Cytotoxicity effect of alkaloidal extract from *Prosopis juliflora* Sw. D.C. (Algaroba) pods on glial cells

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Abstract

Prosopis juliflora is largely used for feeding cattle and humans. Neurological signals have been reported in cattle due to intoxication with this plant. In this study, an alkaloidal fraction (AF) obtained from *P. juliflora* pods was tested on astrocyte primary cultures. Astrocytes display physiological functions essential to development, homeostasis and detoxification in the central nervous system (CNS). These cells are known for their role on energetic support and immune response in the CNS. Concentrations between 0.03 to 30 μg/ml AF were assayed for 24 - 72 h. The mitochondrial activity, assayed by MTT test, showed cytotoxicity at 30 μg/ml AF after 24 h. At concentrations ranging between 0.3 – 3 μg/ml, the AF induced an increase on mitochondrial activity, indicating cell reactivity. Immunocytochemistry assay for GFAP cytoskeletal protein, revealed alterations on cell morphology after treatment with 0.3 – 3 μg/ml AF for 72 h. This result corroborates with western blot analysis when cells treated with 0.3 – 3 μg/ml AF for 72 h showed GFAP upregulation. The vimentin expression was not significantly altered in all tested concentrations. These results suggest that alkaloids induce astrocyte reactivity and might be involved in the neurotoxic effects induced by *P. juliflora* consumption.

Key-words:
Alkaloids.
Astrocytes.
Gliosis.
Prosopis juliflora.

Introduction

Prosopis juliflora is a shrub that grows abundantly in the Sind and Punjab, provinces of Pakistan[1]. This plant was introduced in the states of Pernambuco and Rio Grande do Norte, Northeast Brazil, in 1942 and 1948, respectively, with seeds from Peru and Sudan[2]. Due to their palatability and nutritional value, pods of *P. juliflora* or its bran are largely used for feeding dairy and beef cattle with good nutritional and economic results[3]. Products from this plant have also been used for human consumption in bread, biscuits, sweeties, syrup and liquors[4].

Extracts of *P. juliflora* seeds and leaves have several *in vitro* pharmacological effects such as antibacterial[5,6,7,8,9,10], antifungal[6,7,11], and anti-inflammatory properties[12]. These properties have been attributed to piperidine alkaloids[6,13]. These compounds could be potentially used as drugs, however toxicological studies should be performed specially because this plant has been found to cause disease in animals when it is used as the sole sustenance. Intoxication with *P. juliflora* has been reported in the USA[14], Peru[15], and also in Brazil. In the latter, the illness is called "cara torta"[16]. This disease is characterised by emaciation, neuro-muscular alterations, including muscular atrophy of the masseter,

and histologic lesions like spongiosis, gliosis, the loss of Nissl substance and fine vacuolation of the perikaryon of neurons from trigeminal motor nuclei[17]. However, despite of the neurological disease in animals induced by *P. juliflora* there is no data about its cytotoxicity toward glial cells.

Glial cells, mainly astrocytes, are essential to development, homeostasis and detoxification in the CNS[18]. Moreover, these cells are known for their role on energetic support and immune response in the CNS against chemical, infectious or traumatic challenges[19]. They actively control synaptogenesis, synapse function, synaptic plasticity[20], uptake of neurotransmitters and maintenance of extracellular ion levels. Astrocyte primary cultures are largely used to evaluate astrocyte functions in CNS disorders[19,21]. It has been demonstrated that astrocytes react to chemical or physical insults by undergoing activation, a phenomena known as astrogliosis[22,23,24,25,26]. *In vitro* studies have clearly demonstrated that reactive astrocytes in primary cultures modify their morphology from enlarged to rounded cell bodies, retract their monolayer with distinct cellular process and accumulate the intermediate filaments containing the astrocyte specific marker, the glial fibrillary acidic protein (GFAP).

In this work effects of alkaloids total fraction obtained from *P. juliflora* pods on astrocyte primary cultures was studied, evaluating astrocyte viability, morphological structure and GFAP expression.

Materials and Methods

Pods gathering and Alkaloids Extraction

Ripe pods of *Prosopis juliflora* were harvested in Salvador (BA) at the experimental fields of the Federal University of Bahia (UFBA). Air-dried pods were grounded and immediately flooded in hexane. The alkaloid fraction from *Prosopis juliflora* pods was obtained by an acid/basic modified extraction[27], with some modifications. In brief, the air-dried plant material was exhaustively extracted using hexane (1.5 l/kg) to eliminate apolar constituents, at room temperature with occasional shaking. The extract was then filtered and the residue was flooded with methanol (1.5 l/kg) using the above process. The methanol extract was concentrated under low pressure, and this concentrated residue was stirred with 0.2 N HCl and filtered after 16 h. The solution was shaken with chloroform to remove the non-basic material. The aqueous layer was basified with ammonium hydroxide until it reached pH 11 and then was extracted with chloroform. The resulting solution was evaporated to dryness to yield total alkaloidal fraction (AF) of *P. juliflora*. The presence of alkaloids in AF was confirmed using the Dragendorff reagent identification test[28]. The dried AF was dissolved in dimethylsulfoxide (DMSO, Sigma, St Louis, MO) at a concentration of 3 mg/ml, and stored in the dark at –20 ºC.

Cell culture and treatments

One-day-old postnatal Wistar rat pups used in this study were obtained from the animal house facility of the Instituto de Ciências da Saúde, UFBA. Astrocyte cultures were prepared according to the modified procedure[29]. Briefly, cerebral hemispheres of newborn Wistar rat pups were isolated aseptically and meninges were removed. Neopallia were dissected out and then gently forced through a sterile 75 mm Nitex mesh. Cells were suspended in Dulbecco's Modified Eagle's Medium (DMEM, Cultilab, SP, Brazil), supplemented with 100 UI/ml penicillin G, 100 mg/ml streptomycin, 2 mM L-glutamine, 0.011 g/L pyruvate, and 10% foetal calf serum (Cultilab, SP, Brazil) in a humidified atmosphere with 5% CO_2 at 37 ºC. Primary cultures of astrocytes contained more than 95% immuno-positive cells, when labelled with anti-GFAP antibody.

Cells were treated with AF diluted in the medium at exponential final concentrations ranging between 0.03 μg/ml to 30 μg/ml, for 24 h or 72 h. The control

group was treated with DMSO diluted in the culture medium at equivalent volume used in the treated group.

Cytotoxicity and cell membrane integrity assay

The alkaloidal extract was tested for its cytotoxicity towards astrocytes using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT; Sigma, St Louis, MO) test. The experiment was performed in 96 well plates (TPP Switzerland) (1.6 x 10^6 cells/plate) after cells had become confluent (95%). The cell viability was quantified by the conversion of yellow MTT by mitochondrial dehydrogenases of living cells to purple MTT formazan[30]. 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) SDS, 50% (v/v) DMF (pH 4.7), and plates were kept overnight at 37 ºC in order to dissolve formazan crystals. The optical density of each sample was measured at 560 nm using a BIO-RAD 550PLUS spectrophotometer. Four replicate wells were used for each analysis. Results were shown as percentages of the viability of the control group.

Membrane integrity was evaluated by Trypan Blue staining. Cells were treated with AF for 24 or 72 h and after this time of exposure cells were trypsinized, harvested, pooled with floating cells and centrifuged at 1300 *g* for 10 minutes. Cells were then suspended in 200 µl DMEM without supplementation and stained with 0.1% (w/v) Trypan blue (n = 5). The number of viable and non-viable cells was determined in a Neubauer chamber. Results were expressed as a mean of the percentage of the ratio dead cells/total cells ± standard deviation (S.D.).

Astrocytes reactivity

Morphological changes were studied analysing cells by phase microscopy and immunocytochemistry. Western blotting was performed for the cytoskeletal proteins GFAP and vimentin.

Immunocytochemistry and chromatin staining

All cells, control and treated, were rinsed thrice with PBS and fixed with cold methanol at –20 ºC for 10 minutes. Cells were incubated under slow agitation with rabbit polyclonal anti-GFAP (1/500, DAKO, Denmark) overnight and then with tetramethylrhodamine isothiocyanate conjugated goat anti-rabbit antibody (1/250, Biomakor, Israel) for 30 min at room temperature. Chromatin integrity or nuclear fragmentation/condensation was investigated staining nuclear chromatin of fixed cells with the fluorescent dye Hoechst 33258 (Sigma, St Louis, MO), at a final concentration of 5 µg/ml in PBS, for 10 minutes at room temperature in a dark chamber. Thereafter, cells were analysed by fluorescent microscopy (Olympus AX70).

Protein assay and Western blot

After treatment with AF, cells were rinsed twice with PBS, harvested, and lysed in 2% (w/v) SDS, 2 mM EGTA, 4 M Urea, 0.5% (v/v) Triton X-100, 62.5 mM Tris-HCl buffer (pH 6.8) supplemented with 1µl/ml of a cocktail of proteinase inhibitors (Sigma, St Louis, MO). Protein content was determined by the suggested method[31]. For GFAP and vimentin analysis, 18 µg protein was loaded onto a discontinuous 4% stacking and 8% running SDS polyacrilamide gel (SDS-PAGE). Electrophoresis was performed at 200 V for 45 minutes. Proteins were then transferred onto a polyvinylidene fluoride membrane (PVDF, Immobilon-P, Millipore), at 100 V for 1 h. Thereafter, membranes were blocked for 1 h at room temperature in 20 mM Tris-buffered saline (pH 7.5), containing 0.05% Tween 20 (TBS-T) and 1% BSA. Subsequently, membranes were incubated with mouse anti-GFAP (1:10,000, Boehringer, Mannheim) or mouse anti-vimentin (1:10,000, clone V9, Boehringer, Mannheim) monoclonal antibodies for 1 h. Conjugated alkaline phosphatase anti-mouse IgG (1:5,000, Promega Corporation, WI, USA) was used as secondary antibody. Immunoreactive

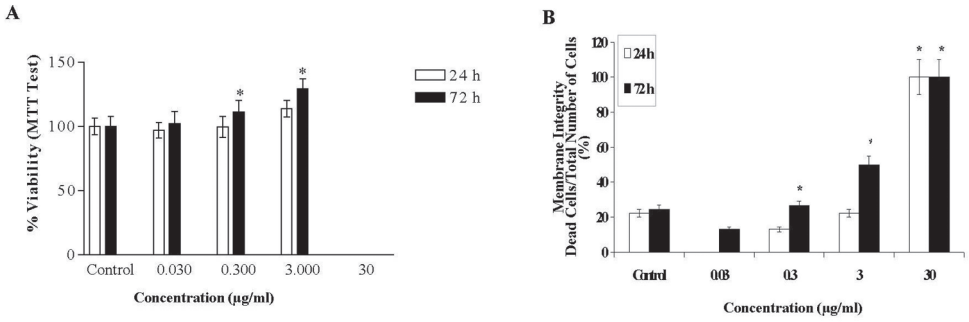

Figure 1 – Cytotoxicity assays. **A)** MTT test on astrocyte primary culture treated with alkaloidal fraction (AF) at various concentrations (0.03 – 30 µg/ml) and evaluated at 24 and 72 h posttreatment. Results were shown as percentages of the viability of the control group. **B)** Membrane integrity assayed by Trypan blue. Results were expressed as a mean of the percentage of the ratio dead cells/total cells ± standard deviation (S.D.). * $P < 0.05$

bands were visualised using Protoblot II AP System Kit (Promega Corporation, WI, USA), according to manufacturer's instructions. Quantification was obtained by scanning densitometry (ScanJet 4C – HP) and analysed using ImageQuant TL software (Amersham). Antibody specificity and linearity of the densitometric analysis system was assessed by serial dilutions of total protein from cells in control conditions within a range of 5-20 ìg of protein *per lane*.

Statistical analysis

ANOVA one way test was performed followed by the Student-Newmann-Keuls test to determine the significance of differences in multiple comparisons. Values of $P < 0.05$ were considered as statistically significant.

Results

The effects of alkaloidal fraction (AF) from *P. juliflora* pods upon the cell viability were assessed by the MTT test (Figure 1A). No apparent cytotoxic effect was observed in cells treated with concentrations ranging between 0.03 to 3 µg/ml for 24 h. Moreover, the mitochondrial activity increased significantly in cells treated with 0.3 to 3 µg/ml AF for 72 h ($P < 0.05$). However, 30 µg/ml AF killed all cells at 24 h evidenced by MTT assay and Trypan Blue staining (Figure 1B). Although MTT assay showed no toxicity after treatments with 0.3 to 3 µg/ml for 72 h, Trypan Blue staining showed alterations in membrane integrity ($P < 0.05$).

The morphology in control conditions analysed by immunocytochemistry for GFAP protein showed a monolayer of large flat cells (Figure 2A). The alkaloidal extract induced morphological changes after 72 h exposure to 0.3 to 3 µg/ml. Cell bodies began to retract after treatment with 0.3 µg/ml (Figure 2B) and presented long and dense processes with a star-like shape expressing GFAP when treated with 3 µg/ml (Figure 2C). Nuclei also showed condensation of the chromatin, which increased with AF concentration.

Vimentin and GFAP expression examined by western blotting showed that AF from *P. juliflora* pods induced an increase in GFAP levels of cells treated with 0.3 to 3 µg/ml for 72 h (Figure 3). On the other hand, the level of vimentin was not modified in astrocytes treated with these same concentrations. The specificity GFAP up-regulation was demonstrated by a significant increase in GFAP expression relative to vimentin.

Discussion

The biological activity of alkaloids extracted from pods of *P. juliflora* was assessed to test whether these molecules act

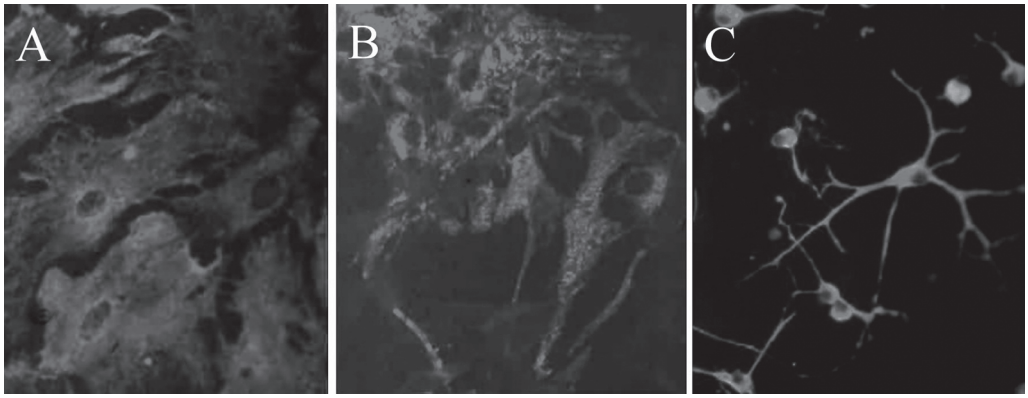

Figure 2 - Immunofluorescence labelling of GFAP in Astrocytes treated with alkaloidal fraction (AF) extracted from *P. juliflora* pods for 72 h. GFAP staining of Astrocytes grown in control conditions (A) and with 0.3 μg/ml AF (B) or with 3 μg/ml (C)

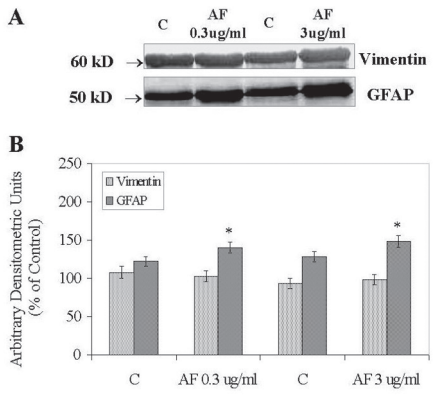

Figure 3 - Western blotting analysis of vimentin **(A)** and GFAP **(B)** protein expression from astrocytes in control conditions (lane 1 and 3) or treated with 0.3 or 3 μg/ml AF from *P. juliflora* pods (lane 2 and 4) for 72 h. Samples containing 18 μg of protein were electrophoretically separated through 8% polyacrylamide gels containing 0.1% SDS in running buffer. This result is representative of three independent experiments

directly on astrocytes and if they might be related on neurotoxic phenomenons observed in other early animal studies[16,17]. Astrocyte-enriched primary glial cell cultures from the rat cerebral cortex were exposed to exponential concentrations of an alkaloidal fraction (AF) and its effects on cell viability and reactivity were investigated. The cleavage of tetrazoline ring in MTT involve the mitochondrial succinate dehydrogenase and depends on the activity of the respiratory chain and the redox state of the mitochondria[32,33]. Short time exposure (24 h) to the AF, as shown by MTT test, causes cytotoxicity only at the highest concentration (30 μg/ml). Such effect was demonstrated previously on erythrocytes subjected to alkaloids from *P. juliflora,* showing significant hemolysis due to membrane injury[34]. Treatments with 0.03 - 3 μg/ml AF for 24 h did not compromise the astrocytes mitochondrial activity. However, an increase of MTT reduction was observed in astrocytes exposed to intermediary concentrations (0.3 - 3 μg/ml AF), which indicates a significant increase in mitochondrial activity and cell reactivity. This is consistent with studies that revealed an increase in metabolic activity of astrocytes that survive after stressing conditions[23,35,36].

The major role of astrocytes is the control of neurotoxins within the central nervous system. These cells possess the remarkable characteristic of responding to apparently all forms of neurological damage, included those induced by toxicants, by undergoing activation, known as astrogliosis[22]. The astrogliosis is associated with altered phenotype due to upregulation of a large number of molecules, specially the accumulation of intermediate filaments containing the astrocyte specific GFAP. Several studies have shown that GFAP is upregulated after exposure to diverse set of toxic chemicals that includes, kainic acid, mercury chloride, aluminium chloride, toluene, ethanol, dibutyryl-cAMP and trimethyltin[23,24,37,38]. This study demonstrated

that treatment of astrocytes exposed to concentrations of 0.3 or 3 μg/ml AF changed their morphology and increased the GFAP content. These increases in GFAP content were reflected in an increased staining in GFAP cells presenting a multipolar extending processes, visualised by immunofluorescence and by an increase in GFAP immunoreactive bands, measured by western blotting. The intermediate filament protein vimentin was the other marker of astrocyte reactivity protein examined. Vimentin expression in astrocytes was not directly affected by AE from *P. juliflora*. It is in accordance with studies *in vitro* both on primary cultures[23] and on glial cell lines[24,39] exposed to chemical challenges, and after some types of whole brain insults, especially when permanent damage occurs[40]. However, increases in cell number or generalised protein synthesis were not observed.

The data presented in this study has not addressed the mechanism (or mechanisms) that underlies the GFAP increases in astrocytes after *P. juliflora* AF exposure. However, concentrations of AF which cause GFAP increasing correlates well with the loss of Trypan blue exclusion. This suggests that the GFAP component of the response to alkaloids from *P. juliflora* may be mediated throughout membrane effects

The neurological disorders observed in intoxication by *P. juliflora* pods by goats[17] are similar in some aspects to that observed in some acquired storage diseases, including intoxication by *Swainsona sp*. This plant contains swainsonine, an indolizidine alkaloid, which inhibits a-manosidase and also b-glycosidade. Such disorders are characterised by neurological signs of cerebellar dysfunction, including staggering and incoordination, with severe cellular vacuolation and degeneration of Purkinje cells in the brain[41]. It is known that piperidine alkaloids identified in the *P. juliflora* pods (juliprosopine and juliprosine) are weakly active as b-glycosidase inhibitors[42], but until now it is unknown if they induce neuronal vacuolation. These alkaloids have in its molecular structure not only a piperidine ring, but also an indolizidine ring, which might be inhibiting the a- and b-glycosidases. This study observed that the astrocyte membrane were damage after AF *P. juliflora* exposure, suggesting that these alkaloids may have the ability to permeate the plasma membrane, changing its conformation and promoting the vacuolation[17].

Taken together, our data show that the AF from *P. juliflora* act directly on astrocytes triggering astrogliosis, may be because membrane damages improve the mitochondrial function. These cells might be acting on the control of toxic effects involved in neurological damages induced by alkaloids from *P. juliflora*.

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Citotoxicidade do extrato alcaloidal das vagens de *Prosopis juliflora* Swartz. D.C. (Algaroba) em células gliais

Resumo

A *Prosopis juliflora* é amplamente utilizada na alimentação humana e de várias espécies animais, especialmente bovinos. Quadros de intoxicação por esta planta, nesta espécie, têm sido relatados,

principalmente quando a mesma é oferecida como única fonte alimentar, desencadeando uma doença de sintomatologia nervosa. Neste estudo, objetivou-se avaliar os efeitos *in vitro* da fração de alcalóides totais (FA) extraída das vagens da *Prosopis juliflora* utilizando cultura primária de astrócitos obtidos do córtex cerebral de ratos como modelo de estudo. A avaliação da atividade mitocondrial pelo teste do MTT demonstrou a citotoxicidade em 30 μg/ml da FA após 24 h. As concentrações de 0,3 e 3 μg/ml da FA induziram um aumento da atividade mitocondrial, indicando reatividade celular. Testes imunocitoquímicos para a GFAP, principal proteína de citoesqueleto de astrócitos, revelaram alterações morfológicas nas células após tratamento com 0,3 e 3 μg/ml da FA por 72 h. Tais resultados são consoantes à análise desta proteína por westernblot, quando as culturas foram tratadas com 0,3 e 3 μg/ml da FA por 72 h, demonstrando interferências na regulação da expressão da GFAP. A expressão de vimentina não foi significativamente alterada em nenhuma das concentrações testadas. Estes resultados sugerem que os alcalóides da *P. juliflora* induzem a reatividade astrocitária, o que pode estar envolvido nos efeitos neurotóxicos providos pelo consumo desta planta.

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