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Juliprosopine and Juliprosine from *Prosopis juliflora* Leaves Induce Mitochondrial Damage and Cytoplasmic Vacuolation on Cocultured Glial Cells and Neurons

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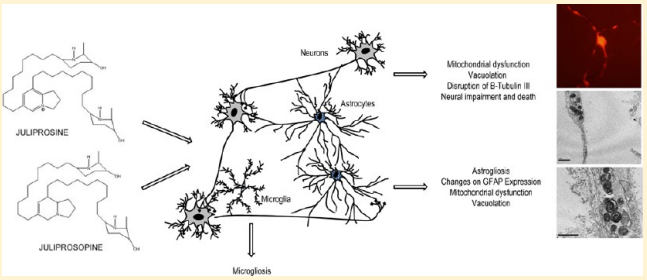
ABSTRACT: *Prosopis juliflora* is a shrub largely used for animal and human consumption. However, ingestion has been shown to induce intoxication in animals, which is characterized by neuromuscular alterations induced by mechanisms that are not yet well understood. In this study, we investigated the cytotoxicity of a total alkaloid extract (TAE) and one alkaloid fraction (F32) obtained from *P. juliflora* leaves to rat cortical neurons and glial cells. Nuclear magnetic resonance characterization of F32 showed that this fraction is composed of a mixture of two piperidine alkaloids, juliprosopine (majority constituent) and juliprosine. TAE and F32 at concentrations between 0.3 and 45 μg/mL were tested for 24 h on neuron/glial cell primary cocultures. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide test revealed that TAE and F32 were cytotoxic to cocultures, and their IC_{50} values were 31.07 and 7.362 μg/mL, respectively. Exposure to a subtoxic concentration of TAE or F32 (0.3−3 μg/mL) induced vacuolation and disruption of the astrocyte monolayer and neurite network, ultrastructural changes, characterized by formation of double-membrane vacuoles, and mitochondrial damage, associated with changes in β-tubulin III and glial fibrillary acidic protein expression. Microglial proliferation was also observed in cultures exposed to TAE or F32, with increasing levels of OX-42-positive cells. Considering that F32 was more cytotoxic than TAE and that F32 reproduced *in vitro* the main morphologic and ultrastructural changes of "cara torta" disease, we can also suggest that piperidine alkaloids juliprosopine and juliprosine are primarily responsible for the neurotoxic damage observed in animals after they have consumed the plant.



INTRODUCTION

Prosopis juliflora Sw. D.C. (mesquite) is a shrub that was introduced to northeast Brazil in the 1940s.[1] Northeast Brazil has an area characterized as the Drought Polygon. This is due to poorly distributed rains during the year and an average annual rainfall of just 500 mm, which has led to a vegetation type known as *Caatinga* characterized by low productivity and low species diversity, compared to that found in the rainforest. *P. juliflora* is a very resilient plant, can grow in the adverse conditions of *Caatinga*, and constitutes the main alternative food source for humans and animals in this region, because of its high rate of production of pods and high palatability and nutritional value.[2−4] Because of their palatability and nutritional value, the pods of *P. juliflora* or its bran is typically used for feeding dairy and beef cattle.[5]

However, *P. juliflora* causes disease when it is the sole or main source of sustenance for the animal.[6,7] The illness induced is called "cara torta", characterized in cattle and calves by emaciation, neuromuscular alterations (including muscular atrophy of the masseters), and histological lesions such as spongiosis, gliosis, the loss of Nissl substance, and fine vacuolation of the perikaryons of neurons from trigeminal motor nuclei.[8,9] Neuromuscular alterations were observed in experimentally intoxicated goats and cattle fed with rations containing high concentrations of *P. juliflora* pods (>50%), especially after chronic exposure (>200 days). These alterations were also recently observed in cattle reared in an extensive system with free access to forage and *P. juliflora*.[10] Histological lesions were also characterized by fine vacuolation of the perikaryons of neurons, reactive astrocytes with vesicular dilated nuclei, and scant eosinophilic cytoplasm from trigeminal motor nuclei. Occasionally, damage to neurons of the oculomotor nuclei and Wallerian degeneration in mandibular and trigeminal nerves were observed.[9,11]

In vitro studies have shown that alkaloids from *P. juliflora* pods and leaves have a direct action in central nervous system cells, causing toxic and inflammatory effects and suggesting that some alkaloids or one alkaloidal fraction (F32) is the most effective in inducing cytotoxicity and reactivity in glial cells.[12,13] However, little is known about which alkaloids are present in this

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toxic fraction, and it is unknown whether these alkaloids have potential to induce mitochondrial disorders or vacuole formation on neuronal perikaryon that are the main neurohistologic and ultrastructural lesions visualized in intoxicated animals.[9−11]

Neuronal vacuolization is not a specific finding of intoxication by *P. juliflora* and occurs in other diseases caused by eating herbivores plants of the genera *Solanum*,[14−17] *Swainsona*, *Oxytropis*, *Astragalus*,[18] and *Ipomoea*.[19] During intoxication with many of these plants, for example, *Solanum fastigiatum*, cytoplasm vacuoles are a result of the accumulation of nonmetabolized substrates in lysosomes, which results in lysosomal storage disease (DSL). Others such as *P. juliflora* still have not had their structures characterized, which are important for understanding the mechanism of action of toxic compounds of this plant.

This study complements the previous *in vitro* studies that suggest the alkaloids are the toxic agents of animal intoxication by *P. juliflora*. We characterized the alkaloids present in the most active fraction (F32) from *P. juliflora* leaves and induced and characterized *in vitro* the main morphologic and ultrastructural changes of "cara torta" disease using an extract and the F32 fraction in models of neuron/glial cell primary cocultures.

MATERIALS AND METHODS

Extraction and Characterization of Alkaloids. Leaves of *P. juliflora* were harvested in Salvador (BA) in the experimental fields of the Federal University of Bahia (UFBA). The alkaloid extract (TAE) was obtained by an acid/basic modified extraction, as described by Ott-Longoni and co-workers,[20] with minor modifications.[13] *P. juliflora* leaves were dried in a greenhouse at 50 °C, and the air-dried plant material (874 g) was extracted three times with hexane (2.0 L/kg) for 48 h at room temperature with occasional shaking to eliminate nonpolar components. The extract was then filtered, and the residue was flooded with methanol (1.5 L/kg) using the process described above. The methanol extract was concentrated in a rotary evaporation system at 40 °C, and this concentrated residue was stirred with 0.2 N HCl for 16 h, followed by filtration. The solution was shaken with chloroform to remove the nonbasic material. The aqueous layer was basified with ammonium hydroxide until it reached pH 11 and was then extracted with chloroform. The chloroform phase was evaporated, leading to the production of 4.4 g of TAE. This extract was fractionated by chromatography in a silica gel column using a chloroform/methanol mixture (99:1 to 1:1) as the solvent system, with a subsequent 100% methanol elution. Thirty-six fractions were obtained from the TAE, and after thin-layer silica gel chromatography, they were developed with iodine and tested for the presence of alkaloids using Dragendorf's test.[21] More details about the TAE and fractions may be found in our previous publication.[13] Considering that in our previous experiments F32 was the fraction most toxic to glial cells in this study, we investigated the cytotoxic effect of TAE and F32 in neurons and glial cells.[13] F32 corresponded to 51 mg, an equivalent of 1.16% from TAE and 0.0058% from the leaves, and it was characterized by nuclear magnetic resonance of ^{1}H (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD).

Treatments. For treatments, TAE and F32 were dissolved in dimethyl sulfoxide (DMSO) (Sigma, St. Louis, MO), forming 30 mg/mL stock solutions, and stored at −20 °C. Cells were treated with concentrations ranging from 0.3 to 45 μg/mL for 24 h. The negative control group was treated with DMSO diluted in the culture medium at the higher equivalent volume used in the treated groups (0.1%) and showed no significant effect on the analyzed parameters compared to those of cells that did not receive diluents.

Cell Cultures. Cell cultures were prepared from cerebral hemispheres from Wistar rats, obtained from the Department of Physiology of the Health Sciences Institute of the Federal University of Bahia, and performed according to the local Ethical Committee for Animal Experimentation.

Neuronal Primary Cultures. Cerebral hemispheres of 15−18-day-old Wistar rat embryos were isolated aseptically, and the meninges were removed. Each cortex was dissected out and then gently forced through a sterile 75 μm Nitex mesh. Cells were suspended in DMEM HAM F12 medium (Cultilab, SP, Brazil), supplemented with 100 IU/mL penicillin G, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.011 g/L pyruvate, 10% fetal calf serum (FCS), 3.6 g/L Hepes, and 33 mM glucose (Cultilab), plated at a density of 1000 cells/mm^2, and then incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 8 days.

Glial Cell Primary Cultures and Neuron/Glial Cell Primary Cocultures. Glial cell primary cultures were prepared according to the method of Cookson and Pentreath.[22] Briefly, cerebral hemispheres from 1-day-old postnatal Wistar rat pups were isolated aseptically, and the meninges were removed. The cortex was dissected out and then gently forced through a sterile 75 μm Nitex mesh. Cells were suspended in DMEM HAM F12 medium (Cultilab), supplemented with 100 IU/mL penicillin G, 100 μg/mL streptomycin, 2 mM L-glutamine, 0.011 g/L pyruvate, 10% FCS, 3.6 g/L Hepes, and 33 mM glucose (Cultilab), and cultured in 10 cm diameter dishes in a humidified atmosphere of 5% CO_2 at 37 °C. The culture medium was changed every 2 days, and cells were cultured for 15 days. Cells were then trypsinized (Trypsin EDTA) and plated at a density of 1000 cells/mm^2 to glial cell culture or 670 cells/mm^2 to neuron/glial cell primary cocultures and maintained for 48 h for culture stabilization before treatment or neuron/glial cell cocultures. To make the neuron/glial cell cocultures, neurons obtained from cerebral hemispheres of 15−18-day-old Wistar rat embryos using the same method described above were suspended in supplemented DMEM HAM F12 medium and seeded at half the amount of glial cells (335 cells/mm^2) onto the astroglial monolayer. Cells were incubated in a humidified atmosphere of 5% CO_2 at 37 °C for 8 days, when treatments were performed.

Cell Viability. *Concentration−Effect.* Concentration−effect curves were determined by the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) (Sigma) test. The experiment was performed on 96-well plates (TPP Switzerland) with neuron/glial cell cocultures. The cells were incubated with 1.5−45 μg/mL TAE or F32 or 0.1% DMSO (control) for 24 h. The cell viability was quantified using the conversion of yellow MTT by mitochondrial dehydrogenases of living cells to purple MTT formazan as an index.[23] Control and treated cells were incubated with MTT at a final concentration of 1 mg/mL for 2 h. Thereafter, cells were lysed with 20% (w/v) sodium dodecyl sulfate (SDS) and 50% (v/v) dimethylformamide (DMF) (pH 4.7). Plates were incubated overnight at 37 °C to dissolve formazan crystals. The optical density of each sample was measured at 492 nm using a spectrophotometer (Thermo Plate-Reader). Three independent experiments were conducted with eight replicate wells for each analysis. Results from the MTT test were expressed as percentages of the viability of the treated groups compared to the control groups. A nonlinear regression was performed, using GraphPad Prism version 3.0, to fit concentration−effect curves and to calculate the IC_{50} values of TAE and F32, which were effective concentrations that killed 50% of the cells.

Induction of Membrane Damage. The membrane integrity was evaluated by Trypan blue staining. The experiment was performed on 4 cm diameter dishes (TPP Switzerland) with neuron/glial cell cocultures exposed for 24 h to 30 μg/mL TAE or 7.5 μg/mL F32. After this exposure, the medium was exchanged for one with 0.2% Trypan blue and incubated for 10 min in a humidified atmosphere of 5% CO_2 at 37 °C. The number of cells not stained was analyzed in an optic phase microscope (Nikon TS-100). Cells were quantified in three fields, and the results are expressed as a percentage according to the total amount of cells in the field.

Determination of Morphological Changes. *Rosenfeld's Staining.* Morphological changes and vacuolization were primarily assessed by analysis of Rosenfeld's staining. The experiment was performed on 4 cm diameter dishes (TPP Switzerland). The cells were rinsed three times with PBS (without Ca^{2+} and Mg^{2+}) and fixed for 10 min with methanol at −20 °C. Fixed cells were stained. Rosenfeld's reagent (1 mL) was added and the mixture incubated for 20 min at room temperature. Thereafter, the plates were rinsed with water, air-dried, analyzed in an optic microscope (Olympus BX70), and photographed using a digital camera (CE Roper Scientific). Vacuolation was quantified

Table 1. ^{1}H (500 MHz, CD_3OD) and ^{13}C (125 MHz, CD_3OD) NMR Spectra and DEPT 135 from F32 Obtained from the Methanol Extract of *P. juliflora* Leaves

H/C	δ ^{1}H(a)	δ ^{13}C(a)	δ ^{1}H(lit)	δ ^{13}C(1)	δ ^{1}H(b)	δ ^{13}C(b)	δ ^{1}H(1)	δ ^{13}C(1)
2, 2′	3.25	57.6	3.19 dq	51.5	3.11 m	54.2	3.13 q (7.8)	50.5
3, 3′	3.84	65.9	3.75 ddd (4.8)	68.4	3.84	65.9	3.66 br s	68.9
4, 4′	1.82−1.80	27.2	1.70 m, 1.59 m	26.7	1.76−1.67	27.3	1.72 m, 1.62 m	27.9
5, 5′		25.5	1.82 m, 1.21 m	28.2	1.59−1.54	24.9	1.49 m, 1.22 m	26.9
6, 6′	2.80 m	47.7	2.80 m	49.0	3.00 m	47.9	2.85 m	50.1
7, 7′	1.45−1.42	14.5	1.14 d(7)	11.9	1.29	14.5	1.12 d (6.5)	15.3
1″, 1‴	1.33 m	29.0	1.37 m	29.3	1.33 m	28.8	1.40 m	28.3
2″, 2‴	1.73−1.76	29.6, 29.2	1.35−1.27 m	30.7, 29.8	1.34−1.33	29.0	1.39−1.27 m	27.0
3″, 8″	1.73−1.76	29.3, 29.1	1.35−1.27 m	29.4, 29.2	1.34−1.33	31.9, 31.7	1.39−1.27 m	30.5, 30.1
3‴, 8‴	1.73−1.76	28.9, 28.9	1.35−1.27 m	29.1, 29.0	1.34−1.33	30.3, 30.2	1.39−1.27 m	30.0, 29.8
9″, 9‴	1.73−1.76	27.2	1.35−1.27 m	26.3	1.71 m	29.0, 29.1	1.43 m	29.8, 29.7
10″	2.80 m	33.8	2.80 m	32.1	2.4 m	33.3	2.00 t (7.5)	35.4
10‴	2.80 m	31.4	2.80 m	31.7	1.76−1.67	29.5	1.39−1.27 m	29.8
1‴″	2.80 m	34.5	2.80 m	31.6	1.56 m	33.8	1.52 m	33.4
2‴″	2.51 m	20.0	2.50 m	20.9	2.44 m	22.2	2.05 m, 1.80 m	21.8
3‴″	nd[a]	52.8	4.88 m	59.4	3.10−2.40	52.7	3.13 dd (18, 7.8) 2.10 dd (18, 9)	54.7
5‴″	8.62 s	138.9	8.64 s	137.3	3.10	59.1	3.29 d (15)	55.5
6‴″	–	140.8	–	139.1	2.50	134.0	2.64 d (15)	136.1
7‴″	8.16 s	145.9	8.18 s	144.3	5.59 (s)	125.7	5.37 s	123.8
8‴″	–	143.0	–	141.3	1.71 m	52.8	2.00 m	42.8
8a‴″	–	nd[a]	–	155.0	1.80 m	66.1	1.82 m	65.8

[a]Not detected under MeOH water.

in 10 fields. Astrocytes with more than 10 vacuoles in the cytoplasm were considered to be vacuole-possessing astrocytes.[24]

Immunocytochemistry. Morphological changes in astrocytes and neurons were also studied by immunocytochemistry for cytoskeletal proteins glial fibrillary acidic protein (GFAP) and β-III-tubulin, respectively. All control and treated cocultures were seeded on 4 cm diameter dishes (TPP Switzerland) as neuron/glial cell cocultures. The cells were rinsed three times with PBS and fixed with cold methanol at −20 °C for 10 min. Nonspecific binding of antibody reagents was blocked by preincubating the plates with 3% bovine serum albumin (BSA) in PBS. Cells were incubated with the mouse monoclonal anti-β-tubulin antibody conjugated with Cy3 (1:500 in PBS, Sigma). For GFAP immunocytochemistry, fixed cells were incubated with rabbit polyclonal anti-GFAP (1:100 in PBS, DAKO) overnight and then with the tetramethylrhodamine isothiocyanate-conjugated goat anti-rabbit IgG antibody (1:250 in PBS, Sigma) for 30 min at room temperature. Nuclear chromatin of fixed cells was stained with the fluorescent dye DAPI, at a final concentration of 5 μg/mL in PBS, for 10 min at room temperature in a dark chamber. Thereafter, cells were analyzed by fluorescence microscopy (Olympus BX70) and photographed using a digital camera (CE Roper Scientific).

To identify amoeboid activated microglia, immunocytochemistry for OX-42 (CD11B) was performed before Rosenfeld's staining. First, the endogenous peroxidase activity was blocked for 10 min with 3% hydrogen peroxide. The coculture was incubated for 1 h with the mouse monoclonal anti-OX 42 (CD11b/c) antibody (1:200, CALTAG, Burlingame, CA). Cells were then incubated with the goat anti-mouse IgG peroxidase-conjugated antibody (1:1000, Sigma) for 1 h. Microglial cells were labeled brown after incubation with the substrate, a 0.3% 4-Cl-α-naphthol/methanol solution diluted in PBS buffer (1:5), and H_2O_2 (0.33 mL/mL), at room temperature for 30 min. These cells were analyzed (Olympus BX70) and photographed (CE Roper Scientific) in a light optic phase microscope using a digital camera. The number of immunoreactive cells was counted under the microscope using 20× magnification in a 0.29 mm^2 field. Ten randomized representative fields were analyzed, and the proportion of OX-42-positive cells was presented as the percentage of labeled cells among the total number of cells counted.

Protein Assay and Western Blot. GFAP expression and β-III-tubulin expression were investigated by Western immunoblotting. After being treated, cells were rinsed twice with PBS, harvested, and lysed in 2% (w/v) SDS, 2 mmol/L EGTA, 4 mol/L urea, 0.5% (v/v) Triton X-100, and 62.5 mmol/L Tris-HCl buffer (pH 6.8) supplemented with 0.1% (v/v) protease inhibitor cocktail (Sigma, catalog no. P8340). The protein content was determined by a method adapted from Lowry et al. with a DC protein assay reagent kit (Bio-Rad, Hercules, CA). For analysis, 50 μg of protein, prepared as described above, was loaded onto a discontinuous 4% stacking and 8% running SDS−polyacrylamide gel. Electrophoresis was performed at 200 V for 45 min. Proteins were then transferred onto a polyvinylidene fluoride membrane (PVDF, Immobilon-P, Millipore) at 100 V for 1 h. Equal protein loading was confirmed by staining the membranes with Ponceau red (Sigma). Thereafter, membranes were blocked for 1 h at room temperature in 20 mmol/L Tris-buffered saline (pH 7.5), containing 0.05% Tween 20 (TBS-T) and 5% powdered skim milk. Subsequently, membranes were incubated with rabbit anti-GFAP (1:5000, DAKO), diluted in TBS-T containing 1% powdered skim milk, overnight. Alkaline phosphatase-conjugated goat anti-rabbit IgG or goat anti-rabbit IgG (1:5000 in TBS-T, Bio-Rad) was used as a secondary antibody. Immunoreactive bands were visualized using the AP-conjugated substrate kit (Bio-Rad), according to the manufacturer's instructions. Quantification was achieved by scanning densitometry (ScanJet 4C, Hewlett-Packard) in three independent experiments and analyzed with ImageJ versiono 1.33u (W. Rasband, National Institutes of Health, Bethesda, MD). The antibody specificity and linearity of the densitometric analysis system were assessed by serial dilutions of total protein from cells under control conditions, with 5−20 μg of protein per lane.

Cytoplasm Ultrastructural Analysis. Ultrastructural changes were evaluated by transmission electron microscopy. The experiment was performed on 4 cm diameter dishes (TPP Switzerland) with neuron/glial cell cocultures incubated with 3 μg/mL TAE or F32 or 0.1% DMSO. The cells were fixed with 2.5% glutaraldehyde (Sigma) in 0.1 M cacodylate buffer (pH 7.2) for 2 h at room temperature, washed in 0.1 M cacodylate buffer, and postfixed with 1% osmium tetroxide and 0.8% potassium ferricyanide and 5 mM $CaCl_2$ in the same buffer for 1 h at room temperature. They were then scraped off, dehydrated in an

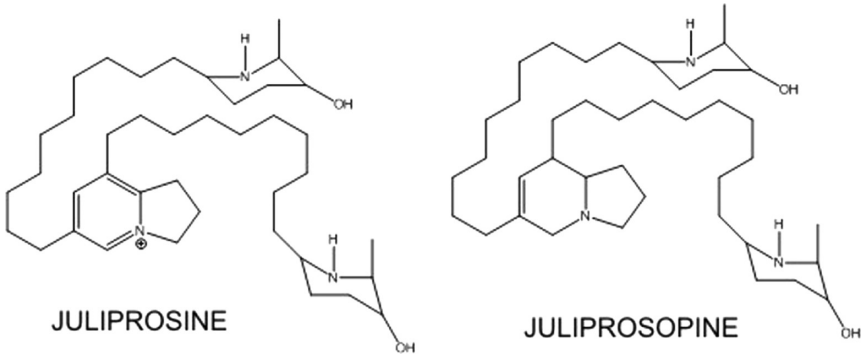

Figure 1. Chemical structures of piperidine alkaloids juliprosine and juliprosopine.

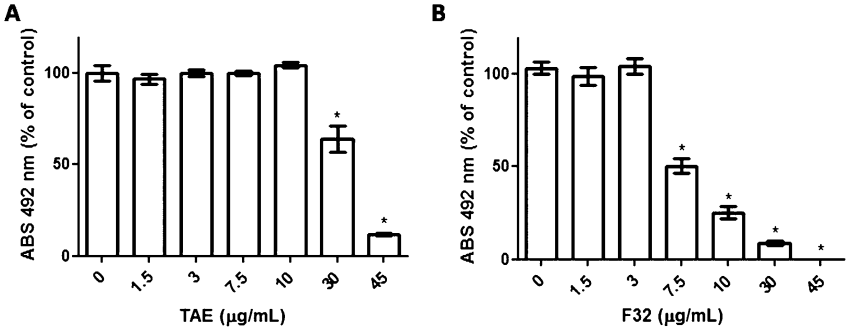

Figure 2. Effect of alkaloids from *P. julifora* on the succinate dehydrogenase activity of neuron/glial cell cocultures. Cells were incubated in the absence (control, 0.1% DMSO) or presence of TAE (A) or F32 (B) and evaluated 24 h post-treatment. Values of control samples were set at 100%. $^*p < 0.05$.

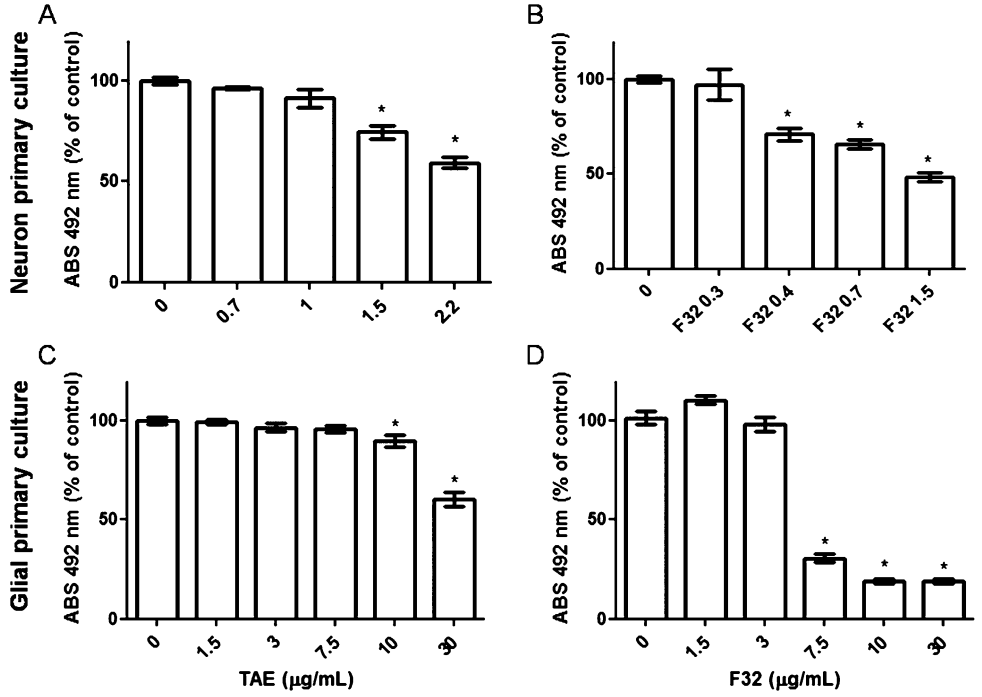

Figure 3. Effect of alkaloids from *P. julifora* on the succinate dehydrogenase activity of isolated neurons (A and B) and glial cells (C and D). Cells were incubated in the absence (control, 0.1% DMSO) or presence of TAE (A and C) or F32 (B and D) and evaluated 24 h post-treatment. Values of control samples were set at 100%. $^*p < 0.05$.

acetone series, and embedded in Polybed resin. Thin sections were stained with uranyl acetate and lead citrate and observed under a Zeiss EM109 transmission electron microscope.

Statistical Analysis. Results are expressed as means ± the standard deviation. One-way ANOVA followed by the Student−Newmann−Keuls test was used to determine the statistical differences among groups differing in only one parameter. The Student's *t* test was used for comparisons between two groups. *p* values of <0.05 were considered to be significant. This statistical analysis was evaluated with GraphPad Prism version 5.

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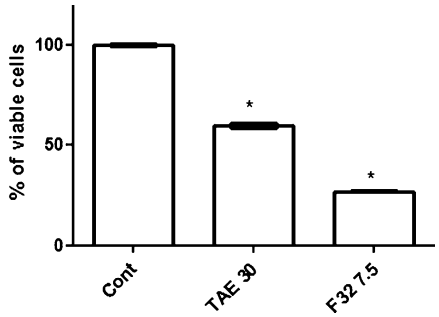

Figure 4. Effect of alkaloids from *P. juliflora* on the membrane integrity of neurons and glial cells in neuron/glial cell primary cocultures. Cells were incubated in the absence (control, 0.1% DMSO) or presence of 30 μg/mL TAE or 7.5 μg/mL F32 and evaluated 24 h post-treatment. Results are expressed as means of the percentage of the ratio of viable cells to total cells ± SD. *$p < 0.05$.

RESULTS

Characterization of Alkaloids in F32. F32 was obtained from the methanol extract of *P. juliflora* leaves as a dark gum. The ^{1}H (500 MHz, CD_3OD) and ^{13}C (125 MHz, CD_3OD) NMR spectra and DEPT 135 experiment displayed a mixture of 1,2,3,5,8,8a-hexahydroindolizine and 2,3-dihydro-1*H*-indolizinium heterocycles. The hexahydroindolizine moiety can be observed through hydrogens $\delta_{H\text{-}1'''}$ 1.56 (m), $\delta_{H\text{-}2'''}$ 2.44 (m), $\delta_{H\text{-}3'''}$ 3.10–2.40 (m), $\delta_{H\text{-}5'''}$ 3.10 (m), $\delta_{H\text{-}6'''}$ 2.50 (m), $\delta_{H\text{-}7'''}$ 5.59 (s), $\delta_{H\text{-}8'''}$ 1.71 (m), and $\delta_{H\text{-}8a'''}$ 1.82 (m) and carbons $\delta_{C\text{-}1'''}$ 33.8 (CH_2), $\delta_{C\text{-}2'''}$ 22.2 (CH_2). $\delta_{C\text{-}3'''}$ 52.7, $\delta_{C\text{-}5'''}$ 52.7 (CH_2), $\delta_{C\text{-}6'''}$ 134.0 (C), $\delta_{C\text{-}7'''}$ 125.7 (CH), $\delta_{C\text{-}8'''}$ 52.7, and $\delta_{C\text{-}8a'''}$ 66.1 (CH). The hydrogens of the indolizinium nucleus appear at $\delta_{H\text{-}1'''}$ 2.80 (m), $\delta_{H\text{-}2'''}$ 2.51 (m), $\delta_{H\text{-}5'''}$ 8.62 (s), and $\delta_{H\text{-}7'''}$ 8.16 (s) and the carbons at $\delta_{C\text{-}1'''}$ 34.5 (CH_2), $\delta_{C\text{-}2'''}$ 20.0 (CH_2), $\delta_{C\text{-}3'''}$ 52.8 (CH_2), $\delta_{C\text{-}5'''}$ 138.9 (CH), $\delta_{C\text{-}6'''}$ 140.8 (C), $\delta_{C\text{-}7'''}$ 145.9 (CH), and $\delta_{C\text{-}8'''}$ 143,0 (C). Signals such as $\delta_{C\text{-}7,7'}$ 14.5 (CH_3), $\delta_{C\text{-}3,3'}$ 65.9 (CH), and $\delta_{c\text{-}6,6''}$ 47.7 (CH) are distinguished in the piperidine ring. The methylenes of the aliphatic portion are at δ 29.6–28.8 (Table 1). The presence of a single group methyl substituent at δ 14.5 denotes the same stereochemistry at C7,7′ for both structures. Analysis of these data and comparison with the literature allowed us to deduce that this fraction is composed of a mixture of alkaloids, juliprosopine (majority constituent) and juliprosine (Figure 1).[25,26]

Cell Viability. *Concentration–Effect.* The toxic effects of TAE and F32 obtained from *P. juliflora* leaves on cell viability were assessed by the MTT test, which measures the reduction of the tetrazolium salt (MTT) to the purple formazan by cellular dehydrogenase enzymes in living cells. After a 24 h

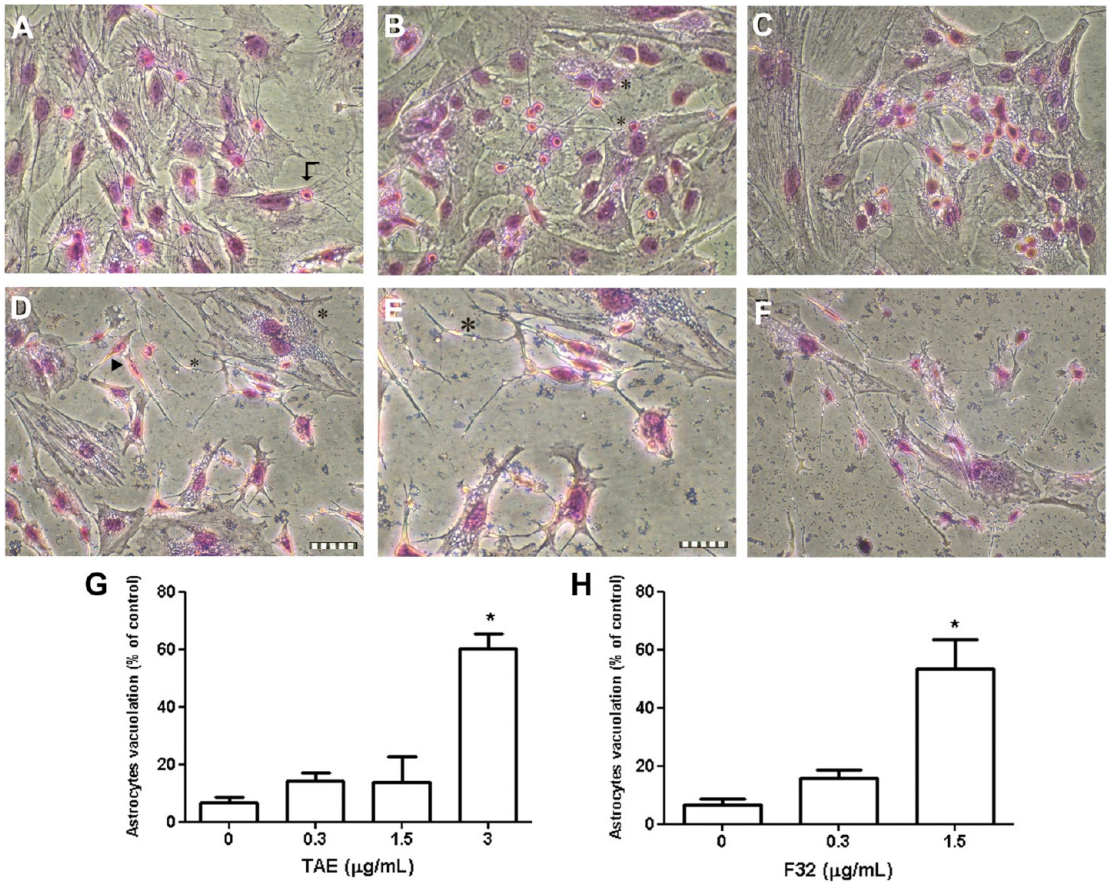

Figure 5. Effect of alkaloids from *P. juliflora* on the cell morphology and vacuolization of astrocytes in neuron/glial cell cocultures after Rosenfeld's staining. Neuron/glial cell cocultures under control conditions (A) and after a 24 h treatment with 1.5 μg/mL TAE (B), 3 μg/mL TAE (C), 1.5 μg/mL F32 (D and E), or 3 μg/mL F32 (F). The arrow indicates a neuronal cell body. The arrowhead indicates a disrupted neuron. The asterisk indicates astrocytes with cytoplasmic vacuolation and vacuoles in neurites. The objective was 20 × 0.70; the scale bars are 10 μm. (G and H) Quantification of astrocytes in vacuolization in neuron/glial cell cocultures under control conditions (0.1% DMSO) and after a 24 h treatment with TAE (0.3–3 μg/mL) or F32 (0.3–1.5 μg/mL). Results are expressed as means of the percentage of the ratio of vacuole-possessing astrocytes to total astrocytes ± SD. *$p < 0.05$.

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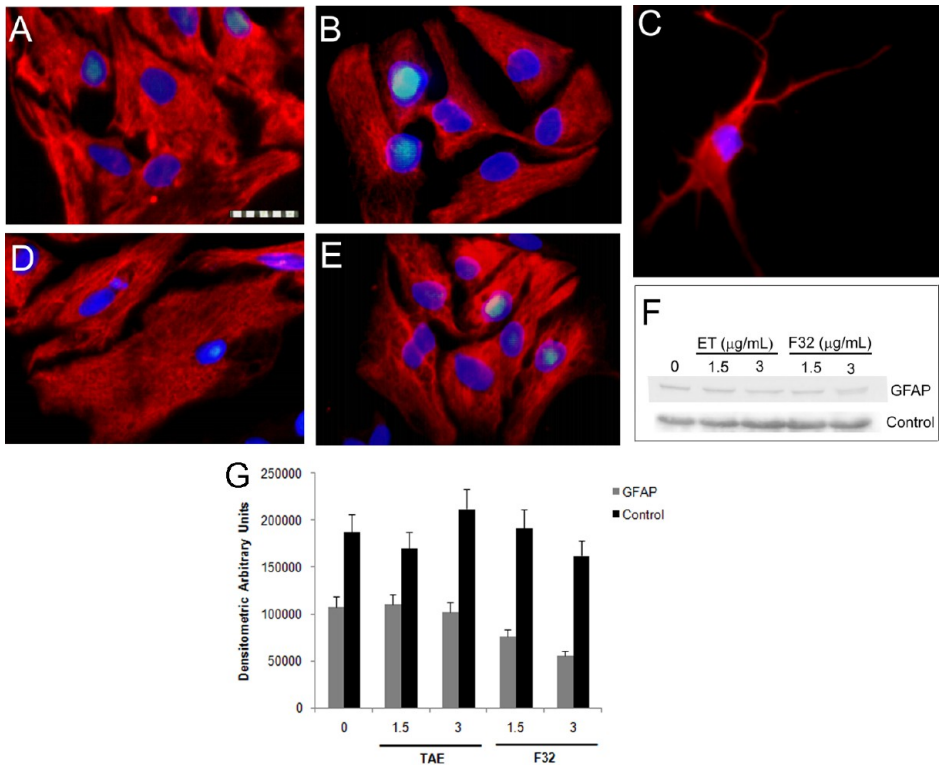

Figure 6. Effect of alkaloids from *P. juliflora* on the astrocyte morphology and GFAP expression in neuron/glial cell primary cocultures. Photomicrographs of cells under control conditions (A) and after a 24 h treatment with 1.5 μg/mL F32 (B), 3 μg/mL F32 (C), 1.5 μg/mL TAE (D), or 3 μg/mL TAE (E). Nuclear chromatin of neuron/glial cell cocultures was stained with Hoechst 33258. The objective was 20 × 0.70; scale bars are 10 μm. (F) Immunoreactive bands after Western blot analysis of GFAP expression in cocultures after a 24 h treatment with alkaloids. Results are representative of three independent experiments. (G) Densitometric arbitrary units of GFAP immunoreactive bands and controls.

exposure, a dose-dependent decrease in dehydrogenase activity was induced by 1.5−45 μg/mL TAE or F32 in neuron/glial cell cocultures (Figure 2A,B). The median inhibitory concentrations (IC_{50} values) were 31.07 μg/mL for TAE and 7.362 μg/mL for F32.

To differentiate neuronal death from glial cell death, we performed the same test for toxicity in isolated cultured cells (Figure 3). A 24 h exposure to 1.5−2.2 μg/mL TAE and 0.4−1 μg/mL F32 induced a dose-dependent decrease in mitochondrial activity in neuron cultures (Figure 3A,B). Glial cell cultures were more resistant than neurons to the toxicity of alkaloids from *P. juliflora* and presented dose-dependent decreases in mitochondrial activity only after exposure to 10 μg/mL TAE (89% of viable glial cells) and 7.5 μg/mL F32 (30% of viable glial cells) (Figure 3C,D).

Moreover, effects of alkaloids from *P. juliflora* on membrane integrity were also determined by counting viable and nonviable cells after Trypan blue staining. We observed a significant reduction in the proportion of viable cells in neuron/glial cell cocultures exposed to 30 μg/mL TAE (41.5%) or 7.5 μg/mL F32 (63%) (Figure 4).

To make possible the analysis of morphological and ultrastructural alterations, we used neuronal toxic but not glial toxic concentrations in other tests (1.5 and 3 μg/mL).

Effect of Alkaloids on Glial Cells and Neuron Morphology. To investigate the effects of *P. juliflora* alkaloids on cell morphology, Rosenfeld's staining, and GFAP, β-III-tubulin, and OX-42 immunocytochemistry were performed in neuron/glial cell cocultures. Rosenfeld's staining in untreated, control cocultures revealed neurons homogeneously distributed over the dense astroglial monolayer, with cells presenting a flat/polygonal phenotype (Figure 5A). In cultures exposed to 1.5 μg/mL TAE the neurite network was maintained, but some astrocytes presented cytoplasmic vacuolation (Figure 5B). However, in cultures exposed to 3 μg/mL TAE, the proportion of astrocytes with cytoplasmic vacuolation increased significantly (Figure 5C,G), with a reduction in the integrity of the neurite network; some giant astrocytes were also observed. Exposure of cells to 1.5 μg/mL F32 was sufficient to disrupt the astrocyte monolayer and the neurite network. Intense cytoplasmic vacuolation in astrocytes and large vacuoles in neurites was also observed (Figure 5D,E,H). These effects were amplified in cultures treated with 3 μg/mL F32 (Figure 5F); however, it was not possible to quantify vacuole-possessing astrocytes, because there are few adherent cells under this condition.

Neuron/glial cell primary cultures analyzed by immunocytochemistry for the astrocyte cytoskeletal protein GFAP showed a monolayer of large, flat cells, with protein distributed throughout the cell bodies (Figure 6). However, dramatic changes in astrocyte morphology were observed only in cultures treated with 3 μg/mL F32. The remaining adherent astrocytes presented thin and multipolar GFAP filaments (Figure 6C), suggesting astrocyte activation. GFAP steady-state levels in the control and in cultures treated with 1.5−3 μg/mL TAE and 1.5 μg/mL F32 remained similar, as determined by a 49 kDa immunoreactive band on the Western blot. However, in cultures exposed to 3 μg/mL F32, a change in the pattern of GFAP migration was observed. GFAP appeared

as an extended protein band of very similar molecular weights, suggesting protein degradation (Figure 6F).

Immunocytochemistry analyses conducted for β-III-tubulin revealed that the neurons exhibited dysfunctional protein polymerization in cultures treated with 3 μg/mL TAE and 3 μg/mL F32 (Figure 7B,C).

The few activated microglial cells immunostained with OX-42 (1.3%) appeared as small, black round cells in neuron/glial cell primary cocultures under control conditions (Figure 8A). However, exposure to 1.5−3 μg/mL TAE or F32 increased the proportion of OX-42-positive microglial cells by up to 5-fold (Figure 8B−E).

Cytoplasm Ultrastructural Analysis. The ultrastructural analysis revealed that the cells under control conditions (0.1% DMSO) (Figure 9A) presented a normal morphology for the cytoplasm, mitochondria, and nucleus. However, cells treated for 24 h with 3 μg/mL TAE presented a range of mitochondrial shapes and sizes. These changes in mitochondrial morphology suggested a mitochondrial fusion. In addition, cells treated for 24 h with 3 μg/mL F32 presented mitochondria with shortened and disintegrating cristae and cytoplasmic vacuoles, characterized by double-layer membranes or multilayer membranes. This morphology of cytoplasmic vacuoles suggested autophagic vacuoles formatio (Figure 9B,C)

DISCUSSION

In our previous studies, we demonstrated that the alkaloidal extract (TAE) and some alkaloidal fractions from *P. juliflora* leaves were cytotoxic and induced activation of glial cells in primary cultures. Glial cells have important functions that impact neuronal cell health and integrity.[27] Considering a growing body of evidence that astrocytes and microglia play important roles in the physiology of the CNS and in the mechanisms of pathogenesis of various neurological diseases,[28−30] it is important to investigate the reactions of glial cells interacting with neurons to various stimuli, including chemical agents. In this study, we investigated the effects of TAE and the more cytotoxic alkaloidal fraction for glial cells [F32 fraction (for review, see ref 13)] in a system of neuron/glial cell cocultures to induce and characterize the main morphologic and ultrastructural changes in central nervous system cells visualized in "cara torta" disease. NMR characterization of F32 showed that this fraction is composed of a mixture of two alkaloids, juliprosopine (majority constituent) and juliprosine. In a phytochemical investigation of the pods of *P. juliflora* cultivated in the semi-arid region of the State of Paraiba of Brazil, Tabosa and co-workers found that the toxic activity, observed in laboratory animals, is chemically related to the piperidine alkaloids juliprosopine and juliprosine, present in the pods of this Leguminosae.[25]

We determined the cytotoxic concentrations of TAE and F32 obtained from *P. juliflora* leaves in neuron/glial cell cultures by the MTT test. MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide] is a water-soluble tetrazolium salt, which is converted to an insoluble purple formazan by cleavage of the tetrazolium ring by succinate dehydrogenase within the mitochondria in healthy cells.[31,32] More recent evidence suggests that reduction of MTT can also be mediated by NADH or NADPH within the cells and out of mitochondria.[33] We observed by the MTT test that a 24 h exposure to low concentrations of alkaloids from F32 and TAE (3 μg/mL) was not sufficient to reduce succinate dehydrogenase activity on neuron/glial cell cultures. However, this subtoxic concentration

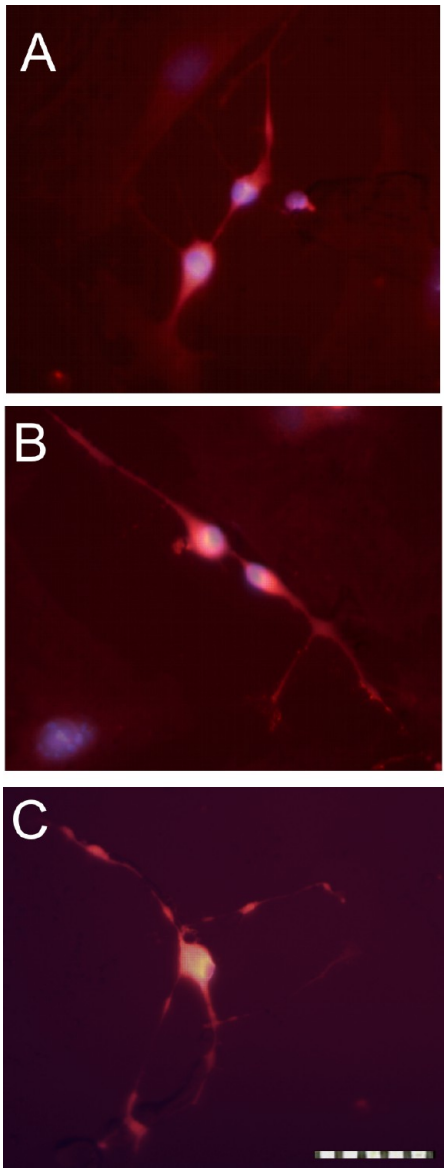

Figure 7. Effect of alkaloids from *P. juliflora* on the neuron morphology and β-III-tubulin expression in neuron/glial cell primary cocultures. Photomicrographs of cells under control conditions (A) and after a 24 h treatment with 3 μg/mL TAE (B) or 3 μg/mL F32 (C). Nuclear chromatin of neurons and glial cells in cocultures was stained with Hoechst 33258. The objective was 20 × 0.70; scale bars are 10 μm.

induced mitochondrial disorder visualized by electron microscopy, suggesting the involvement of extramitochondrial NADH or NADPH on the reduction of MTT to formazam. Moreover, to clarify a cell death induced by F32 and TAE, we also investigated whether these compounds induce membrane damage at IC_{50} concentrations in neuron/glial cell primary cocultures of ~59.5 and ~27%, respectively. To clarify the sensibility of the cell types to the toxins, we also evaluated the activity of succinate dehydrogenase on neuron culture and glial cell culture isolated systems. We observed that only at higher concentrations of F32 (7.5 μg/mL) and TAE (30 μg/mL) was the succinate dehydrogenase activity reducted in cultures of glial cells; however, low concentrations of alkaloids from F32 and TAE (0.4−1.5 μg/mL) were sufficient to reduce succinate dehydrogenase activity in cultures of cortical neurons,

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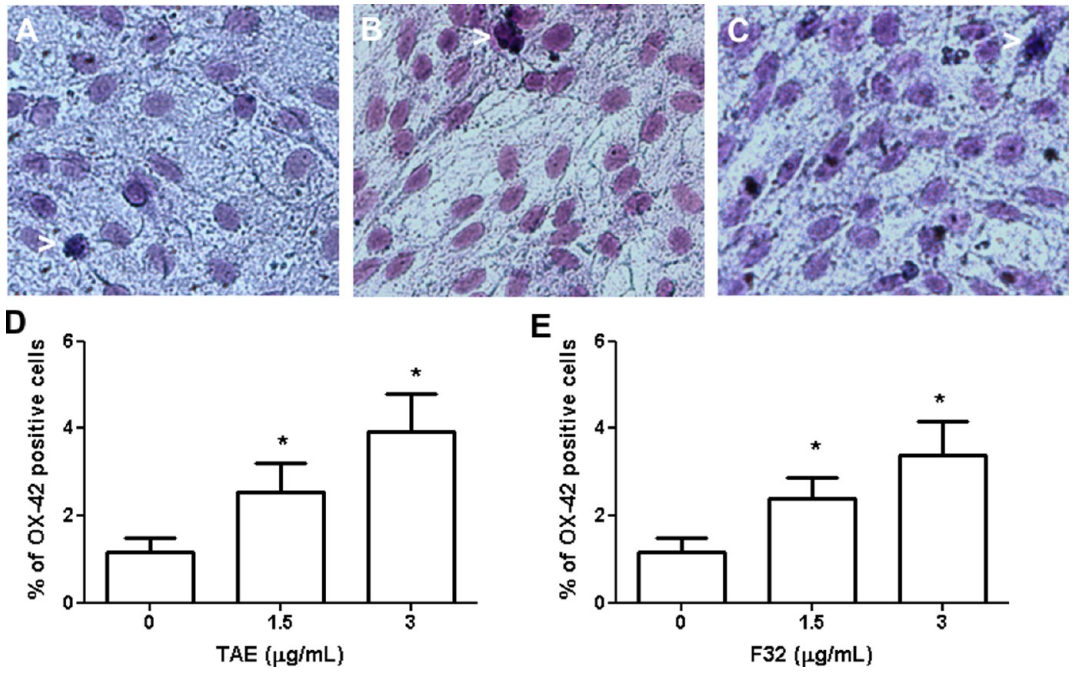

Figure 8. Effect of alkaloids from *P. juliflora* on the microglial morphology and activation in neuron/glial cell primary cocultures after immunocytochemistry for OX-42 protein and after Rosenfeld's staining. Photomicrography of OX-42-positive round microglial cells (arrows) under control conditions (A) or after a 24 h treatment with 1.5 μg/mL TAE (B) or 1.5 μg/mL F32 (C). The objective was 20 × 0.70; scale bars are 10 μm. Quantification of OX-42-positive cells in cultures under control conditions or treated with 1.5−3 μg/mL TAE (D) or F32 (E). *$p < 0.05$.

indicating that isolated neurons were more sensible them glial cells. Our previous study, developed in astrocyte primary cultures, also showed that, after a 24 h exposure, a crude extract containing alkaloids, obtained from *P. juliflora* pods, induced only cytotoxicity related to reducing succinate dehydrogenase activity and induction of membrane damage when administered at 30 μg/mL.[12] However, in another study, conducted in astrocyte primary cultures enriched with microglia, significant reductions in mitochondrial activity and a loss of membrane integrity were observed after exposure to 3 μg/mL TAE or F32 obtained from *P. juliflora* leaves.[13] In the central nervous system, astrocytes are the cells that respond to insults and metabolize xenobiotics as a mechanism of neuronal protection and stand out by having enzymatic machinery that allows them to develop both the phase I metabolism enzyme isoforms by system cytochrome P450 metabolism and phase II by glutathione transferase isoforms.[34−36] It is possible that the resistance of glial cell cultures and neuron/glial cell cocultures to alkaloids is a result of the metabolism of the astrocytes, and further studies may be performed to improve our understanding its relation with neurotoxicity.

Cytoplasmic vacuolization upon exposure to a variety of chemicals and bioactive substances has been extensively reported (for a review, see ref 37). We also observed, after Rosenfeld staining, that exposure of neuron/glial cell cultures to alkaloids from *P. juliflora* induced structural changes in astrocytes and an important dose-dependent cytoplasmic vacuolation. Moreover, a disruption in the neurite network and intense vacuolation in neurites were observed. In the field of cell pathology, cell deterioration characterized by cytoplasmic vacuolation is called vacuolar degeneration,[38] and the vacuolation induced by alkaloids from *P. juliflora* in neuron/glial cell primary cultures may be a type of vacuolar degeneration. Similar morphological changes in CNS cells were reported in experimental and spontaneous ingestion of *P. juliflora* among cattle.[10,11] We observed gliosis, vacuolation, and neuronal loss in trigeminal motor nuclei and other motor cranial nerve nuclei to be the main histologic lesions after *P. juliflora* intoxication, with Wallerian-like degeneration in the cranial nerves.

Autolysosomes are not true vacuoles that sometimes expand to fill the cytoplasm and therefore are often termed autophagic vacuoles, which could be observed via light microscopy.[37] Ultrastructural analysis suggested autophagic vacuole formation on cells treated for 24 h with 3 μg/mL F32. The autophagy is an intracellular bulk degradation process whereby cytoplasmic proteins and organelles are degraded and recycled through lysosomes. It plays an important role in the elimination of damaged organelles such as mitochondria and protects against cell death programmed by mitochondrial dysfunction on neuronal cells.[39,40] In addition, cells treated for 24 h whit 3 μg/mL F32 presented mitochondria with shortened and disintegrating cristae. However, cells treated for 24 h with 3 μg/mL TAE presented changes in mitochondrial morphology that suggested a mitochondrial fusion that is the combination of two mitochondria into a single organelle.[41] Studies indicated that fusion is related to changes in the mitochondrial function and protects mitochondria from autophagosomal degradation.[41,42] These ultrastructural results reported apply to all cell types of neuron/glial cell cocultures and corroborate the findings for succinate dehydrogenase activity and morphological analyses that suggest that both neurons and glial cells had mitochondrial dysfunction and vacuolation after being exposed to alkaloids.

Intermediate filaments (IFs) are structures that, together with microtubules and microfilaments, form the cytoskeleton that is present in nearly all eukaryotic cells. The major IF protein in astrocytes is glial fibrillary acidic protein (GFAP). Recent studies have revealed the presence of multiple isoforms of GFAP, which may be differentially expressed in reactive and resting astrocytes (for a review, see ref 43). Changes in the expression of GFAP illustrate astroglial reactivity (astrogliosis).

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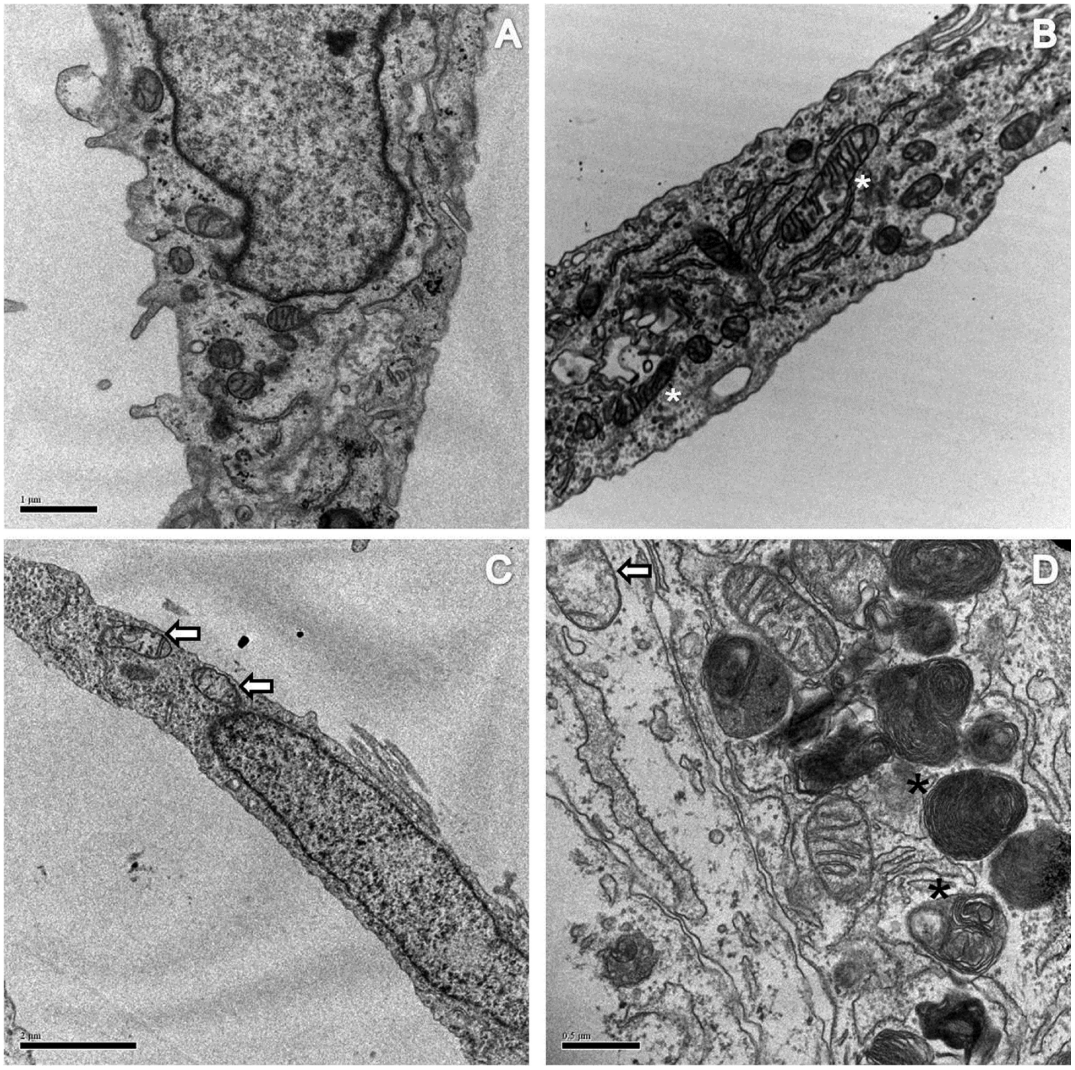

Figure 9. Effect of alkaloids from *P. juliflora* on ultrastructural changes in neuron/glial cell primary cocultures. Transmission electron microscopy of the cytoplasm from cells under control conditions (A) and after a 24 h treatment with 3 μg/mL TAE (B) or 3 μg/mL F32 (C and D). The white asterisk in panel B denotes changes in mitochondrial morphology. The arrows in panels C and D denote mitochondria with shortened and disintegrating cristae. The black asterisk in panel D denotes cytoplasmic vacuoles.

Some astrocytes reacted to the largest dose of F32 tested (3 μg/mL) with a strong retraction of the cell body and emission of thick and ramified processes. This phenomenon was associated with the appearance of two GFAP-immunoreactive bands (as revealed by Western blotting), suggesting protein instability. In our previous study of glial cell cultures, we observed that astrocytes exposed to 3 μg/mL TAE developed compact cell bodies, with many processes overexpressing GFAP, but exposure to 3 μg/mL F32 induced GFAP disruption.[13] Although an increase in the level of GFAP production may be a sign of astrogliosis, reactive injury, and even neurodegeneration,[27,44,45] a decrease in its levels may signify abnormal synaptogenesis and neurotransmission.[46,47]

Exposure of neuron/glial cell cocultures to alkaloids from *P. juliflora* induced disruption of the astrocyte monolayer and neurite network, with intense vacuolation in both cell types. These phenomena were also associated with changes in the expression of β-tubulin III, the main component of the neuronal cytoskeleton. Interruptions in β-III-tubulin staining were typically observed in cultures exposed to 3 μg/mL F32, suggesting a failure in the polymerization of this protein in neurons, a phenomenon that may be associated with vacuolation. Published studies report that astrocytes are capable of producing molecules that affect axonal growth, such as the components of extracellular matrix laminin. Furthermore, disrupted astrocyte integrity and astrogliosis affect neurite outgrowth.[44,48,49] The response of astrocytes to alkaloids from *P. juliflora*, characterized by changes in morphology and GFAP expression, may be associated with changes in the expression of β-III-tubulin and the integrity of the neurite network.

Microglia, the resident immune cells of the brain, function like tissue macrophages in other organs, serving as tissue phagocytes (when required) and constituting the first line of defense against invading pathogens and other challenges.[50] However, their activation may interfere with neuronal cell health and integrity.[27,51] Activated microglia have been observed to transform into small round cells without processes that express OX-42.[52,53] An enhanced number of OX-42-positive microglia cells were observed in neuron/glial cell primary cultures exposed to both TAE and F32. This effect appeared to be enhanced in the presence of the highest concentration of TAE and F32 (3 μg/mL). This observation

indicates that alkaloids from *P. juliflora* can also induce microgliosis. Among several factors released by activated glia, NO seems to play a critical role in stress-induced brain damage.[29,54−56]

Considering that F32 was more cytotoxic than TAE and that F32 reproduced *in vitro* the main morphologic and ultrastructural changes of "cara torta" disease, we can suggest that piperidine alkaloids juliprosopine and juliprosine are primarily responsible for the neurotoxic damage observed in animals after consumption of the plant. We suggested that the potential mechanism of action of these alkaloids is induction of mitochondrial dysfunccion, which results in neuronal death, gliosis, and autophagic vacuolation. More studies should be conducted to characterize the mechanism of vacuole formation and to determine if the vacuoles have a protective effect against programmed cell death induced by mitochondrial damage.

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Notes

The authors declare no competing financial interest.

ABBREVIATIONS

CNS, central nervous system; F32, alkaloid fraction obtained from *P. juliflora* leaves; GFAP, glial fibrillary acid protein; MTT, 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide; TAE, total alkaloid extract obtained from *P. juliflora* leaves; OX-42 antibody, antibody that reacts with the CR3 complement (C3bi) receptor found on most monocytes, granulocytes, and macrophages; β-III-tubulin, microtubule element of the tubulin family (class III) found almost exclusively in neurons

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