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Amburana cearensis seed extract protects brain mitochondria from oxidative stress and cerebellar cells from excitotoxicity induced by glutamate

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ABSTRACT

Ethnopharmacological relevance: *Amburana cearensis* (Allemao) A.C.Sm. is a medicinal plant of the Brazilian Caatinga reported to present antioxidant and anti-inflammatory activity. This study aimed to evaluate the neuroprotective effect of the extracts obtained from the seeds of *A. cearensis* in primary cultures of cerebellar cells subjected to excitotoxicity induced by glutamate and brain mitochondria submitted to oxidative stress.

Materials: and methods: Primary cultures of cerebellar cells were treated with the ethanol (ETAC), hexane (EHAC), dichloromethane (EDAC) and ethyl acetate (EAAC) extracts of the seeds of *A.cearensis* and subjected to excitotoxicity induced by glutamate (10 μM). Mitochondria isolated from rat brains were submitted to oxidative stress and treated with ETAC.

Results: Only the EHAC extract reduced cell viability by 30% after 72 h of treatment. Morphological analyses by Immunofluorescence showed positive staining for glutamine synthetase, β-III tubulin, GFAP and IBA1 similar to control cultures, indicating a better preservation of astrocytes, neurons and microglia, after excitotoxic damage induced by glutamate in cerebellar cultures treated with the extracts. The ETAC extract also protected mitochondria isolated from rat brains from oxidative stress, reducing the swelling, dissipation of the membrane potential, ROS production and calcium influx.

Conclusion: Thus, this study suggests that the seed extracts from *A. Cearensis* exhibit neuroprotective potential against oxidative stress and excitotoxicity induced by glutamate and can be considered a potential therapeutic agent in the treatment of neurodegenerative diseases.

1. Introduction

Glutamate is the predominant excitatory neurotransmitter in the central nervous system (CNS). Increased amounts of glutamate are associated with excitotoxicity a process plays a critical role in the development of neurological and neurodegenerative diseases (Lewerenz and Maher, 2015; Stamoula et al., 2015). Compounds that regulate the intracellular signalling cascades of glutamate excitotoxicity that lead to calcium influx and mitochondrial dysfunction can minimize the main causes of neuronal death. Among these compounds are metabolites present in medicinal plants with antioxidant and anti-inflammatory properties. The *Amburana cearensis* (Allemao) A.C.Sm. is a species of the family of Fabaceae (Papilionoideae, Leguminoseae) that is naturally widespread in the Caatinga of northeastern Brazil (Bravo et al., 1999). The use of preparations from different parts of the plant, such as the stem bark, leaves and seeds, is common, especially for the treatment of headache, muscular aches, constipation, urinary tract infections and as anti-inflammatory and spasmolytic in the respiratory tract diseases (Leal et al., 2003; Agra et al., 2007; Roque, 2009; Lima, . et al., 2013). Several compounds with antioxidant properties were isolated from the seeds of *A. cearensis* including coumarins, methyl esters, phytoesterols (γ-sitosterol, stimasterol, campesterol) (Pereira et al., 2016). Pereira et al. (2014) have observed the increase of the activity of the antioxidant enzymes glutathione reductase and glutathione peroxidase in the seeds of *A. cearensis*, when subjected to water stress, related to total phenols content in methanolic

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extracts. In Parkinson's disease model, it was found that the glucoside amburoside A isolated from *A. cearensis* did not show any citotoxicity and exhibited neuroprotective and antioxidant effect against 6-hydroxydopamine on rat mesecephalic cells culture (Leal et al., 2005). This study aims to evaluate the neuroprotective effect of the extracts from the seeds of *A. cearensis* in primary cultures of cerebellar neural cells subjected to excitotoxicity induced by glutamate and in mitochondria isolated from rat brains subjected to oxidative stress.

2. Materials and methods

2.1. Amburana cearensis seed extracts

The seeds of *Amburana cearensis* (Allemao) A.C.Sm. were obtained in popular trade in the city of Feira de Santana, Bahia, Brazil, and had its authenticity recognized, when compared with the identification (13734) according to Guedes et al. (2007), which are deposited in the Herbarium of the Biology Institute of the Federal University of Bahia. The extractions of the total ethanol (ETAC), hexane (EHAC), dichloromethane (EDAC) and ethyl acetate (EAAC) extracts of the seeds of *A. cearensis* were performed as previously described (Pereira et al., 2016).

The preparation of extracts was performed by the soaking method. The seeds (1 kg) were ground in a knife mill and dried for a period of 24 h in an exhaust hood. Then, the material was placed in a glass container, covered with ethyl alcohol for 72 h and successively shaken every 24 h. After this period, the supernatant was collected and filtered. Additional ethanol was added to the obtained pellet. This process was repeated three times. The resulting supernatant from the three macerations was collected and placed on a rotary evaporator at 40–50 °C. Then, the extract was dried in an oven at 40 °C for three days. The final product was named the crude ethanolic extract of the seeds of *A. cearensis* (ETAC). The preparation of the fractions occurred using the lowest solvent polarity to the highest polarity. Fractionation of the other extracts using the partition method was performed using the ethanolic extract. The dry crude ethanolic extract (ETAC, 2 g) was solubilized in 100 mL of ethanol (PA) with a 20% volume of distilled water (20 mL H_2O). A volume of 50 mL hexane solvent was added to this mixture. The mixture was stirred and placed at rest until the phases separated. The hexanic phase was then collected. This process was repeated three times to obtain a final volume of 150 mL for the hexane partition. The same technical procedure was performed for the solvents methylene chloride and ethyl acetate. The resulting extracts were placed on a rotary evaporator and dried in an oven at 40 °C for three days. The resulting extracts were named the hexane extract of the seeds of *A. cearensis* (EHAC), the dichloromethane extract of the seeds of *A. cearensis* (EDAC) and the ethyl acetate extract of the seeds of *A. cearensis* (EAAC).

The stock solutions for the bioassays were prepared by dissolving each extract in water to a final concentration of 100 mg/mL. The solutions were then stored at 4 ◦C.

2.2. Primary cultures of cerebellar neural cells

The neural cerebellar cell cultures were obtained according to (Whittemore et al., 1995) with adaptations. The postnatal Wistar rats, 8 days of age (P8), were decapitated, and the cerebellum was removed and placed in DMEM HAM F12 (Cultilab, Brazil) medium supplemented with 10% (v/v) fetal bovine serum (FBS) (CULTILAB, Brazil), L - (+) - glutamine (2 mM), 10% (v / v) glucose, KCl (25 mM), penicillin (100 IU / mL) and streptomycin (100 mL) (CULTILAB, SP, Brazil). Then, the cerebellum was mechanically perforated with a Pasteur pipette previously polished to reduce the opening of the pores and facilitate the separation of the cells. The cell suspension was filtered using a cell strainer. Subsequently, the culture was centrifuged at 37 °C for 4 min at 1000 × g; the supernatant was discarded; and the cells were resuspended in fresh medium (described above). The cells were counted in a Neubauer chamber, seeded on polystyrene culture plates (TPP, Trasadingen, Switzerland) according to the experimental design, and maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C. The animals were obtained from the vivarium of the School of Veterinary Medicine and zootechnology at the Federal University of Bahia (UFBA). The procedures were performed according to the rules of the Ethics Committee on Animal Experimentation (CEUA) of the Health Sciences Institute of UFBA and approved with registration No. 0272012.

2.3. Cell viability analysis

The cytotoxicity of *A. cearensis* seed extracts on cerebellar cells was determined using 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). Primary cultures of neural cerebellar cells were cultured in 96-well culture plates (TPP, Trasadingen, Switzerland) at a density of $1x10^5$ cells / well and exposed to ETAC, EAAC, EDAC and EHAC extracts at concentrations of 0.1, 1.0, 10 and 100 µg/mL dissolved in sterile Milli Q water for a period of 72 h. For the neuroprotective assay, the cerebellar cultures in 96-well plates were exposed to a glutamate excitotoxic agent at a concentration of 10 µM. After 4 h, the cultures were treated with the ETAC, EAAC, EDAC and EHAC extracts at concentrations of 0.1, 1.0, 10 and 100 µg/mL and maintained in a humidified chamber until the completion total of 24 h of treatment with glutamate. Cultures without any treatment were included as control. Two hours before the end of exposure time, the MTT solution was added to each well (100 µL / well) at a final concentration of 1 µg/mL, diluted in DMEM F12 (Cultilab, Brazil). Thereafter, the cells were lysed with a volume of 100 µL/well lysis buffer containing 20% sodium dodecyl sulfate (SDS) and 50% dimethylformamide (DMF) at pH 4.7 at 37 °C overnight to complete dissolution of the formazan crystals. The next day, the optical absorbance of each sample was measured using the spectrophotometer at a wavelength of 595 nm (Thermo Varioscan, Finland). The cell cytotoxicity was quantified by measuring the conversion of yellow MTT into purple MTT formazan by mitochondrial dehydrogenases of living cells. Each experimental condition was performed in eight replicate wells in at least three independent experiments, and the results were presented as the percent viability of the control, considered as 100%.

2.4. Analysis of neuronal degeneration

The degeneration of neurons was analysed by the Fluoro-Jade B reagent (FJB), a fluorescent marker with a high affinity for locations with neuronal degeneration (Oh et al., 2015). The cerebellar cells were cultured in 96-well black bottom plates (TPP, Trasadingen, Switzerland) at a density of $1x10^5$ cells/well and treated with glutamate at a concentration of 10 µM for 4 h. Then, the ETAC, EAAC, EDAC and EHAC extracts were added at a concentration of 0.1 µg/mL, and cells were maintained in a humidified chamber until the completion of a total of 24 h of treatment with glutamate. Cultures without any treatment were considered as control. The culture supernatant was removed, and cells were fixed with ethanol at 4 °C for 10 min. The cultures were rinsed 3 times with PBS and permeabilized with PBS / Triton X-100 0.3% for 10 min. After this time the cultures were rinsed 3 times with distilled water and incubated with 0.001% Fluoro-Jade B solution for 30 min at 24 °C under slow stirring and protected from light. Next, the cultures were rinsed 3 times with PBS and incubated with a fluorescent DNA intercalating agent, 4', 6-diamidino-2-phenylindole dihydrochloride (DAPI, Molecular Probes, Eugene, OR), for 5 min and rinsed again with PBS. Two readings were taken in the fluorometer (Varioskan Thermo, Finland): the first filter, 480/530 nm, for quantification of Fluoro-Jade B and the second filter at 350/470 nm for quantification of DAPI. The result of the quantitation of neuronal degeneration was assessed by correlation between the fluorescence intensity of Fluoro-Jade B and DAPI and presented as a percentage of

fluorescence intensity of Fluoro-Jade B / DAPI. Each experimental condition was performed in eight replicate wells in at least three independent experiments.

2.5. Morphological evaluation of neurons and glia

The evaluation of glial and neuronal morphology of cerebellar cultures were performed using immunofluorescent staining against the following proteins: the enzyme, glutamine synthetase (GS), exclusively found in astrocytes; beta-III tubulin, a neuronal cytoskeleton protein; glial fibrillary acidic protein (GFAP), an intermediate filament protein exclusive to the cytoskeleton of differentiated astrocytes; and calcium binding adapter molecule 1 (Iba1), a marker of microglial morphology. Primary cultures of neural cerebellar cells were seeded at a density of $3x10^5$ cells/well in 24-well plates with cover slips pretreated with poly-D-ornithine (10 μg/mL) and laminin (5 μg/mL). These cells were exposed to the cytotoxic agent glutamate (10 μM). After 4 h, the cells were treated with the extracts (ETAC, EAAC, EDAC and EHAC) at a concentration of 0.1 μg/mL and maintained in a humidified chamber to complete a total of 24 h of treatment with glutamate. Cultures without any treatment were considered as control. Then, these cultures were washed three times with PBS at pH 7.4 and fixed with 4% paraformaldehyde for 20 min at 0 °C. Excess paraformaldehyde was discarded, and the plates were allowed to dry at 24 °C. Cells were then rehydrated with PBS, permeabilized with PBS / Triton X-100 (0.2%) and blocked with PBS and 5% bovine serum albumin (BSA) for 1 h. Next, the cultures were incubated separately with the following antibodies: rabbit polyclonal antibody against GS (1: 300 Abcam), rabbit polyclonal antibody against Iba1 (1: 200, Wako), monoclonal mouse antibody against beta-III-tubulin (1: 1000, Sigma), and polyclonal rabbit antibody against GFAP (1: 300, Dako), all diluted in PBS / BSA (1%) and kept in a humid chamber at 4 °C overnight. The next day, the cells were rinsed 3 times with PBS and then incubated with the secondary antibodies: Alexa Flour 488 green sheep secondary antibody against rabbit IgG (1:500, Life Tecnologic) or Alexa Flour 594 red sheep secondary antibody against mouse IgG (1: 500, Life Tecnologic), both diluted in PBS and incubated with slow stirring for 2 h at 24 °C. Control cultures were not incubated with secondary antibodies. Then, the nuclear chromatins were stained with DAPI (Molecular Probes, Eugene, Oregon, USA) at a concentration of 5 μg/mL for 10 min at 24 °C. The cultures were rinsed 3 times in PBS and mounted on slides containing the anti-fading agent N-propyl gallate. Then, the cultures were observed and photographed under a fluorescence microscope (Olympus AX70, Olympus) and confocal microscope (DMI6000 B, Leica) with filters suitable for each fluorescent antibody. To quantify cells of the immunofluorescence for Iba1, Image J software was used. Each experimental condition was performed photographs of 8 fields of two replicate wells, in two independent experiments.

2.6. Evaluation of mitochondrial function

To investigate whether mitochondria are the targets of *A. cearensis* seed extracts and have antioxidant activity, the effects of the total ethanol extract ETAC (100 μg/mL) were evaluated in isolated brain mitochondria from adult rats subjected to oxidative stress. Each experimental condition was performed in eight replicate wells in at least three independent experiments.

2.6.1. Mitochondria isolation and treatment

The mitochondria were isolated using a differential centrifugation method, as described by (Nuñez-Figueredo et al., 2014). Male Wistar rats of approximately 200 g were sacrificed. The brains were immediately removed and transferred to a Potter homogenizer tube containing the isolation buffer composed of 75 mmol/L sucrose, 1 mmol/L EGTA, 225 mmol/L mannitol, 0.1% BSA and 10 mmol/L HEPES-KOH, pH 7.2 and homogenized mechanically. The suspension was then centrifuged at 2.000 g for 3 min, and the resulting supernatant was collected and centrifuged at 12.000 g for 8 min. The pellet of this centrifugation was resuspended in 10 mL of isolation buffer containing 20 μL of 10% digitonin, which was used to release synaptosomes of the mitochondria, and centrifuged again at 12.000 g for 10 min. The supernatant was discarded and the final pellet was gently washed and resuspended in isolation buffer devoid of EGTA at a protein concentration of approximately 30–40 μg/mL. All isolation procedures were performed at 4 °C (Nuñez-Figueredo et al., 2014). The determination of the protein concentration was employed using the Lowry method (Lowry et al., 1951), and the reaction medium was used as a standard. After isolation, the mitochondria were maintained in incubation medium containing 130 mmol/L KCl, 1 mmol/L $MgCl_2$, and 2 mmol/L HEPES-KOH phosphate, pH 7.4, at 30 °C. In subsequent experiments, to evaluate mitochondrial activity, mitochondria (referred to as 1 μg protein/mL) underwent the energization process with 5 mmol/L potassium succinate, oxidative damage induced by 5 mmol/L rotenone, and treatment with the total ethanol extract of the *A. cearensis* seeds at one concentration of 100 μg/mL. The control was not treated with the extract. For breath assays, the medium was supplemented with 0.5 mmol/L EGTA and 10 mmol/L potassium phosphate.

2.6.2. Mitochondrial membrane potential

The mitochondrial membrane potential was determined immediately after incubation with the different treatments by spectrofluorimetric analysis. To this end, the fluorescent marker safranin O (10 μM), a lipophilic cation that accumulates in the mitochondrial membrane in proportion to the membrane potential (Zanotti and Azzone, 1980; Kowaltowski and Vercesi, 2002) was used, and the measurements were made using a fluorometer (Varioskan Thermo, Finland) with a 495/586 nm excitation / emission.

2.6.3. Reactive oxygen species (ROS) production

The amount of H_2O_2 generated by the mitochondria was measured by oxidation of the reagent Amplex Red (50 μM) in the presence of peroxidase (Horse Radish Peroxidase, - HRP, 1 U/mL) to a fluorescent compound called resorufin. The measurements were made using a fluorometer (Varioskan Thermo, Finland), with excitation wavelength and emission of 563 and 587 nm, respectively.

2.6.4. Mitochondrial swelling

Mitochondrial swelling was estimated by changes in light scattering due to the uptake of K^+ ions. It is known that the entry of K^+ into mitochondria is accompanied by phosphate intake and water (Kowaltowski and Vercesi, 2002). Because of this, there is a decrease in light scattering of the suspended mitochondria after swelling. Assessment of the activity of mitochondrial K^+ channel ATP-sensitive (myth KATP) was also performed by measuring the light scattering, that is, the higher the light scattering difference, the greater the channel activity. After the determination of the mitochondria protein concentration, the mitochondrial swelling was measured with the addition of 100 μM $CaCl_2$. Then, after 10 min, the absorbance reading was measured at 540 nm in a spectrophotometer (Thermo Varioscan, Finland).

2.6.5. Mitochondrial calcium

To determine the total mitochondrial calcium concentration, 1 μM red ruthenium and 1 μM oligomycin, to prevent Ca^{2+} influx, inhibit ATP synthesis and maintain the membrane potential of organelles, and 1 μM cyclosporin, to prevent flow of calcium through the transition pores of the permeable mitochondria, were added to the isolation buffer. After isolation, 2 mg/mL of mitochondrial suspension was subjected to hypo-osmotic shock with ultrapure water and three consecutive cycles of ultrasound for 5 min to release the full intramitochondrial calcium. Ca^{2+} influx was monitored by spectrofluoro-

metry at the excitation/emission of 506/531 nm (F4500 Hitachi Fluorescence spectrophotometry, Tokyo, Japan) (Radjev and Reynolds, 1993).

2.7. Statistical analysis

Statistical analyses were performed using GraphPad Prism 5.0 software. To analyse the results from cytotoxicity assays, the ANOVA followed by Tukey's post-test was used. The neuroprotection assays were analysed by ANOVA followed by Dunn's post-test. Testing of isolated mitochondria was analysed by ANOVA followed by Tukey's post-test. The results were expressed as the mean ± standard deviation relative to the control, which was considered to be 100%. In the mitochondria assay, the negative control with rotenone was considered to be 100%. P values < 0.05 were considered significant.

3. Results

3.1. Cytotoxicity of Amburana cearensis seed extracts

The cytotoxicity of the ETAC, EAAC, EHAC and EDAC extracts was determined by the MTT test in cerebellar cell cultures exposed for 72 h. The EAAC EDAC and EHAC extracts did not reduce cell viability at the tested concentrations ranging from 1 to 1000 μg/mL after 24 h of exposure compared to the control culture. The ETAC extract induced a significant increase (50%) in cell viability at the concentration of 1000 μg/mL (data not shown).

The results of the experiments after 72 h of exposure are presented in Fig. 1. The ETAC, EAAC and EDAC extracts showed no cytotoxicity to cerebellar cells at concentrations evaluated from 0.1 to 1000 μg/mL after 72 h of treatment. A 30% reduction in viability was observed when the cells were treated with EHAC only at the highest concentration of 1000 μg/mL (Fig. 1).

3.2. Evaluation of neuronal degeneration and glial reactivity

The analysis of neuronal degeneration of cerebellar cultures by Fluoro-Jade B after treatment with *A. cearensis* extracts at concentrations of 0.1 μg/mL showed an absence of FJB-positive cells, indicating neurons remained viable after 24 h (Fig. 2A). However, treatment with glutamate (10 μM) induced a significant increase in the number of FJB-positive cells and 31% cell death (Fig. 2B). On the other hand, treatment with EAAC and EDAC extracts significantly reduced the number of FJB-positive cells induced by glutamate, indicating protection from neuronal damage.

In cultures in control conditions and those exposed to the EAAC, EDAC and EHAC extracts, the double staining for β-III tubulin and GFAP revealed a preservation of neuronal morphology and a typical network of astrocytes, respectively. However, in cultures treated with the ETAC extract, a lower preservation of cells in the network was observed. Cultures treated with EDAC extract showed a better preservation of cells (Fig. 3D).

In cultures treated with glutamate (10 μM) and subsequently treated with the extracts, a reduction in the labelling of β-III tubulin and GFAP was observed, and this effect was worse in cultures treated with ETAC (Fig. 3F). The double staining for β-III tubulin and GFAP in these cultures treated with glutamate showed that the neurite network of neurons was not preserved, and only a perinuclear stain and an increase in the expression of positive GFAP cells was observed. Cultures submitted to glutamate and treated with the EAAC and EHAC extracts maintained the cell network. Moreover, the cultures treated with the extract EDAC showed the best preservation of astrocyte morphology and neuronal integrity, similar to that observed in control conditions (Fig. 3A).

Cerebellar cultures treated with the extracts (ETAC, EAAC, EDAC and EHAC) and control cultures, stained only with GFAP, showed typical astrocytes of the cerebellum, including Bergmann glia, with a hyperplasic nucleus and many thin processes stained by GFAP (Fig. 4A-E). In cultures treated with glutamate (10 μM), a reduction in the number of cells stained by GFAP was observed, with some hyperplasic cells (Fig. 4F-G).

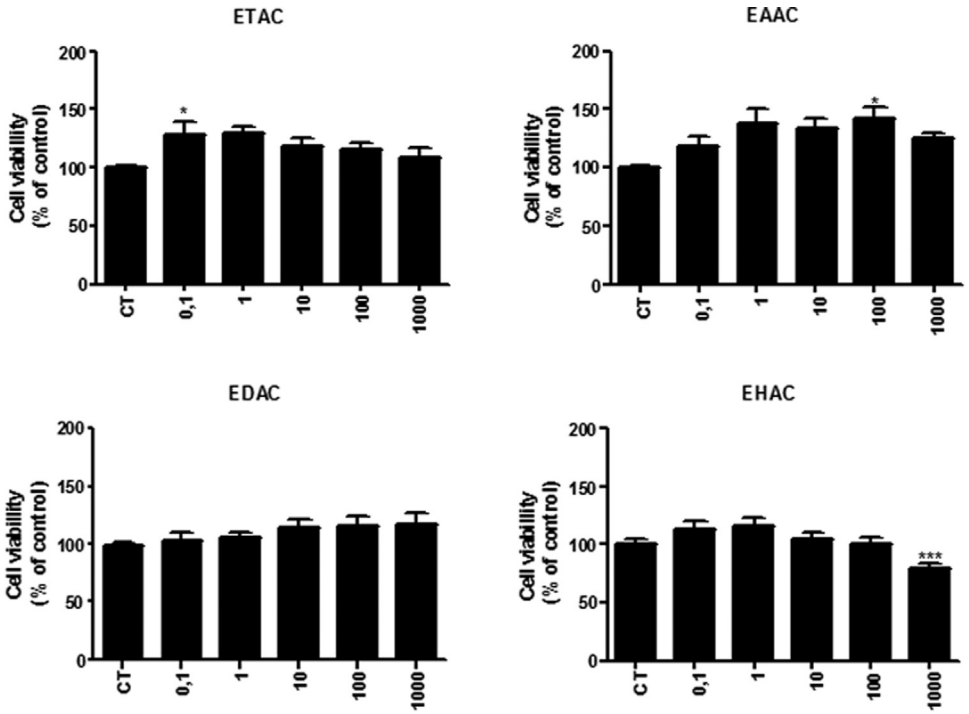

Fig. 1. Cytotoxicity of *A. cearensis* seed extracts in cerebellar primary culture treated for 72 h. Evaluation of cell viability by the MTT assay in cultures treated with ethanol (ETAC), dichloromethane (EDAC), ethyl acetate (EAAC) and hexane (EHAC) extracts of the seeds of *A. cearensis* in concentrations from 1 to 1000 μg/mL. The results are expressed as the percent absorbance at 595 nm of the control (CT), considered as 100% of cell viability. (n=8 per experimental condition, ANOVA followed by Tukey's post-test: *p < 0.05; ***p < 0.0001).

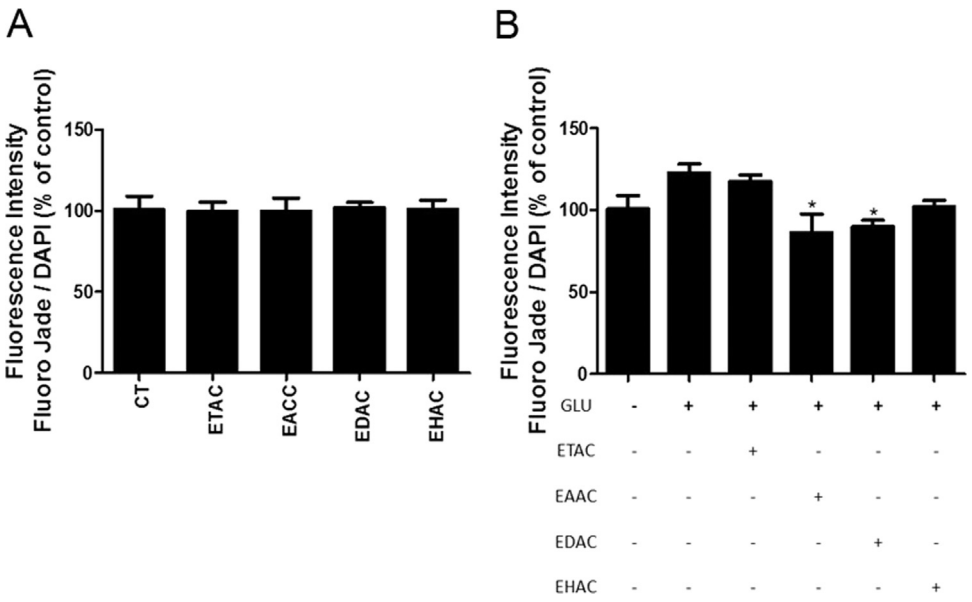

Fig. 2. Evaluation of neuronal degeneration by Fluoro-Jade B assay in primary cultures of cerebellar cells. (A) Cultures treated with ethanol (ETAC), dichloromethane (EDAC), ethyl acetate (EAAC) and hexane (EHAC) extracts from seeds of *A. cearensis* at concentrations of 0.1 μg/mL. (B) Cultures treated with the excitotoxic agent glutamate (GLU, 10 μM) and, after 4 h, treated with the extracts at a concentration of 0.1 μg/mL for a period of 24 h. The results are expressed in relation to the percentage of Fluoro-Jade B fluorescence related to DAPI fluorescence of the control cultures (CT), considered as 100% of neuronal degeneration. (n = 8 per experimental condition, ANOVA followed by Dunn's post-test: *p < 0.05).

In the cultures labelled for the enzyme glutamine sintetase (GS), the cells treated with glutamate (10 μM) became stunted (Fig. 4K-O). In the cultures treated with glutamate (10 μM) and subsequently treated with the extracts (ETAC, EAAC, EHAC and EDAC), there was an increase in the intensity of the GFAP and GS staining (Fig. 4E-O). This increase in GFAP and GS expression contributes to glutamate detoxification of the cultures by glia cells and may enhance neuronal survival. However, this increase in GS expression was not observed in cultures treated subsequently with ETAC (Fig. 4G). In control cultures, microglial cells labelled with Iba-1 showed relatively rounded cell body and few thin process, indicating a quiescent state (resting cells). Specific staining for microglia cells revealed less Iba1-positive cells after exposure to the ETAC, EAAC and EHAC extracts compared with the control cultures. Cultures treated with the EDAC extract demonstrated an increase in the expression of Iba1-positive cells (Fig. 5). In cultures treated with glutamate, an increase in the population of Iba1-positive cells with rounded cell bodies and no process was observed (Fig. 5F). Cultures previously exposed to glutamate and treated with ETAC also showed an increase in the number of Iba1-positive cells, suggesting microglial proliferation and activation. On the other hand, in cultures treated with glutamate (10 μM) and then treated with the EAAC, EHAC and EDAC extracts, a reduction in Iba1 staining was observed (Fig. 5). The proliferation and activation of microglial cells is associated to neuronal degeneration and apoptosis. Glutamate increased Iba1-positive cells and EAAC, EHAC and EDAC reduced Iba1 staining, that may contributes for neuroprotection.

3.3. Effects of A. cearensis ETAC extract on mitochondrial function

As noted in Fig. 6, the ETAC extract at a concentration of 100 μg/mL was able to produce a protective effect in rat brain mitochondria, significantly reducing ($p < 0.05$) the dissipation of the mitochondrial potential, mitochondrial swelling and ROS production (Fig. 6A-C). Furthermore, the ETAC extract inhibited the Ca^{2+} influx to the rat brain mitochondria energized with succinate (Fig. 6D). Therefore, it can be inferred that the components present in ETAC can protect mitochondria isolated from adult rats from oxidative stress and that this mechanism is possibly associated with the neuroprotection observed in neuronal and glial cells of the cerebellar cultures studied.

4. Discussion

The *Amburana cearensis* is a medicinal plant from the Caatinga of northeastern Brazil that is mainly used in folk medicine as anti-inflammatory to treat urinary and respiratory tract infections (Leal et al., 2003; Agra et al., 2007; Roque, 2009; Lima, . et al., 2013). Pereira et al., (2014, 2016) observed several compounds and enzymes with antioxidant properties in the seeds of *A. Cearensis*. The present study demonstrated the neuroprotective and antioxidant potential of extracts obtained from seeds of *A. cearensis* in cerebellar cultures submitted to excitotoxic damage induced by glutamate and in brain mitochondrias subject to oxidative stress.

Excitotoxicity induced by glutamate and oxidative stress are common features present in various neurodegenerative diseases such as Alzheimer, Huntington, amyotrophic lateral sclerosis and ischemic stroke (Lewerenz and Maher, 2015; Rama Rao and Kielian, 2015; Liao et al., 2017). Excitotoxicity is a term proposed by Olney that has been used for several years to describe death induced by glutamate. This process is dependent upon the influx of calcium through NMDA receptors and is associated with neurodegeneration caused by excitatory amino acids (Olney, 1978). Metabolic compounds with antioxidant and neuroprotective effects may be able to combat the cascade of events after the excitotoxic insult. According to Halliwell (2000), an "antioxidant is any substance that, when present at low concentration compared to that of the oxidizable substrate, it regenerates the substrate or substantially prevents the oxidation of the same". Antioxidant agents that possess the ability to detoxify free radicals can be found endogenously or are acquired from the diet, especially from foods rich in phytochemicals. The results of this study showed that ETAC, EAAC, EDAC and EHAC extracts of *A. cearensis* showed no toxicity to primary cultures of cerebellar cells and protected neurons and glia cells against excitotoxicity induced by glutamate. Studies by Leal et al. (2003) and Lima et al. (2013) also evaluated the anti-inflammatory effect of the hydroalcoholic and aqueous extracts obtained from the stem bark and seeds of *A. cearensis*, respectively, and showed that they did not exhibit any toxic effect.

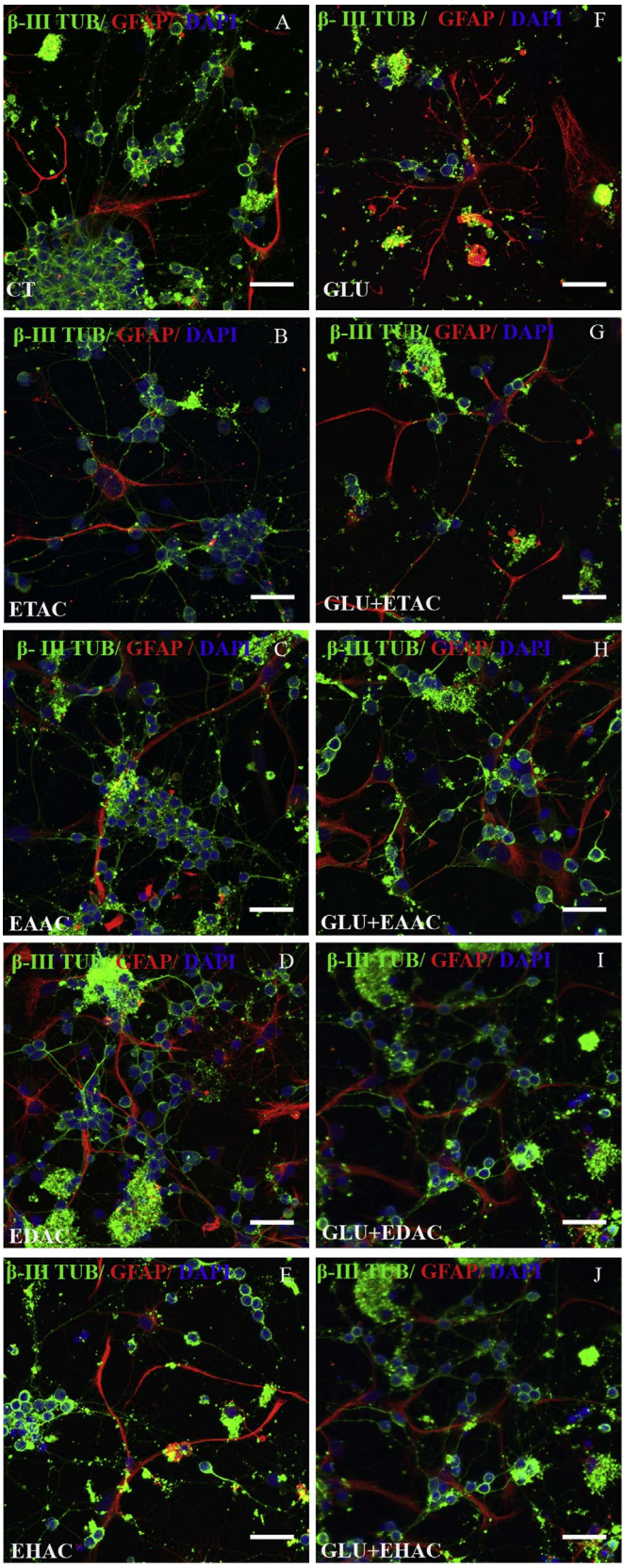

Fig. 3. Double immunofluorescence labelling of β-III tubulin (green) and GFAP (red) cytoeskeleton proteins in primary cultures of cerebellar neural cells analysed by confocal microscopy. (A-E) Cultures in control conditions (CT) and those treated with ethanol (ETAC), ethyl acetate (EAAC), dichloromethane (EDAC) and hexane (EHAC) extracts obtained from the seeds of *A. cearensis* at a concentration of 0.1 μg/mL for a period of 24 h. (F-G) Cultures treated with the excitotoxic agent glutamate (GLU, 10 μM) and, after 4 h, treated with ethanol (ETAC), ethyl acetate (EAAC), dichloromethane (EDAC) and hexane (EHAC) extracts from the seeds of *A. cearensis* at a concentration of 0.1 μg/mL to complete the treatment with glutamate for a period of 24 h. In blue: nuclear chromatin stained with 4', 6-diamidino-2-phenylindole dihydrochloride (Dapi). Objective 63x. Scale bars = 100 μm. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

In a previously study to characterize the constituents of the seeds of *A. Cearensis* coumarins were shown to be concentrated in the EDAC extract (Pereira et al., 2016). Coumarins are the main component of *A. cearensis* (Leal et al., 2006) and are directly related to its antioxidant potential. In addition, in the present study, EDAC showed the greatest neuroprotective potential, reducing the neuronal degeneration induced by glutamate, as shown in the test with Fluoro-Jade B, a specific marker of neuronal degeneration. Immunofluorescence for beta III-tubulin also demonstrated that the extracts (EAAC, EDAC and EHAC) increased neuronal survival.

The *A.cearensis* extracts appear to contribute to neuronal and glial cell survival in various ways. Many studies have demonstrated the involvement of astrocytes as a fundamental element for the survival of neurons (Sontheirmer, 1999; Ahlemeyer, 2000; for review see Gomes et al., 2013; Ospina et al., 2014). This contribution is related to the ability of astrocytic detoxification, where glutamate uptake by astrocytes normally prevents high concentrations of glutamate to remain in the extracellular space, making this process a critical determinant of neuronal survival (Swanson et al., 2004). The detoxification function of glutamate by astrocytes occurs by the enzyme glutamine synthetase, which converts glutamate to glutamine with in glial cells (Lemberg and Fernandez, 2009). The increase in immunofluorescence for the glutamine synthetase enzyme and the glial fibrillary acidic protein (GFAP), which is exclusive to astrocytes, after treatment with *A. cearensis* extracts, suggests that the astrocytes change their morphology and metabolic state in response to glutamatergic damage, and the extracts can positively modulate this response, enhancing glutamate detoxification. Evidence suggests that astrocytes have machinery that not only responds to neuronal activity but also modulates synaptic transmission and plasticity, demonstrating the existence of a mutual communication between neurons and astrocytes (Zorec et al., 2012). Moreover, the immunofluorescence to Iba1 demonstrated a significant reduction of microglia cells, after treatment with the extracts (EAAC, EDAC and EHAC) in control conditions and in cultures treated with glutamate. These results suggested that the seeds extracts of *A. cearensis* can reduced the activation of microglial cells, which in turn may contribute to its neuroprotective potential (see Costa et al., 2016). Microglial proliferation and activation occurs in response to insults and are normally associated with apoptosis and neuronal degeneration following brain damage (Nobili, 2017)

Many of the mechanisms involved in the pathogenesis of neurodegenerative diseases such as ischemic lesions, glutamate excitotoxicity, oxidative stress, Alzheimer's and Parkinson's disease are associated with mitochondrial dysfunction (Sanderson et al., 2013). The results demonstrated a protective effect of the ethanol extract (ETAC) on brain mitochondria of rats subjected to oxidative stress. In the assays with isolated brain mitochondria, the ETAC extract (100 μg/mL) reduced the mitochondrial membrane potential, ROS generation, mitochondrial swelling and calcium influx compared to that seen in the control group not treated with the extract. For there to be a loss in mitochondrial potential, swelling or rupture of the outer mitochondrial membrane must occur, causing a sudden increase in permeability of the inner mitochondrial membrane. This process is called mitochondrial membrane permeability transition (Tsujimoto and Shimizu, 2007). Mitochondrial dysfunction has been reported to act as an important production source of free radicals in neurodegenerative diseases (Prentice et al., 2015). Mitochondrial defects may lead to defects in energy metabolism and, depending on the disease, may be related to losses in complex I, II, III and IV of the electron transport chain (Damiano et al., 2010; Alleyne et al., 2012; Prentice et al., 2015). Calcium influx is closely related to glutamate excitotoxicity and

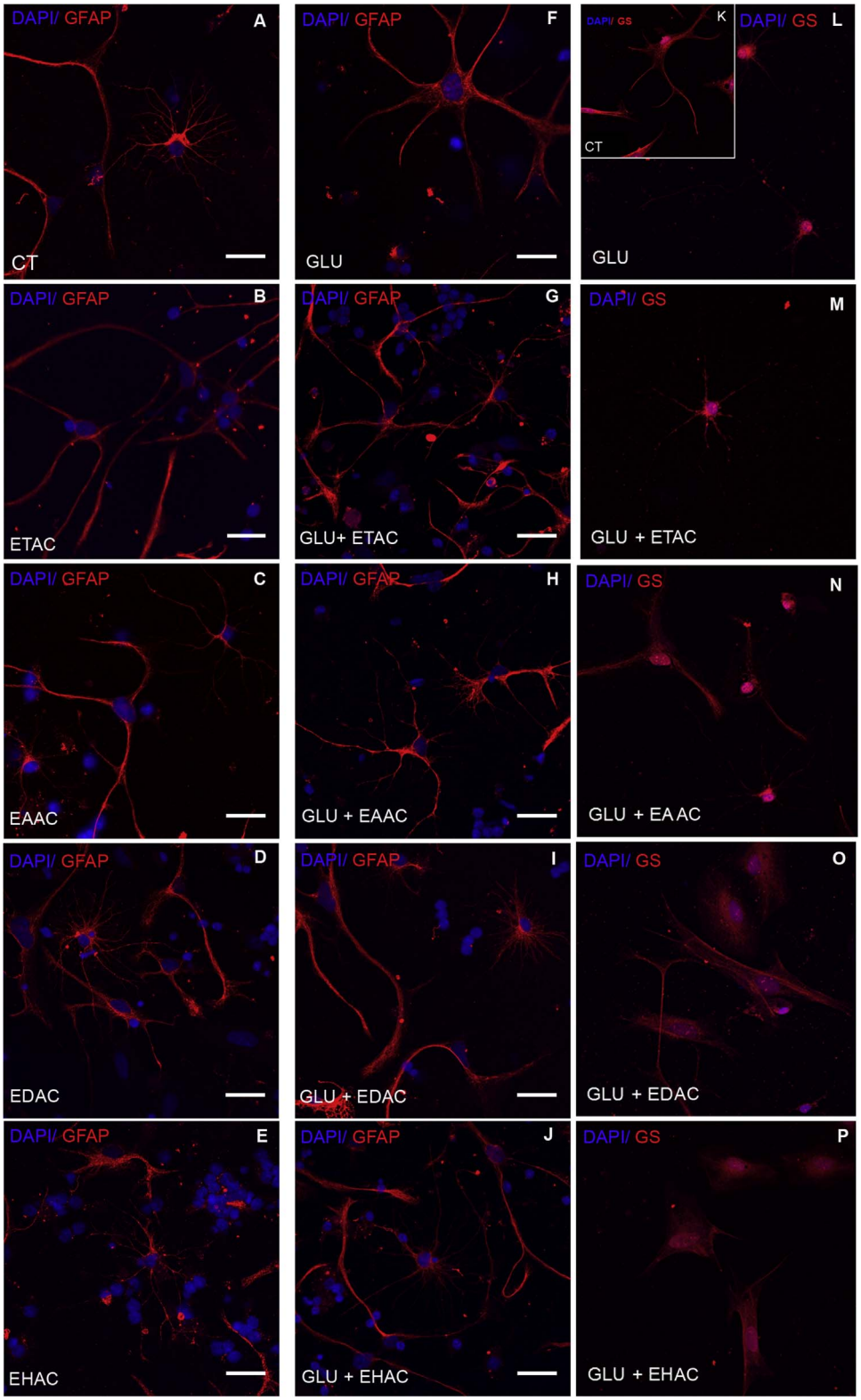

Fig. 4. Immunofluorescence labelling for GFAP and GS proteins in primary cultures of cerebellar neural cells analysed by confocal microscopy. (A-E) Cultures labelled for GFAP in control conditions (CT) and those treated with ethanol (ETAC), ethyl acetate (EAAC), dichloromethane (EDAC) and hexane (EHAC) extracts from the seeds of *A. cearensis* at a concentration of 0.1 μg/mL for a period of 24 h. (F-J) Cultures labelled for GFAP or (L-P) labelled for GS treated with the excitotoxic agent glutamate (GLU, 10 μM) and, after 4 h, treated with ethanol (ETAC), ethyl acetate (EAAC), dichloromethane (EDAC) and hexane (EHAC) extracts at a concentration of 0.1 μg/mL for a period of 24 h. (K) Cultures labelled for GS in control conditions. In blue: nuclear chromatin stained with 4', 6-diamidino-2-phenylindole dihydrochloride (Dapi). Objective 63x. Scale bars = 100 μm.

consequent cell death. Treatment with the ETAC extract showed a reduction in mitochondrial Ca^{2+} influx, corroborating the evidence of the neuroprotective potential of seed extracts from *A. Cearensis*. Most likely, the neuroprotective effects observed in the *A. cearensis* seed extracts are related to the antioxidant activity and decreased ROS production by the mitochondria. But, the mechanisms of actions of *A. cearensis* seed extracts in reducing the mitochondrial calcium influx still need to be further investigated in future studies.

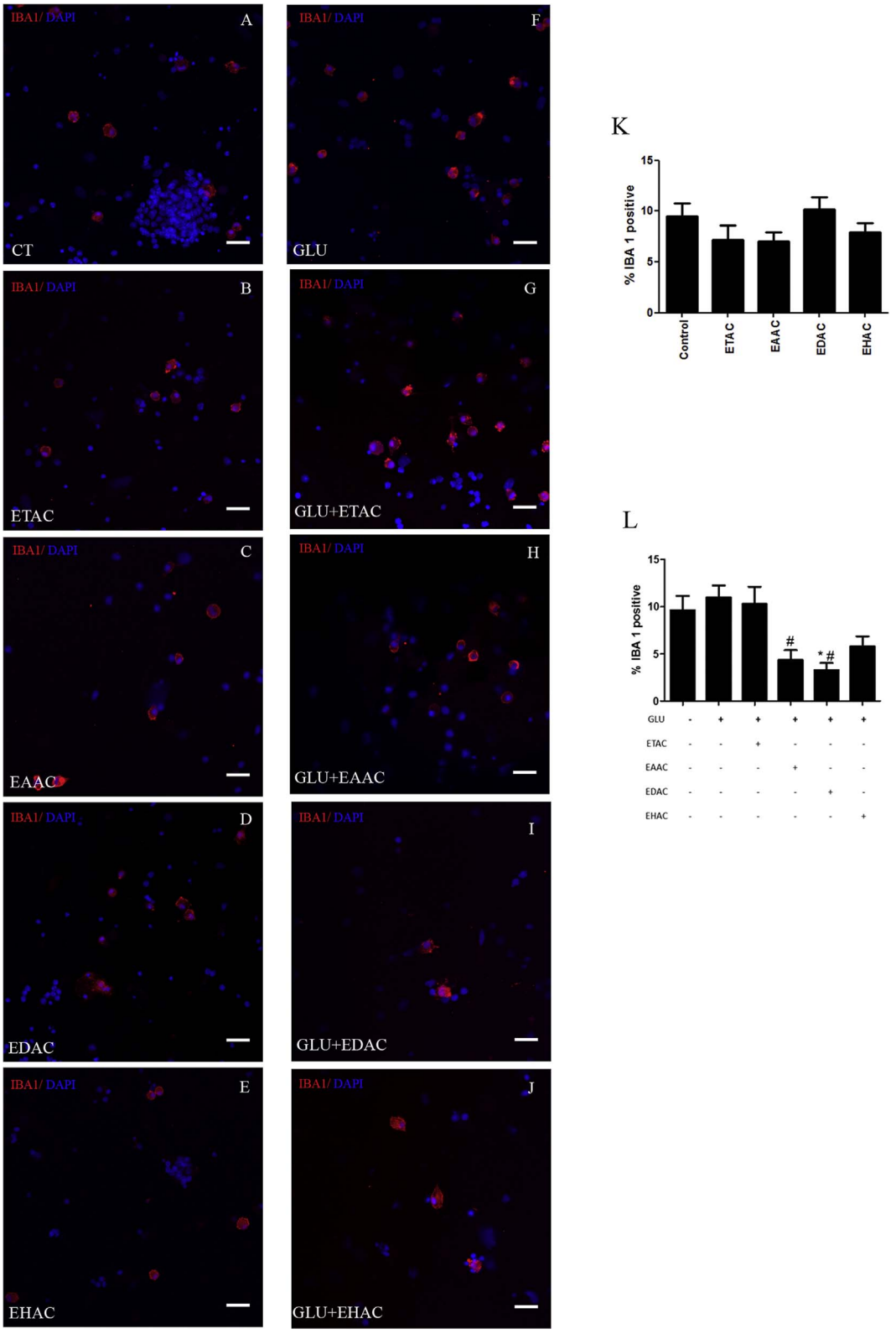

Fig. 5. Immunofluorescence labelling for Iba1, a microglial-specific marker, in primary cultures of cerebellar cells analysed by confocal microscopy. (A-E) Cultures in control conditions (CT) and those treated with ethanol (ETAC), ethyl acetate (EAAC), dichloromethane (EDAC) and hexane (EHAC) extracts from the seeds of *A. Cearensis* at a concentration of 0.1 µg/mL for a period of 24 h. (F-J) Cultures treated with the excitotoxic agent glutamate (GLU, 10 µM) and, after 4 h, treated with ethanol (ETAC), ethyl acetate (EAAC), dichloromethane (EDAC) or hexane (EHAC) extracts at a concentration of 0.1 µg/mL for a period of 24 h. In blue: nuclear chromatin stained with 4', 6-diamidino-2-phenylindole dihydrochloride (Dapi). Objective 63x. Scale bars = 100 µm. (K-L) The bar graphs represent the average percentage (± standard deviation) of immunofluorescence staining of Iba1-positive cells relative to the control, considered as 100%. (n = 8 fields per experimental condition, ANOVA: $^{*}p < 0.001$; $^{\#}p < 0, 05$).

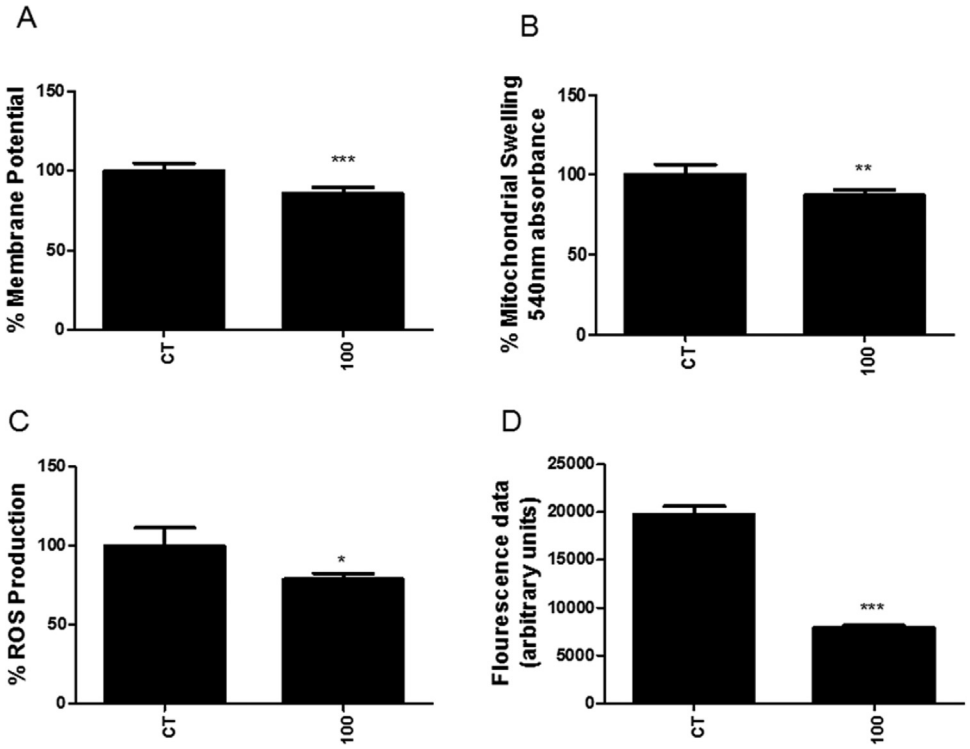

Fig. 6. Effects of ETAC seed extract of *A. cearensis* (100 μg/mL) in isolated rat brain mitochondria incubated with rotenone. (A) Mitochondrial membrane potential measured by the fluorescence intensity at 495/586 nm excitation / emission; (B) mitochondrial swelling measured by absorbance at 540 nm and (C) production ROS measured by fluorescence intensity at 563/587 nm excitation / emission; (D) Ca^{2+} influx measured by the intensity of 506/531 nm excitation / emission. In the graphs A, B and C the results are expressed as a percentage relative to the control, considered as 100%. (n = 8 fields per experimental condition, ANOVA followed by Tukey's post-test: * $p < 0.03$; **$p < 0004$; ***$p < 0{,}0001$).

5. Conclusions

The extracts (EDAC, EAAC, EDAC and EHAC) obtained from *A. cearensis* seeds showed no toxicity for cultures of neural cells of the cerebellum. The EDAC and EHAC extracts demonstrated better neuroprotective effects against neural damage induced by glutamate in cerebellar cultures than the other extracts. The neuroprotective effect may be associated with increased expression of the astrocytic enzyme glutamine synthetase, the glial fibrillary acidic protein (GFAP) and β-III tubulin, indicating glial and neuronal preservation, respectively. Moreover, the seeds extracts of *A. cearensis* reduced microglial cells, stained by Iba-1 and neuronal degeneration, showed by Fluoro-Jade B. According to the present results, the ETAC extract was also capable of protecting isolated rat brain mitochondria from calcium influx and oxidative stress. Together, the results provide evidence for the neuroprotective effects from *Amburana cearensis* seeds extracts, indicating that *Amburana cearensis* may be regarded as a potential therapeutic agent in the treatment and prevention of neurodegenerative diseases.

Conflicts of interest

The authors declare no conflicts of interest.

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