices during 15 min of OGD or during the 2 h re-oxygenation period. PMME treatment significantly reduced cell death in OGD or OGD+R-induced hippocampal slices (Fig. 3). Therefore, not only is PMME

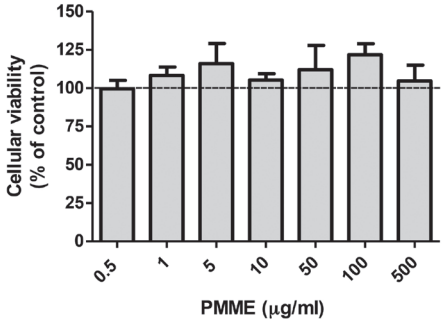

Fig. 1 - Effect of crescent concentrations of PMME on cellular viability of hippocampal slices incubated in control conditions. Slices were incubated for 2 h and 15 min in KRB (100% of cellular viability, dashed lines) or in KRB with crescent concentrations of PMME (0.5, 1, 5, 10, 50 100 and 500 µg/ml). These results represent the means ± SEM of five experiments carried out in triplicate. There was no significant difference between the groups.

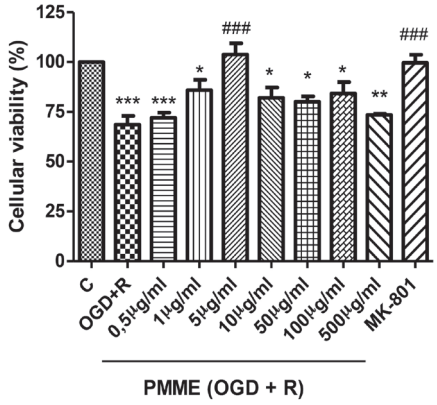

Fig. 2 - Effect of preincubation with crescent concentrations of PMME (0.5, 1, 5, 10, 50, 100 and 500 µg/ml) on cellular viability reduction induced by oxygen/glucose deprivation and re-oxygenation (OGD+R). The control group (dashed lines) was considered to be 100%. The results represent the means±SEM of five experiments carried out in triplicate.
*** ($p \leq 0.001$), ** ($p \leq 0.01$) and * ($p \leq 0.05$) indicate that the mean is significantly different from the control (dashed lines), PMME 5 µg/ml or MK-801 groups; ### ($p \leq 0.001$; F = 8.809) indicates that the mean is significantly different from the OGD+R group, (ANOVA followed by Tukey's test).

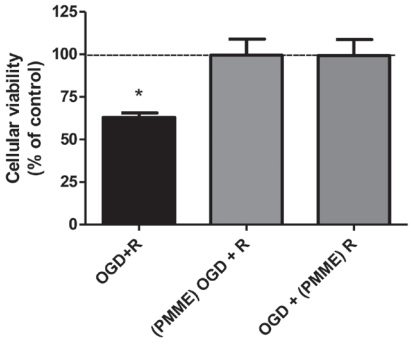

Fig. 3 - Effect of PMME on oxygen/glucose deprivation and re-oxygenation (OGD+R)-induced cell death. PMME (5 µg/ml) was incubated during the 15 min OGD time period or during the 2 h re-oxygenation (R) period. The control group (dashed lines) was considered to be 100%. The results represent the means ± SEM of five experiments carried out in triplicate. * Indicates that the mean is significantly different from all other groups; $p \leq 0.05$; F = 7.292 (ANOVA followed by Tukey's test).

neuroprotective as a pretreatment but, more importantly, it can also reverse cellular injury when it is already been activated. This experiment was performed in a condition that is clinically relevant to the treatment of ischemic injury.

Impairment of glutamate transport and excessive activation of glutamate receptors are involved in ischemia-induced damage *in vitro* and *in vivo* (Dal-Cim et al., 2011; Puyal et al., 2013). Because MK-801, an NMDA subtype receptor antagonist, afforded the same level of neuroprotection as PMME extract (Fig. 2), we evaluated the ability of PMME extract to counteract OGD+R-induced glutamate uptake impairment. OGD+R significantly decreased glutamate uptake, and the addition of PMME during the re-oxygenation period counteracted this OGD+R-induced impairment of glutamate uptake (Figure 4). These data suggest that PMME has an inhibitory effect on ODG+R –induced glutamate transport impairment, affording neuroprotection by inhibiting excitotoxicity.

Adenosine levels rise markedly in response to ischemia, hypoxia, excitotoxicity or inflammation, as it is a neuroprotectant under these conditions (Lopes et al., 2011). In hippocampal glutamatergic nerve terminals, A1 and A_{2A} receptors co-localise and functionally interact with a subset of these terminals (Rebola et al., 2005); (Ciruela et al., 2006). In this study, the neuroprotective effect of PMME was abolished in the presence of A1 or A_{2A} adenosine receptor antagonists (Figs. 5 and 6), suggesting that A1 and A_{2A} receptors mediate the neuroprotective effects of PMME extract in hippocampal slices. In contrast, when the antagonists were added to the media during the re-oxygenation period (in the absence of PMME extract), they did not alter cell death, which indicates that blocking adenosine receptors was not detrimental toward hippocampal slices compared to OGD+R *per se*.

Due to their low density in the brain, the possible involvement of A3 and A_{2B} receptors in brain function and neuroprotection are poorly understood. However, both A3 and A_{2B} are present in neural tissue and can be activated under pathological conditions (Popoli and Pepponi, 2012). As shown in Figs. 7 and 8, the presence of A_{2B} or A_3 receptors antagonists (respectively) did not alter the PMME-induced increase in cell viability . Therefore, neither the A_{2B} nor A3 receptors are involved in the neuroprotective effect of PMME against OGD+R in mouse hippocampal slices. Moreover, antagonists added to the media during the re-oxygenation period

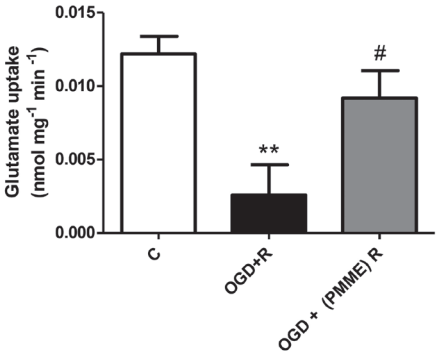

Fig. 4 - Effect of PMME on glutamate uptake in hippocampal slices subjected to oxygen/glucose deprivation and re-oxygenation (OGD + R). PMME (5 µg/ml) was added during the re-oxygenation period (R). The values are expressed as nmol L-[^{3}H] glutamate/mg protein/min and represent the means ± SEM of five experiments carried out in triplicate. * Indicates that the mean is significantly different from the control group ($p \leq 0.01$; F = 8.004); # indicates that the mean is significantly different from the OGD + R group ($p \leq 0.05$; F = 8.004) (ANOVA followed by Tukey's test).

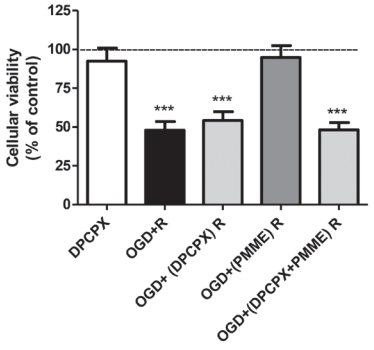

Fig. 5 - Effect of the adenosine A1 receptor antagonist (DPCPX, 100 nM) on the neuroprotective effect of PMME. The control group (dashed lines) was considered to be 100%. The values are expressed as the percentage of the control and the data represent the means ± SEM of five experiments carried out in triplicate. *** indicates that the mean is significantly different from the control, DPCPX and OGD + (PMME) R groups, $p \leq 0.001$; F = 17.64 (ANOVA followed by Tukey's test).

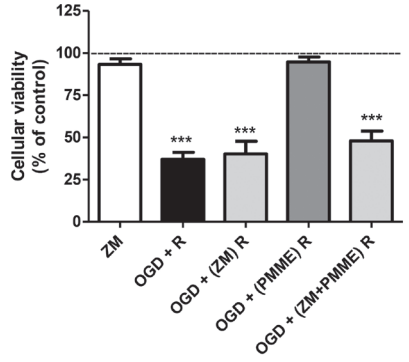

Fig. 6 - Effect of the adenosine A_{2A} receptor antagonist (ZM 241385, 50 nM) on the neuroprotective effect of PMME. Control group (dashed lines) was considered to be 100%. The values are expressed as the percentage of the control and the data represents the mean s± SEM of four experiments carried out in triplicate. *** Indicates that the mean is significantly different from the control, ZM and OGD + (PMME) R groups, $p \leq 0.001$; F = 42.05 (ANOVA followed by Tukey's test).

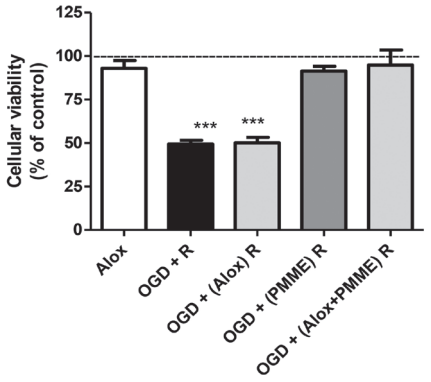

Fig. 7 - Effect of the adenosine A_{2B} receptor antagonist (alloxazine 0,1 µM) on the neuroprotective effect of PMME. Control group (dashed lines) was considered to be 100%. The values are expressed as the percentage of the control and the data represent the means ± SEM of seven experiments carried out in triplicate. *** Indicates that the mean is significantly different from the control, alox, OGD + (PMME) R and OGD + (alox + PMME) R groups, p ≤ 0.001; F = 28.67 (ANOVA followed by Tukey's test).

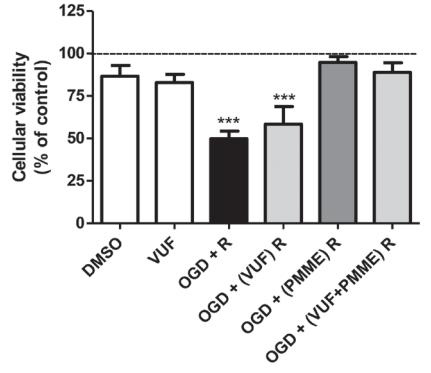

Fig. 8 - Effect of the adenosine A3 receptor antagonist (VUF 5574, 1 µM) on the neuroprotective effect of PMME. Control group (dashed lines) was considered to be 100%. The values are expressed as the percentage of the control and the data represent the means ± SEM of seven experiments carried out in triplicate. *** indicates that the mean is significantly different from the control, DMSO, VUF, OGD + (PMME)R and OGD+(VUF+PMME)R groups, $p \leq 0.001$; F = 10.45 (ANOVA followed by Tukey's test).

did not alter cell death, which indicates that blocking adenosine receptors was not detrimental toward hippocampal slices compared to OGD+R per se.

Taken together, these results indicate that PMME-induced neuroprotection involves modulation of the adenosine A1 and A_{2A} receptors but not of the A_{2A} or A3 receptors. Presynaptic A_{2A} receptors heterodimerise with A1 receptors and tightly regulate glutamate release (Ciruela et al., 2006). Thus, we hypothesise that PMME extract acts as an agonist of adenosine receptors, or it may increase adenosine release into the synaptic cleft, which in turn can act on the A1 or A_{2A} receptors and provide neuroprotection by decreasing the release of glutamate and counteracting neuronal cell death.

Polyphenols are natural substances with variable phenolic structures that are found in vegetables, fruits, grains, bark, roots, tea, and wine (Landete, 2012). Dietary phenols are important antioxidants (Yang et al., 2009) that are neuroprotective against glutamate-induced excitotoxicity (Lee et al., 2003; Martini et al., 2007) and ischemia-induced neurodegeneration (Lin, 2011; Panickar and Jang, 2013; Simonyi et al., 2005). The literature demonstrates that extracts containing polyphenols (Cho et al., 2010; Gupta et al., 2010; Panickar et al., 2009; Shimada

et al., 2000) and isolated polyphenol obtained from Lauraceae species provide neuroprotection against OGD-induced cell death (Lee et al., 2010) by inhibiting glutamate release (Panickar et al., 2012). The *Persea* genus is rich in polyphenols (Rodriguez-Carpena et al., 2011) and has demonstrated neuroprotective effects against ischemia-induced neuronal death (Eser et al., 2011; Yaman et al., 2007). However, to the best of our knowledge, this is the first evidence that a species from the Lauraceae family exerts neuroprotection against OGD by modulating adenosine receptors.

In conclusion, for the first time, this study demonstrates the neuroprotective effect of PMME and its underlying mechanism of action in hippocampal brain slices. Few studies have isolated and identified the chemical constituents of *Persea major*. Batista et al., (2010) reported the presence of flavonoids, sesquiterpenes and lignans. Phytochemical studies are currently in progress in our laboratory to elucidate the principles of PMME and its relevant mechanism of action. The medical management of ischemic stroke is still dependent on thrombolytics, anticoagulants and nonspecific neurotonics (Heitsch and Panagos, 2013); thus, the introduction of new natural products that prevent the effects of glutamate during ischemia opens a new chapter for ischemic stroke management that might provide substantial benefits for patients.

Authorship

MLF (undergraduate student) and FKL (PhD student) contributed to the laboratory work, biological studies, data analysis and drafted the paper. SM and CIT designed the study, supervised the laboratory work and critically read the manuscript. All the authors have read the final manuscript and approved its submission.

Acknowledgments

This research was supported by a grant from Fundação de Amparo à Pesquisa e Inovação do Estado de Santa Catarina (FAPESC). The authors thank Reinaldo Ribeiro for his contribution in collecting plant samples and Clarisse Bolfe Poliquesi for assisting with plant identification and herbarium confection.

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