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Original Article

Amburana cearensis seed extracts protect PC-12 cells against toxicity induced by glutamate

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ABSTRACT

Amburana cearensis (Allemão) A.C. Sm., Fabaceae, has been widely studied for its medicinal activities. Many neurodegenerative disorders are caused by oxidative stress, mitochondrial dysfunction, excitotoxicity induced by glutamate and ultimately cell death. This study describes the chemical profile of the ethanolic, hexane, dichloromethane, and ethyl acetate extracts obtained from seeds of *A. cearensis*. The objective of this study was to investigate the chemical profile of extracts obtained from seeds of *A. cearensis*, as well as their cytotoxicity and neuroprotective effects in cultures of neural PC12 cells. Metabolite profile was performed by GC–MS. PC12 cells were treated with increasing concentrations of the extracts (0.01–2000 μg/ml) and the cell viability was analyzed after 24 and 72 h using an MTT test. For the excitotoxicity assay, PC12 cells were pre-treated with glutamate (1 mM) for 6 h and treated with increasing concentrations (0.1–1000 μg/ml) of the extracts. The chromatographic analysis of the extracts detected various compounds with antioxidant properties, with the majority of peaks corresponding to the isoflavone coumarin. Only the hexane extract showed toxicity after 72 h exposure at the highest concentration (1000 μg/ml). By contrast, all extracts increased the cellular viability of PC12 cells against the toxicity caused by glutamate. Therefore, the extracts from the seeds of *A. cearensis* showed no toxicity and have neuroprotective potential against neuronal damage induced by glutamate, which may be related to their antioxidant properties.

Introduction

Neurodegenerative diseases are among the most serious health problems facing modern society and are characterized by neuronal cell loss in the brain and spinal cord leading to functional or sensory impairment (Uttara et al., 2009). Many of these disorders, including Parkinson's disease (PD), Alzheimer's disease, multiple sclerosis, stroke, amyotrophic lateral sclerosis and many others have become more common with the aging of the population and have been associated with oxidative stress, mitochondrial dysfunction, and excitotoxicity. Finally, cell death is caused by apoptosis (Uttara et al., 2009). Excitotoxicity is caused by the excessive release of glutamate, the most excitatory neurotransmitter in the central nervous system (CNS) of mammals (Fujikawa, 2015). Neuronal damage associated with excitotoxicity is a type of cell death triggered by the over activation of glutamate receptors and the loss of calcium homeostasis due to excessive calcium influx (Camacho and Massieu, 2006). Many studies have evaluated compounds that inhibit cascades that trigger neuronal damage to minimize the primary causes of neuronal insults. Medicinal herbs that have been used in many cultures for thousands of years have played a vital role in the discovery of new drugs (Silva et al., 2012). *Amburana cearensis* (Allemão) A.C. Sm., is a species of the family of Fabaceae (Papilionoideae, Leguminoseae), naturally occurring in northeastern Brazil, specifically in Caatinga. Different parts of the plants, leaves, stem and seeds are used in popular medicine and have many medicinal applications. *A. cearensis* is an antioxidant (Pereira et al., 2014), bronchodilator (Leal et al., 2005) and affects respiratory tract diseases (Agra et al., 2007; Roque, 2009) among others. This plant is listed among endangered plants (IPEF, 2008), and despite its great therapeutic potential, very little is known about the action of its compounds in the CNS. Because

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glutamatergic transmission is related to numerous neurological and psychiatric disorders (Valli and Sobrinho, 2014), the objective of this study was to evaluate the antioxidant and neuroprotective potential of extracts from *A. cearensis* seeds in PC12 cell cultures subjected to excitotoxicity models induced by glutamate.

Materials and methods

Amburana cearensis *seed extracts*

The seeds of *Amburana cearensis* (Allemão) A.C. Sm., Fabaceae, were obtained in popular trade in the city of Feira de Santana, Bahia, Brazil, and had its authenticity recognized when compared with the identification according to Guedes et al. (2007) 13734, the ID are deposited in the Herbarium of the Biology Institute of the Federal University of Bahia, with the number 13734. 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.

Chemical analyses

Metabolite profiling was performed with an Agilent 7809A gas chromatograph (Agilent Technologies, California, USA) coupled to a Triple-Axis detector (Agilent 5975C) using a ZB-5 (Phenomenex; 30 m × 0.25 mm) capillary column (0.25 mm film thickness) with helium as the carrier gas at a flow rate of 1 ml/min as described by Ribeiro et al. (2014). The inlet temperature of the injector was set to 250 °C. The initial oven temperature was 45 °C for 1 min and was increased to 300 °C after 1 min at a rate of 10 °C/min and held at 300 °C for 5 min. A solvent delay of 420 s was set. Stock solutions of the extracts were prepared in a mixture of chloroform: acetone (1:1). An aliquot (50 μl) was transferred to a 2 ml glass vial and a glass insert of a 200 μl with 150 μl of diazomethane solution in ether was added. Octadecane was used as the internal standard, and the samples were injected in a splitless mode. All analyses were performed three times. Metabolites were putatively annotated (e.g., without chemical reference standards, based upon the physicochemical properties and/or spectral similarity with public/commercial spectral libraries) according to Sumner and colleagues (2007).

PC12 cell culture and treatments

The PC12 rat pheochromocytoma cell line was purchased from the ATCC Cell Bank. These cells were cultured in RPMI medium (Cultilab, SP, Brazil), supplemented with L-glutamine, 10% inactivated fetal bovine serum (Cultilab, SP, Brazil), 5% inactivated horse serum (Cultilab, SP, Brazil), 1% penicillin and 1% streptomycin (Cultilab, SP, Brazil). PC12 cells were cultured until confluence in 100 mm polystyrene plates (TPP, Trasadingen, Switzerland), trypsinized and replated in 96-well (7.5×10^3 cells/cm^2) polystyrene culture dishes (TPP, Trasadingen, Switzerland). The cells were maintained in an incubator with a humidified atmosphere of 95% air and 5% CO_2 at 37 °C.

After 24 h, the medium was changed and extracts and/or glutamate diluted in medium without serum were added directly in PC12 cell cultures. Negative control group was exposed to medium without serum.

Cell viability assay

The cytotoxicity of the *A. cearensis* extracts on the PC12 cells was determined using 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St. Louis, MO). The MTT test is based on the principle of converting a yellow color substrate of formazan crystals to a violet color by the mitochondrial dehydrogenases of living cells (Mosmann, 1983). PC12 cells were cultivated for 24 h in 96-well plates (TPP, Trasadingen, Switzerland). The culture medium were removed and the cells were treated with increasing concentrations (0.001, 0.01, 0.1, 1, 10, 100 or 2000 μg/ml) of the extracts ETAC, EAAC, EDAC, or EHAC. Negative control group was culture maintained in medium without serum. The MTT assay was performed 24 or 72 h after the treatments. The MTT solution (100 μl) diluted in RPMI medium was added to each well at a final concentration of 1 μg/ml. The plates were incubated for 2 h in a humidified atmosphere with 5% CO_2 at 37 °C. Thereafter, to complete the dissolution of the formazan crystals, the cells were lysed with 100 μl/well lysis buffer, containing 20% sodium dodecyl sulfate (SDS) and 50% dimethylformamide (DMF), pH 4.7. After 12 h at room temperature, the optical absorbance of each sample was measured using a spectrophotometer at 595 nm. To determine whether the extracts have absorbance or react with MTT, the optic density of the extracts was measured alone and in the presence of MTT at 595 nm in wells without cells. All experiments were performed in eight wells and were repeated at least three times.

Neural excitotoxicity assay

To evaluate the neuroprotective effect of the extracts of the seeds of *A. cearensis*, PC12 cells were cultivated for 24 h in 96-well plates. Then, the culture medium was removed and the cells were treated with 1 mM L-glutamic acid monosodium salt hydrate (Sigma RES5063G) (Ma et al., 2013) diluted in the RPMI medium. After 6 h, the cultures were post-treated with the extracts (ETAC, EAAC, EDAC and EHAC) at final concentrations of 0.1, 1, 10, 100 and 1000 μg/ml with 1 mM glutamate. The cells were maintained in a humidified atmosphere of 95% air and 5% CO_2 at 37 °C for 24 h. All experiments were performed in eight wells and repeated three times independently. The effect of the extracts against the neuronal damage induced by glutamate was measured using the MTT test described above. The morphology of cells in the glutamate excitotoxicity assay was also analyzed by phase contrast microscopy.

Table 1
Compounds identified by GC-MS in extracts ETAC, EAAC, EDAC and EHAC from *Amburana cearensis* seeds.

	ETAC	EDAC	EAAC	EHAC
Coumarin	1.1 ± 0.1 (b)	14.9 ± 1.0 (a, c, d)	1.0 ± 0.0 (b)	1.1 ± 0.0 (a, b)
Methyl hexadecanoate	1.8 ± 0.0 (b, c, d)	5.4 ± 0.2 (a, c, d)	1.0 ± 0.1 (a, b, d)	4.1 ± 0.1 (a, b, c)
Methyl 9-*cis*-11-*trans*-Octadecanoate	1.0 ± 0.0 (b, c, d)	6.9 ± 0.3 (a, c, d)	Nd	2.9 ± 0.2 (a, c, b)
Methyl 13-*trans*-octadecanoate	1.0 ± 0.1 (b, c, d)	11.0 ± 0.7 (a, c, d)	1.0 ± 0.1 (a, b, d)	6.3 ± 0.2 (a, b, c)
Methyl octadecanoate	2.3 ± 0.1 (b, d)	3.9 ± 0.7 (c, d)	1.0 ± 0.1 (a, b, d)	2.8 ± 0.1 (a, b, c)
γ-Sitosterol	1.5 ± 0.0 (b, c, d)	1.1 ± 0.3 (c, d)	Nd	6.1 ± 0.4 (a, b, c)
Ethyl hexadecanoate	Nd	56.8 ± 2.4 (a, c, d)	Nd	1.0 ±0.0 (b)

Ethanolic (ETAC), hexane (EHAC), dichloromethane (EDAC) and ethyl acetate (EAAC) extracts obtained from seeds of *Amburana cearensis*. Data represent means ± s.e.m. Different letters indicate significant differences between samples and ETAC (a), EDAC (b), EAAC (c), EHAC (d) by one-way analysis of variance followed by Tukey–Kramer *post hoc* test ($p < 0.001$). Nd, not detected.

Table 2
Metabolite profiling of *Amburana cearensis* seed extracts.

Compounds	TR	IR	NI
Coumarin	10.32	1445.38	2
Methyl hexadecanoate	13.85	1969.20	1
Methyl 9-*cis*-11-*trans*-octadecadienoate	14.96	2155.41	2
Methyl 13-*trans*-octadecenoate	14.99	2161.73	2
Methyl octadecanoate	15.14	2187.26	1
γ-Sitosterol	23.61	3493.82	2
Methyl 3-coumarin	10.85	1514.87	2
Ethyl hexadecanoate	14.30	2043.52	1
Ethyl 9,12-octadecadienoate	15.37	2225.60	2
Ethyl 9-cis-octadecenoate	15.41	2232.67	1
Ethyl octadecanoate	15.54	2256.47	2
Campesterol	22.57	3373.30	2
Stigmasterol	22.85	3407.43	1
β-amyrin	24.31	3563.39	1

TR, retention time; IR, retention index; NI, identification level.

Statistical analysis

Statistical analyses were performed using the software Graphpad Prism 5.0. The cytotoxicity assay was analyzed by ANOVA followed by the Tukey test. The neuronal excitotoxicity assay induced by glutamate was analyzed by ANOVA followed by Student's *t*-test. The results are expressed as the mean ± standard deviation relative to the negative control, considered 100%. Values of $p < 0.05$ were considered significant.

Results and discussion

Metabolite profiling of Amburana cearensis *seed extracts*

Fourteen metabolites were identified in the extracts, including the isoflavones coumarin and 3-methyl-coumarin, eight fatty acids (methyl hexadecanoate, methyl 9-*cis*-11-*trans*-octadecadienoate, methyl 13-*trans*-methyl-octadecanoate, methyl hexadecanoate, ethyl hexadecanoate, octadecanoate acetate, 9,12-*cis*-9-ethyl octadecanoate, and ethyl octadecanoate), three steroids (γ-sitosterol, campesterol, and stigmasterol) and the triterpenoid β-amyrin (Tables 1 and 2). The isoflavone coumarin was detected in all four extracts. However, its concentration was 14.9-fold higher at the EDAC than at the other three extracts.

Four methyl esters were detected in the extracts. The first, methyl hexadecanoate, was detected in the four extracts and was in different concentrations in the EDAC (44% ± 1.6%), EHAC (32% ± 1.2%), ETAC (14% ± 0.5%), and EAAC (8% ± 0.3%). The second, 9-*cis*-11-*trans*-methyl octadecadienoate, was detected only in three extracts, with different concentrations in the EDAC (63% ± 2.5%), EHAC (26% ± 1.5%), and ETAC (9% ± 0.3%). The third, 13-*trans*-methyl-octadecanoate, was detected in the four extracts and exhibited different concentrations in the EDAC (53% ± 3.3%), EHAC (31% ± 0.7%), ETAC (11% ± 0.2%) and EAAC (5% ± 0.3%). The last methyl ester, ethyl hexadecanoate, was present at similar concentrations the in EDAC (42% ± 7.8%) and in EHAC (30% ± 1.4%), but was present at low concentrations in the ETAC (16% ± 0.3%) and in EAAC (10% ± 1.5%).

The compound of the phytosterol class, γ-sitosterol, was detected in three extracts at different concentrations in the EHAC (69% ± 5%), EDAC (12% ± 3%) and ETAC (18% ± 5%). Furthermore, the ethyl hexadecanoate from the ethylated compounds class was detected in two extracts, with different concentrations in the EDAC (98% ± 4%) and EHAC (2% ± 0. 02%). Several studies showed various biological actions of some of these compounds in the *A. cearensis* extracts, but many were isolated from the stem bark or leaves. Our results are promising and identified the compounds in the extracts of the *A. cearensis* seeds. There are few studies that using the seeds of *A. cearensis* to identify compounds, such as Negri et al. (2004), Canuto and Silveira (2006), and Bezerra et al. (2006), who isolated 6-hydroxycoumarin, *o*-coumaric acid, a derivative of the esterified amburoside, acid-*p*-hydroxy-benzoic acid, glycosylated (*E*)-*o*-coumaric acid and glycosylated (*Z*)-*o*-coumaric. The compound coumarin was identified by Negri et al. (2004) in the stem bark of *A. cearensis*. Additionally, several methylated compounds were identified in the stem bark of *A. cearensis*; however, they were not previously identified in the seeds.

Cytotoxicity assay of the extracts from the seeds of Amburana cearensis

To evaluate the cytotoxicity of ETAC, EAAC, EDAC and EHAC, we performed *in vitro* assays using an MTT test on PC12 cells exposed to these extracts at different concentrations and periods of time. All four extracts did not decrease the cell viability of PC12 cell cultures exposed to concentrations of extract ranging from 0.1 to 2000 μg/ml after 24 h of exposure (Fig. 1). The ETAC extract at 2000 μg/ml increased the mitochondrial activity, suggesting an increase of cell density of the culture visualized by optical microscopy (data not shown). After 72 h of exposure to 1000 μg/ml of ETAC and EHAC, a decrease of cell viability of 10% and 70%, respectively, was observed for the PC12 cells cultures. EAAC and EDAC were not toxic under some conditions (0.1–1000 μg/ml, after 72 h) (Fig. 2). Leal et al. (2003) evaluated the hydro-alcoholic extract obtained from the stem bark of *A. cearensis* and demonstrated that it was not toxic to rats. Moreover, several compounds from *A. cearensis*, such as kaempferol, isokaempferide, amburoside A and protocatechuic acid were evaluated to verify their cytotoxicity on tumor cell lines, and the results showed an inhibition of viability by kaempferol and isokaempferide (Costa-Lantufo et al., 2003). We demonstrated that extracts obtained from the seeds of *A. cearensis* did not toxic to the PC12 cell line.

Neuroprotective effect of the Amburana cearensis *seed extracts against excitotoxicity induced by glutamate*

In the results, it can be seen that the group of cells treated with the EAAC extract, in concentrations ranging from 0.1 to 1000 mg/ml, showed neuroprotective effect against damage induced by glutamate (1 mM) at all concentrations, with a reduction of up to 30% neuronal cell death. In the groups of cells

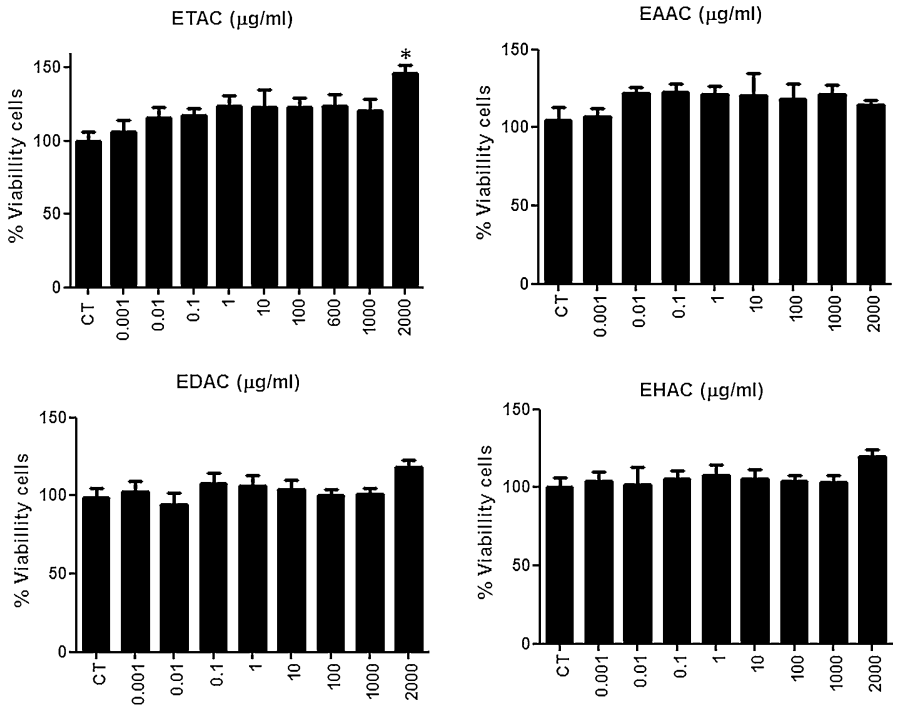

Fig. 1. Cytotoxicity assays of the extracts from *Amburana cearensis* seeds in PC12 cell culture. The ETAC, EAAC, EDAC and EHAC extracts (0.001, 2000 μg/ml) were added to the culture medium. The cell viability was determined using MTT assay 24 h after the treatments. The results are expressed as a percentage of the control, considered 100% (***$p < 0.05$, ANOVA followed by Tukey test).

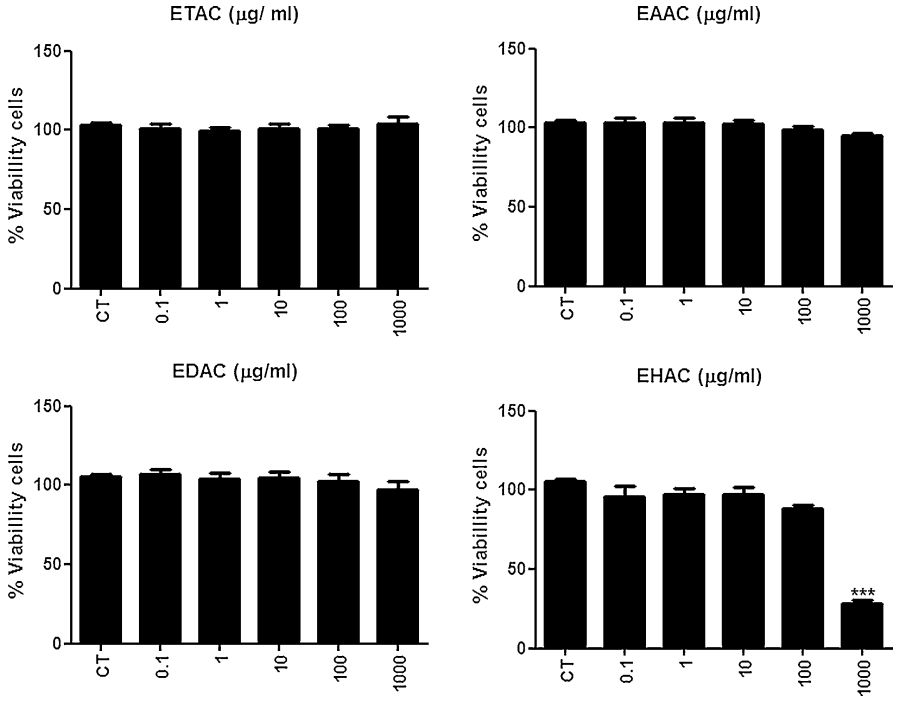

Fig. 2. Cytotoxicity assays of extracts from *Amburana cearensis* seeds in PC12 cell cultures. The ETAC, EAAC, EDAC and EHAC extracts (0.1–1000 μg/ml) were added to the culture medium. The cell viability was determined using the MTT assay 72 h after the treatments. The results are expressed as a percentage of the control, considered 100% (***$p < 0.05$, ANOVA followed by Tukey test).

treated with ETAC and EHAC extracts, reduced death induced by glutamate (1 mM) neuronal PC12 cells, occurred at concentrations of 10 mg/ml, and this reduction by 27% and 29% death cell respectively. In the group of cells treated with EDAC extract only observed front protective effect on the damage induced by glutamate (1 mM), at the highest concentration tested in 1000 μg/ml, a reduction of up to 17% cell death (Fig. 3).

Studies conducted by Leal et al. (2003, 2005) demonstrated that amburoside A presented with hepatoprotective activity related to its antioxidant activity. Its antioxidant activity is a predominant

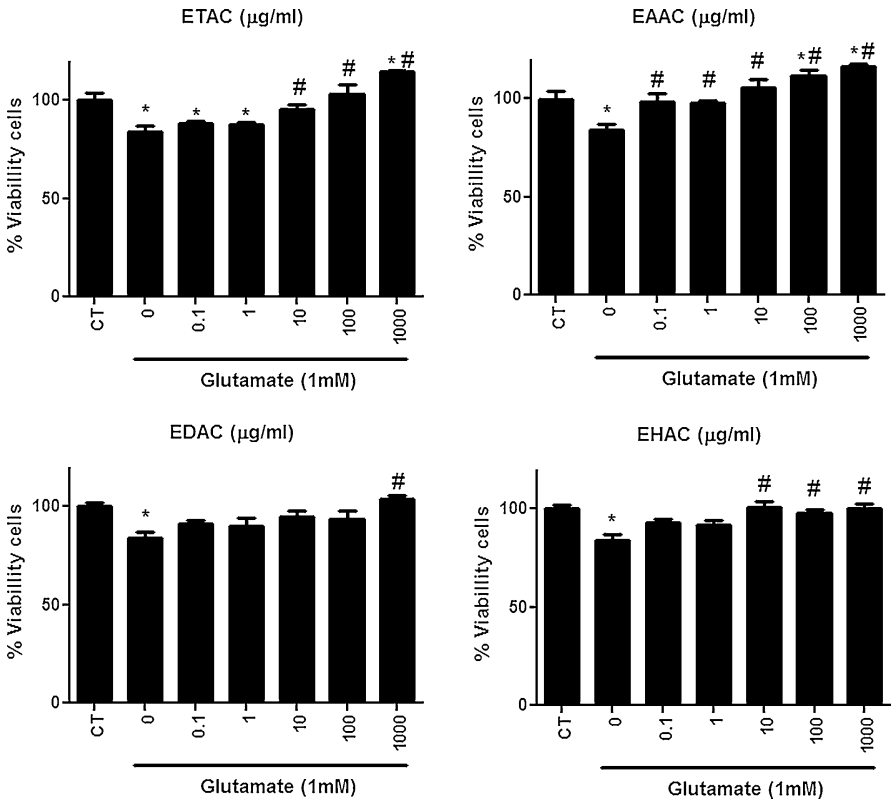

Fig. 3. Neuroprotective effect of extracts from seeds of *Amburana cearensis* in PC12 cells cultures against glutamate-induced toxicity. The cells were pre-treated with glutamate (1 mM), and after 4 h the extracts ETAC, EAAC, EDAC or EHAC (0.1–1000 μg/ml) were added to the culture medium. Cell viability was determined using the MTT assay 24 h after the treatments. The results are statistically significant compared to the control (*) and glutamate (#) ($p < 0.05$, ANOVA).

biological activity of *A. cearensis* and was observed in previous studies evaluating the methanol extract from seeds of *A. cearensis* (Pereira et al., 2014). The results of the present study suggest a therapeutic potential for molecules in the EAAC, which has fewer compounds compared to the other three extracts because it contains only coumarin as the primary component, hexadecanoate methyl, *trans*-13-methyl-octadecanoate and methyl octadecanoate.

Fig. 4 shows the morphological analysis results for neuronal PC12 cells when analyzed by phase contrast microscopy. PC12 neuronal cells in control conditions have a bipolar morphology, refractile, showing cellular viability. In groups treated with the extract (ETAC, EAAC, EDAC and EHAC) cell morphology was similar to the control group cells. In cell cultures treated with 1 mM glutamate can observe cells with cell body and retracted refractile bit indicating loss of viability, and reduced cellularity. However, cultures of cells exposed to glutamate and treated with *A. cearensis* extracts can be verified cells with similar morphology to those in control conditions and with few cells and atypical morphology refractile bit, which indicates damage protection.

Among the compounds isolated from different parts of *A. cearensis* with proven biological activity which may be related to the neuroprotective effect observed in the present study, we can highlight action phenolic compounds such as coumarin. *In vivo* studies conducted by Leal et al. (2006) associated with antioxidant activity antinociceptive effect of phenolic compounds extracted from the bark and stem *A. cearensis*. Lopes, 2010 associated antioxidant activity with anti-inflammatory effect of afrormosina, phenolic compound extracted from the bark and stem *A. cearensis*. Oliveira et al. (2014) observed antioxidant activity *in vitro* 8-methoxypsoralen, synthetic coumarin in astrocytic lineages. In addition, Pereira et al. (2014) observed in free antioxidant system cells of the total methanol extract of *A. cearensis* seeds. The combined findings, it concluded that extracts of seeds of *A. cearensis* contain compounds with neuroprotective potential, particularly the extract in ethyl acetate. Additional studies are needed to characterize the effects of the compounds coumarin methyl ester alone or in combination in cultured neuronal cells.

Our results demonstrate that 24 h after exposure to EAAC, all concentrations tested reduced from 12% to 30% the PC12 cell death induced by 1 mM glutamate. Moreover, for the ETAC and EAAC extracts, concentrations between 10 and 1000 μg/ml also reduced the glutamate induced death of PC12 cells, reducing by 17% and by 17% the cell death, respectively. EDAC only induced neuroprotection at the highest concentration tested (1000 μg/ml), reducing by 17% the cell death induced by glutamate (Fig. 3). Phase contrast microscopy (Fig. 4) shows that treatment with 1 mM glutamate induced retraction of the cell body in the majority of PC12 cells. However, PC12 cells post-treated with *A. cearensis* extracts at 100 μg/ml showed viable adherent cells with different phenotypes and with similar morphology to control cells, Indicating protection against glutamate excitotoxicity. Cell protection was also evidenced in cultures treatment 100 μg/ml EAAC.

Studies conducted by Leal et al. (2003, 2005) demonstrated that amburoside A presented hepatoprotective activity related to its antioxidant activity. The antioxidant activity is a predominant biological activity of *A. cearensis* and was observed in previous studies evaluating the methanol extract from seeds of *A. cearensis* (Pereira et al., 2014). The results of the present study suggest a therapeutic potential for molecules in the EAAC, which has fewer compounds compared to the other three extracts because it contains only coumarin as the primary component, hexadecanoate methyl, *trans*-13-methyl-octadecanoate and methyl octadecanoate.

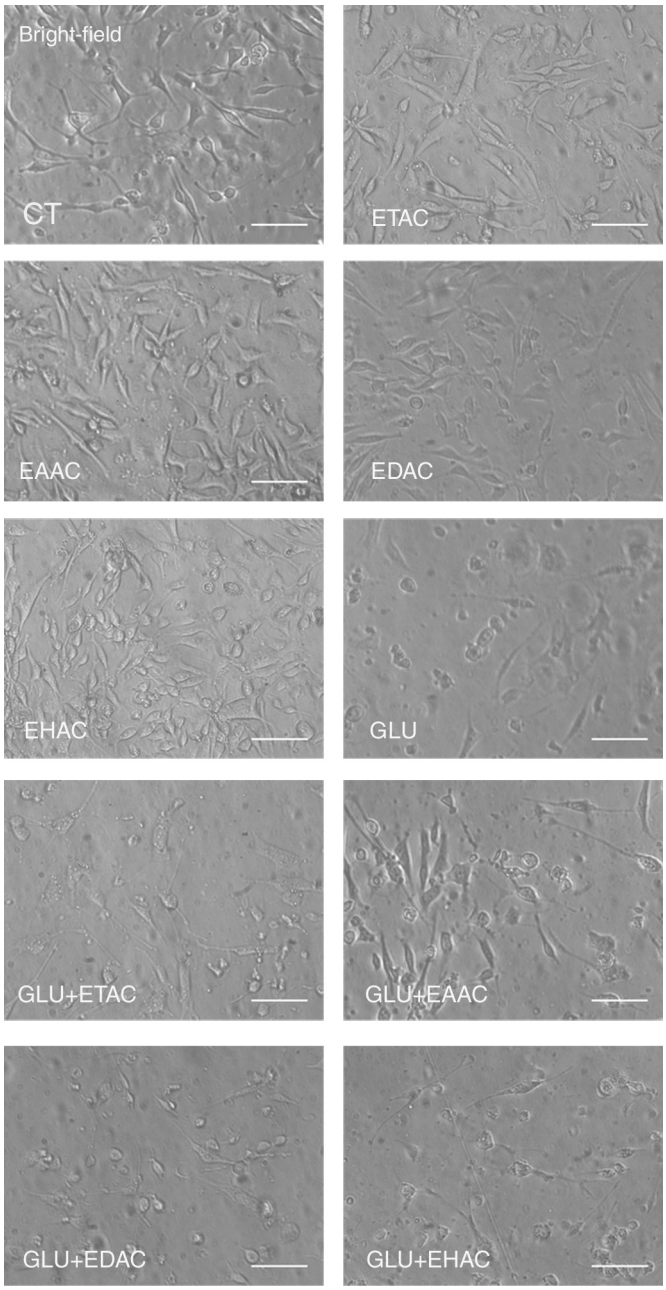

Fig. 4. Micrography analysis of PC-12 cells under control conditions (A) or after 24 h exposure to 1 mM glutamate (B), 0.1 mg/ml EAAC extract (C) or both 1 mM glutamate and 0.1 mg/ml EAAC. Objective ×40. Scale = 100 μm.

Conclusion

The present investigation shows that the extracts obtained to *A. cearensis* seeds contained ethyl esters, methyl esters and coumarins. Moreover ethanolic extract (ETAC) and four extracts derived from it do not present toxic effect to PC12 neuronal cells. Furthermore, a potential neuroprotection effect against glutamate toxicity was determined in this work.

Ethical disclosures

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

Confidentiality of data. The authors declare that no patient data appear in this article.

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

Author contributions

EPP, CCS, LOS, VDAS, SBS, and SLC, participated in study concept and design, acquisition of data, analysis and interpretation of data, and critical revision of the manuscript for important intellectual content. LGF and MDC participated in study concept. EPP, SBS, VDAS and SLC, drafted the manuscript. EPP, MDC and PR carried out the extraction, fractionation and GC-MS analysis, and participated in drafting the manuscript.

Conflicts of interest

The authors declare no conflicts of interest.

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