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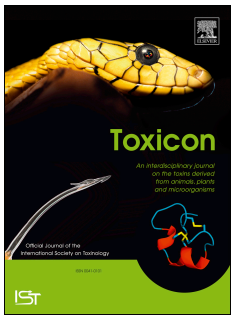

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Cytotoxic activity induced by the alkaloid extract from *Ipomoea carnea* on primary murine mixed glial cultures

Luciana Andrea Cholich[a,e*], María Elena Pistán[a,e], Ana María Torres[b], Hugo Héctor Ortega[c,e], Dale R. Gardner[d], Soledad Bustillo[f]

[a] *Faculty of Veterinary Science, National University of the Northeast, Corrientes, Argentina.*

[b] *Natural Products Laboratory, IQUIBA-NEA CONICET, National University of the Northeast, Corrientes, Argentina.*

[c] *Institute of Veterinary Sciences of Litoral (ICIVET). National University of Litoral (UNL), Esperanza, Santa Fe, Argentina.*

[d] *USDA-ARS Poisonous Plant Research Laboratory, Logan, Utah, USA*

[e] *The National Scientific and Technical Research Council (CONICET), Argentina.*

[f] *Biological and Molecular Investigations Group (GIBYM), IQUIBA-NEA CONICET, National University of the Northeast, Corrientes, Argentina.*

**lucianaandreacholich@gmail.com*

Abstract

The prolonged consumption of *Ipomoea carnea* produces neurologic symptoms in animals and a typical histological lesion, cytoplasmic vacuolization, especially in neurons. The toxic principles of *I. carnea* are the alkaloids swainsonine and calystegines B_1, B_2, B_3 and C_1. In this study, primary brain cultures from newborn mouse containing mixed glial cells were utilized. These cells were exposed **to *Ipomoea* extracts containing between 0 and 250 µM swainsonine for 48 h.** Morphological changes were investigated through Phase Contrast microscopy and Rosenfeld's staining. The extract induced cytoplasmic vacuolization in astrocytes and microglia in a dose dependent manner, being more evident when cultures were exposed to 250 µM of swainsonine. In addition, acridine orange staining evidenced an increase in the number of lysosomes in both microglia and astrocytes cells. Consistent with this, scanning electron microscopy also showed that both types of cells **presented morphological** characteristics of cell activation. Ultrastructurally, cells showed vacuoles filled with amorphous material and surrounded by a single membrane and also multilayer membranes. Taken together, these findings suggest that swainsonine **along with calystegines, are probably responsible for the** activation of glial cells due to a possible lysosomal dysfunction and therefore intracellular storage. Our results demonstrate that this *in vitro* glial cell model is a very good alternative to *in vivo* studies that require several weeks of animal intoxication to observe **similar** neurotoxic effects.

Keywords: Swainsonine; *Ipomoea carnea;* astrocytes; microglia; *in vitro*; cytotoxicity; lysosomal storage disease.

1. Introduction

Ipomoea carnea is found in subtropical and tropical regions of the world, **such as Brazil, Sudan, India, and Mozambique** (Idris et al. 1973; Tartour et al. 1973; Tirkey et al. 1987; de Balogh et al. 1999; Armién et al. 2007). In South America, *I. carnea* naturally grows from Venezuela and Colombia to Argentina. In Argentina, it grows spontaneously in northeastern and central provinces. This area corresponds to the "Chaco Húmedo" ecoregion, which is characterized by a wet and hot climate, with annual rainfall of 1275 mm and average temperatures between 22°C and 33°C in summer and between 12°C and 22°C in winter (Austin 1977; Chiarini and Ariza Espinar 2006).

The prolonged consumption of *I. carnea* produces progressive weight loss and clinical signs related to a nervous disorder, characterized by tremors of head and neck, abnormalities of gait, difficulty in standing, ataxia and wide-based stance. Histological lesions are mainly characterized by vacuolation of different cells, especially neurons of the central nervous system (CNS) (Armién et al. 2007).

I. carnea contains swainsonine, an indolizidine alkaloid, which inhibits the lysosomal α-mannosidase enzyme and Golgi mannosidase II **resulting in altered oligosaccharide degradation and incomplete glycoprotein processing (Elbein 1989;** Malm and Nilssen 2008). This occurs because swainsonine has many similarities to the simple sugar mannose, which it appears to mimic (Colegate et al. 1979; Dorling et al. 1978). The plant also contains calystegines, which are hydroxylated nortropane alkaloids and potent glycosidase inhibitors (Asano et al. 1995; de Balogh et al. 1999). **There is some controversy in the recent literature regarding the participation of these compounds, on the one hand Nunes et al. (2019) propose that calystegines do not contribute to the toxicity of *I. carnea* in goats, but another study evidenced neurological syndrome in these animals associated with the consumption of *I. trifida* and *I. carnea* containing only calystegines, suggesting that swainsonine is not the only toxin involved in these poisonings (Salinas et al. 2019).**

Different animal models have been used to study the experimental poisoning triggered by swainsonine-containing plants. Mice, rats and rabbits were used to study the *in vivo* effects of *Astragalus* sp. (Stegelmeier et al. 1995, **2008**), *Ipomoea carnea* (Hueza et al. 2005), and *Oxytropis* sp. (Li Q et al. 2012 a). Nevertheless, these animals may not be the best options as models for research on swainsonine neurotoxicity. In this sense, **rats and** mice exhibited slight neuronal vacuolization but only with very high doses of swainsonine (Stegelmeier et al. 1995, 2008) and rabbits intoxicated with *Oxytropis* sp. showed only severe microvacuolation of the cerebrum and cerebellum (Li Q et al. 2012 a). On the other hand, guinea pigs were used to study the neuronal intoxication caused by *Swainsona galegifolia* and *I. carnea* exhibiting many of the characteristics found in naturally intoxicated livestock (Cholich et al. 2009, 2013; Huxtable 1969; Huxtable and Gibson 1970).

In natural and experimental intoxication by *I. carnea* , poisoned animals show initially the vacuolation and necrosis confined to the neurons, but **with a longer exposure,** both glia and neurons were affected (Van Kampen and James 1972) with minimal to moderate gliosis (Armién et al. 2007; Nunes et al., 2019). However, the development of these effects *in vivo* requires several weeks of continuous ingestion of toxic plants (Obeidat et al. 2005).

In vitro studies have shown that swainsonine has a direct action in CNS neurons, causing cytotoxicity **in** dopaminergic cells (Li Q et al. 2012 b). However, the contribution of **glial cells** to the neuropathology in this type of poisoning are not yet studied.

It is well known that glial cells are essential to development, maintenance of homeostasis of neurons, forming myelin and detoxification in the CNS (Tanti et al. 2019). Primary glial cell cultures is the most commonly used *in vitro* model for neurobiological studies (Chen et al. 2013; Saura, 2007).

Thus, the aim of this study was to characterize the *in vitro* cytotoxicity and morphological alterations induced by an alkaloid extract from *Ipomea carnea* using a mixed glial cell model.

2. Materials and Methods

2.1 Plant Materials.

Leaf samples of *Ipomoea carnea* were collected from the cultivated plants at the Faculty of Veterinary Sciences, National University of the Northeast, Corrientes, Argentina, in February 2019. A voucher herbarium specimen was deposited in the Institute of Botany, Faculty of Agricultural Sciences (UNNE-CONICET) in Corrientes, Argentina, under number CTES-395. The leaves were dried at 37 °C to a constant weight and finely ground using a FW100 mill.

2.2 Alkaloid extract from *I. carnea.*

An extract of *I. carnea* that is enriched in the alkaloid components of the plant was obtained according to the method described by Hueza et al. 2003. Briefly, the dry leaf sample (200 g) was macerated in 96% ethanol (2 L). After total solvent evaporation under reduced pressure at 50ºC, a dark green extract was obtained, which was suspended in water to remove the waxy residue and consecutively extracted with diethyl ether, ethyl acetate and finally n-butanol Merck (Darmstadt, Germany) saturated with water. The aqueous solution was lyophilized to yield what we call the alkaloid extract (5.7 g).

2.3 Determination of swainsonine concentration in the extract.

A portion of the alkaloid extract was dissolved in 1.0 mL of water and allowed to mix for 16 h, transferred to a tared 7 mL glass vial using methanol for rinsing and transfer. The sample evaporated to dryness under a flow of nitrogen at 60°C, and after cooling to ambient temperature, the vial weighed to determine the weight of the extract. The sample was then dissolved in 5.0 mL of water and 0.050 mL aliquot

was added to 0.950 mL of 20 mM ammonia acetate in a 1.5 mL autosample vial and analyzed by high-performance liquid chromatography and mass spectrometry (HPLC-MS/MS) for quantitative measurement of swainsonine using previously described methods (Gardner et al. 2001). All swainsonine concentrations used in this work were calculated based on the proportion of this alkaloid in the extract of *I. carnea.* Additionally, calystegines (B_3, B_1, B_2 and C_1) were also identified but concentrations of these alkaloids were not determined.

2.4 Primary Mix Glial Cell Culture

Primary glial cell cultures were prepared from 1 - 3 day old neonatal mice (CF-1). Briefly, forebrains free of meninges from five mice (n=5) were removed aseptically and mechanically dissociated by repeated pipetting. Dissociated cells were plated onto poly-D-lysine-coated 25 cm^2 flasks or 24-well plates for experiments using a micro-full medium: Dulbecco's minimum essential medium (DMEM-F12) supplemented with fetal bovine serum (FBS 10%), (Natocor), non-essential amino acids (MEM NEAA 100X-1%), L-Glutamine (1%), **Gentamicin** (10 µg/mL) and Penicillin–Streptomycin (1%). Cells were incubated at 37°C and 5% CO_2, medium was replaced every 5 days and confluency was achieved after 18 ± 3 days *in vitro* (DIV). Unless stated otherwise, reagents were purchased from Gibco (Buenos Aires, Argentina), or Sigma-Aldrich (St. Louis, MO, USA). These studies were approved by the Comité de Bioética de la Facultad de Ciencias Veterinarias-UNNE, Argentina, (Protocol number 0069/2016).

2.5 Immunocytochemistry

Immunocytochemical analysis were performed using ionized calcium-binding adaptor molecule 1 (Iba1) as a marker of microglia and glial fibrillary acidic protein (GFAP) as a marker of astrocytes to check for the purity of the isolated glia. Cells were grown on coverslips at 37°C and 5% CO_2. After 21 days, monolayers were fixed with methanol for 5 minutes, followed by permeabilization with 0.1% Triton X-100 for 5 min. Endogenous peroxidase activity was inhibited by immersion in a peroxidase blocking solution (0.5% v/v hydrogen peroxide/methanol for 10 min) and then rinsed with PBS. To block nonspecific binding, coverslips were incubated in 3% skim milk powder for 15 min and rinsed in tap water. Afterward, cells were incubated with rabbit polyclonal anti-iba-1 (1/250, Abcam, Tecnolab) or mouse monoclonal anti-GFAP (1/500, eBioscience, Invitrogen) overnight in a humidified chamber at 4° C. After being washed with PBS, cells were incubated 30 min at room temperature with super enhancer (Super Sensitive ™ Link Detection System, BioGenex, CA) and another 30 min incubation with polymer-HRP (Super Sensitive™ Label HRP Detection System, BioGenex). Immunostaining was finally developed with DAB (3,3' diaminobenzidine tetrahydrochloride), immersed in de-ionized water to stop the reaction, counterstained with hematoxylin, dehydrated and coverslipped. According to Saura 2007, the use of the term "mixed glial culture" is more

appropriate when microglia content are >10%. Thus, the total number of Iba-1 labeled cells detected at 40X magnification in 10 randomized representative fields was determined.

2.6 Cytotoxicity of alkaloid extract from *I. carnea*

After 18±3 days *in vitro*, glial cells were harvested from subconfluent monolayers after exposure to 0.25 % trypsin/EDTA (1X) (Gibco) at 37°C. The resuspended cells were seeded in 96-well microplates at an approximate initial density of 40×10^3cells per well, in growth medium (DMEM-F12, 10% FBS). When monolayers reached confluence, samples of variable amounts of swainsonine (0, 30, 60, 100 and 250 µM, according to the previously defined proportion by HPLC-MS/MS), diluted in assay medium supplemented with 10% of FBS were added to cells in a total volume of 200 µL/well. After 48 h of incubation, cell viability was quantified by crystal violet staining according to Yamamoto et al. (2001). Briefly, non-adherent cells were removed by washing twice with phosphate-buffered saline (PBS) and adherent cells were fixed with methanol:glacial acetic acid (3:1 ratio), stained with 0.5% crystal violet in 20% (v/v) methanol. The dye was released from the cells by addition of ethanol: glacial acetic acid (3:1 ratio). The optical density of the released dye solution was determined at 620 nm. The percentage of cell viability was determined by comparing the resulting absorbances (620 nm) with the mean absorbance of the control wells (without AE, considered as 100% viability). An additional assay was performed to assess cytolysis determining the release of the cytosolic enzyme lactic dehydrogenase (LDH), as described previously (Lomonte et al. 1994, 1999). Aliquots of the supernatant in culture wells were collected, and LDH activity was determined by using a commercial kit (Wiener, LDH-P UV). Cytotoxic activity was expressed as percentage of LDH release to the medium. Reference controls for 0% and 100% cytolysis consisted of medium alone and medium from cells incubated with 0.1% (v/v) of Triton X-100, respectively. All assays were carried out in triplicates.

2.7 Determination of morphological changes induced by alkaloid extract from *I. carnea.*

Cells were grown on coverslips and treated with 30, 60 and 250 µM of swainsonine from alkaloid extract for 48 h at 37°C and 5% CO_2. After exposure, morphological changes were evaluated methods by described below.

2.7.1 Phase Contrast microscopy

The glial cell morphological changes were investigated qualitatively using a phase contrast microscope (Axiovert 40®, Carl Zeiss Argentina). The photos were taken with a digital camera (Canon CCD 2272×1704, Argentina) before and after treatments.

2.7.2 Rosenfeld's staining

Morphological changes and vacuolization were assessed by analysis of Rosenfeld's staining (Rosenfeld 1947). After incubation, cells were rinsed three times with PBS and fixed for 10 min with methanol at −20 °C. Fixed cells were stained with Rosenfeld's reagent (1 mL) and incubated for 20 min. at

room temperature. Afterward coverslips were rinsed with water, air-dried, analysed in an optic microscope (Primo Star Zeiss), and photographed using a digital camera (AxiocamERc 5s Zeiss).

2.7.3 Fluorescence staining

In order to determine whether the alkaloid extract of *I. carnea* affect lysosome viability in glial cell primary culture, acridine orange (AO) fluorescence staining was made. AO is a metachromatic dye that is capable of staining nucleic acids (DNA, RNA), as well as lysosomes (Kobayashi et al. 1999). After exposure for 48 h treated cultured cells on coverslips were washed twice with PBS and gently mixed with AO (1 µg/mL) for one minute as described by Spector et al. 1997. Coverslips were applied to the slides; afterwards, the sections were observed and photographed under a fluorescence microscope (Axioskop 40®/Axioskop 40 FL®, Carl Zeiss, Argentina).

2.7.4 Scanning electronic microscopy (SEM)

Coverslips from culture cells were fixed for 1 h with ethanol 70% at room temperature and then incubated overnight at 4˚C. After complete dehydration in graded series of alcohol, coverslips suspended in 100% ethanol were air-dried. They were exposed to gold-palladium coating for 3 min. Samples were observed under a scanning electronic microscope (JeoL JSM-5800 LV) and images (original magnification 500x and 2000X) were obtained at different time points of the experimental study.

2.7.5 Transmission electron microscopy (TEM)

Immediately after treatment, the culture medium was removed, and the cell monolayer was fixed in 2% glutaraldehyde in a 0.1 M phosphate buffer (pH 7.2-7.4) for 1 h at 4° C. Then centrifuged at 1,000 rpm for 5 min and the resulting pellet was washed in buffer, post-fixed in osmium tetroxide and embedded in epoxy resin. Semi-thin sections (1 µm thick) were stained with 1% toluidine blue in 1% borax. Ultra-thin sections (60–80 nm) of selected areas were stained with 2% uranyl acetate and lead citrate and examined with a JEOL EM 1200EX II, Tokyo, Japan TEM.

3. Results

3.1 Determination of swainsonine concentration in the extract.

The alkaloid extract was determined to have a swainsonine concentration of 2.83 mg/g. *Ipomoea carnea* typically contains 0.03% swainsonine (dry weight), so the alkaloid extract was approximately 10 times more concentrated than the dry plant material.

3.2 Primary Mix Glial Cell Culture. Immunocytochemistry

Morphological assessment at 21 DIV by phase-contrast microscope showed that cells were homogeneously distributed on cultured field. Microglial cells were clearly visible on top of the astrocyte monolayer, as round refringent cells, and even occupied spare gaps in the culture substrate. Cells showed distinct morphologies, involving fusiform shape, with no visible extensions, to small cells, with numerous ramified extensions (Fig. 1). After establishing mixed glial cell cultures, microglia were identified by immunocytochemistry for Iba1 (Fig. 2A) and astrocytes with its cell specific marker (GFAP) (**Fig. 2B**) demonstrating minimal contamination. Immunocytochemistry analyses revealed a microglia content of **25±1.80 %** in cultures.

3.3 Cytotoxicity of alkaloid extract

Cristal violet staining assay was performed to assess the cytotoxicity of alkaloid extract from *I. carnea* leaves against primary mix glial cells. All swainsonine concentrations tested (30 to 250 µM) did not show any cytotoxic activity after 48h incubation. In addition, not significant release of cytoplasmic lactic dehydrogenase (LDH) was observed, indicating that there was no disruption of cell membranes (data no shown).

3.4 Morphological changes induced by alkaloid extract from *I. carnea*:

3.4.1 Phase Contrast microscopy

To assess alterations of cell morphology subsequent to treatments, glial cells were grown on glass coverslips. Untreated glial cells were distributed on cultured field; they exhibited a normal morphology as shown in detail in Fig. 1. However, after 48 h of incubation with different doses of swainsonine from alkaloid extract, a dose-dependent cytoplasmic vacuolation was observed. This effect was moderate in both astrocytes and microglia exposed to 60 µM of swainsonine, but when the cells were treated with the maximum dose (250 µM), the observed vacuolization was much more severe (Fig. 3).

3.4.2 Rosenfeld's staining

The morphological changes on glial cells were confirmed by microscopy after staining the cells with Rosenfeld's dye (Fig. 4). Under control conditions, astrocytes formed a monolayer and the microglia presented different morphology in spare gaps in the culture substrate.

Exposure of the culture to the lowest dose (30 µM) did not show significant changes with respect to the control. However, incubation of glial cells with 60 µM of swainsonine induced significant morphological changes like small vacuoles, which gave the cytoplasm a foamy appearance. This effect was severe in both microglia and astrocytes when cells were treated with 250 µM of swainsonine, were large vacuoles occupied most of the total cell volume.

230

231 3.4.3 Fluorescence staining

232 In order to determine whether swainsonine from alkaloid extract induced morphological alterations, mix
233 glial cells were stained with the nucleic acid-binding fluorochrome, acridine orange (AO). Control untreated
234 cells showed that astrocytes exhibited a green fluorescence, a light green nucleus with intact structure and
235 presented some punctuate orange red fluorescence in the cytoplasm homogenously distributed, representing
236 lysosomes. Microglial cells were clearly visible on top of the monolayer of astrocytes and, because of the far
237 higher number of lysosomes present in these cells; the organelle staining is far more intense (Fig. 5. Control).
238 The total number of lysosomes increased in direct proportion to the dose tested. With the lower concentration
239 of swainsonine, a slight increase of these organelles was evidenced mainly in astrocytes (Fig. 5. 30 μM). After
240 treatment with 60 μM and 250 μM of swainsonine, the number of lysosomes augmented in both microglia and
241 astrocytes, being more evident at the highest dose assayed (Fig. 5. 60 and 250 μM).

242

243 3.4.4 Scanning electron microscopy (SEM)

244 To assess the swainsonine-induced change in cell morphology, cells were examined by scanning
245 electron microscopy. As shown in Fig. 6A, the majority of cultured cells were microglia and astrocytes glial
246 cells. Microglial cells appeared as smooth cells with few spines while astrocytes showed a smooth upper
247 surface and typically hexagonal shape. Significant changes were evident from exposing the cells to 60 μM of
248 swainsonine, showing predominantly microglia a marked increase in cell size (Fig. 6B). Both cells types
249 showed features of activated cells with the highest concentration tested (250 μM of swainsonine) (Fig. 6C-D).

250 3.4.5 Transmission electron microscopy (TEM)

251 Transmission electron microscopy of primary glial cells treated with alkaloid extract for 48 h, revealed a
252 massive cytoplasmic vacuolization. The vacuoles were filled with amorphous or electron-dense material and
253 were surrounded by a single membrane. Additionally, multilayer membranes observed suggests autophagy
254 vacuoles formation (Fig. 7).

255 4. **Discussion**
256

257 Poisoning with this plant induces a lysosomal storage disease in which α-mannosidase is inhibited,
258 resulting in cellular dysfunction. As mentioned previously, swainsonine is one of the active principles of *I.*
259 *carnea*. It is a small molecule that is easily absorbed from the digestive tract of animals and therefore rapidly
260 distributed throughout all tissues. Poisoned animals initially show vacuolization and necrosis confined to
261 neurons, but with a longer exposition, both glia and neurons are affected (Van Kampen and James 1972).
262 Despite this, the role of glial cells in this type of intoxication is poorly documented and understood (Armién et
263 al., 2007). As the toxicological properties of *I. carnea* are attributed primarily to swainsonine (Armién et al.,

2007; Nunes et al., 2019), we investigated the cytotoxic effects of an extract containing this alkaloid on a mix glial cell primary culture.

Results obtained in this study demonstrated that the alkaloid extract from *I. carnea* did not decrease cell viability at doses of swainsonine or time assayed. Furthermore, it did not induce cytolysis according to the negative result obtained for lactate dehydrogenase (LDH) activity. In contrast, Li Q et al. (2012 b) demonstrated toxic changes in dopaminergic neurons including membrane compromise with 10 times lower concentrations of commercial swainsonine. Similarly, previous studies carried out with the alkaloid extract from another neurotoxic plant, *Prosopis juliflora*, evidenced a higher cytotoxic effect in cortical neuron cultures in comparison with glial cell cultures (Silva et al., 2013). Therefore, it is suggested that glial cells might be less susceptible than neurons to swainsonine effects.

In vivo, the main cellular morphological change observed in **goats** is the cytoplasmic vacuolization of neurons, in association with astrogliosis, axonal degeneration and necrosis (de Balogh et al., 1999; Armién et al., 2007). These lesions are mainly found in Purkinje cells and neurons of the granular cell layers of the cerebellum and basal ganglia (Nunes et al., 2019). In Argentina, intoxication of guinea pigs with *I.* carnea demonstrated by histopathological analysis the presence of vacuoles throughout the tissues (Cholich et al. 2009; García et al. 2015), particularly evident in neurons of different brain regions (Cholich et al. 2013).

It is important to note that in animals poisoned by swainsonine-containing plants, glial changes are minimal to moderate and have historically been considered as secondary to neuronal degeneration (Stegelmeier et al., 1995; Armien et al., 2007, Nunes et al., 2019).

To date, the neurotoxic mechanisms *in vitro* of swainsonine are still unclear and have been poorly investigated. Li Q et al. (2012 b) reported *in vitro* structural degeneration in dopaminergic neurons exposed to commercial swainsonine scored as dendritic shortening, shrinkage of cell bodies and minor cytoplasm vacuolization. In addition, a primary culture of cortical neurons served as a model system to study swainsonine toxicity were authors observed that the treated cell bodies shrunk and the axons shortened (Pang et al. (2012).

In this study, and in agreement with results obtained *in vivo*, concentrations above 60 µM of swainsonine from *I. carnea* alkaloid extract induced an important dose-dependent cytoplasmic vacuolization of glial cells observed by phase contrast microscopy and Rosenfeld staining techniques.

Activation of glial cells is prevalent in the brain of patients with lysosomal storage disease, which directly contributes to CNS pathology (Vitner et al. 2010). Microglia, the brain resident macrophages, have revealed to possess a higher number of lysosomes than astrocytes due to their roles in immune response and phagocytosis (Lovelace et al. 2007). In accordance with this, morphological analysis with the fluorochrome acridine orange (AO) showed that microglia display very intense granular orange-red staining whilst astrocytes showed a variable number of lysosomes that was substantially lower than microglia. After swainsonine exposure, both cells evidenced a higher number of lysosomes, indicative of a possible glial

activation. Moreover, AO staining revealed the fusion of adjacent lysosomes. In this sense, lysosomes play a fundamental role in the autophagic pathway by fusing with autophagosomes and digesting their content. Considering the highly integrated function of lysosomes and autophagosomes it was reasonable to expect that lysosomal storage in lysosomal storage diseases (LSDs) would have an impact upon autophagy.

Likewise, Wang et al. (2019) demonstrated that swainsonine induced autophagy by increasing the levels of Beclin1 and LC3-II in renal tubular epithelial cell lines. Other researchers suggest that swainsonine induces apoptosis in neurons *in vivo* and *in vitro* mediated by caspase-dependent pathway (Lu et al., 2013; Lu et al., 2015). However, xenobiotic compounds can cause both apoptosis and autophagy and the two processes are interconnected (Roy et al., 2014; Saiki et al., 2011). More studies should be conducted to determine the neurotoxic mechanism triggered by *I. carnea* alkaloid extract on glial cells.

The results of the scanning electron microscopy also showed that both microglia and astrocytes evidenced characteristics of cell activation. This is in agreement with other studies where minimal to moderate gliosis in poisoned animals by swainsonine-containing plants was observed (Armién et al. 2007; Cholich et al. 2013; Nunes et al., 2019).

Ultrastructural alterations of nervous cells are a typical feature of lysosomal storage disorders (Huxtable and Gibson, 1970; Armién et al. 2007). In this study, transmission electron microscopy demonstrated lysosomal storage in glial cells treated with the alkaloid extract and the consequent accumulation of cytosolic substrates due to this lysosomal dysfunction. Similarly, Wang et al. (2019) found that renal tubular epithelial cells treated *in vitro* with swainsonine showed significant accumulation of autophagic vacuoles in the cytosol.

In conclusion, this is the first report of the morphologic and ultrastructural changes induced by the alkaloid extract of *Ipomoea carnea* leaves on mixed glial primary cultures. This *in vitro* cell model is a very good alternative to *in vivo* studies that require several weeks of animal intoxication to observe **similar** neurotoxic effects.

Our findings suggest that swainsonine, along with other chemical components present in the alkaloid extract like calystegines, act directly on the activation of glial cells due to a possible lysosomal dysfunction. Future molecular studies may further clarify the role of swainsonine in the autophagy process triggered in glial cells.

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Figure captions

Fig. 1 Representative image of mixed glial cells at 21 DIV after the establishment of the mixed glia culture. A monolayer of astrocytes is observed with some refringent microglia-looking cells (black arrows) and in spare gaps on the culture substratum (white arrows). Phase contrast microscopy. Original magnification: × 200.

Fig. 2 Immunocytochemical analysis of primary mixed glial cells in culture. **A.** Microglia labeled with an antibody to microglial markers Iba-1 (brown). **B.** Astrocytes labeled with an antibody to intermediate filament glial fibrillary acidic protein (GFAP) (brown), counterstained with hematoxylin (blue). Original magnification: × 400.

Fig. 3 Morphological analysis by Phase Contrast Microscopy of primary mixed glial cells in culture after treatment with different doses of swainsonine from alkaloid extract for 48 h. Numerous cytoplasm vacuoles were observed in cells treated with 60 µM and 250 µM . Phase contrast microscopy. Original magnification: × 400.

Fig. 4 Evaluation of morphological changes of glial cells with Rosenfeld's staining after 48 h exposure to 60 and 250 µM of swainsonine. Larger number and size vacuoles (arrows) were observed in the cytoplasm of cells incubated with 250 µM of swainsonine. Original magnification: × 400.

Fig. 5 Acridine Orange fluorescence staining of control mixed glial cells reveals a homogenous distribution of dye throughout the cells (green background), specific orange granular AO staining of lysosomes is shown throughout all astrocytes. Microglial cells (white arrows) are clearly visible on top of the monolayer of astrocytes, because of the far higher number of lysosomes present (control and 30 µM of swainsonine). After

493 exposure to 60 - 250 µM of swainsonine a higher number of orange lysosomes was observed. Original
494 magnification: × 400.

495 **Fig. 6** Scanning electron micrographs of glial cells. **A.** Control: astroglial layer and microglia as spherical
496 smooth cells (Scale bars 12 µm, x 300). **B.** Microglia-like macrophages in presence of 60 µM of swainsonine
497 (Scale bars 15 µm, x 250). **C.** Microglia in control cultures evidenced short spinous processes (Scale bars 5
498 µm, x 1500). **D.** Activated microglia in treated cultures with numerous delicate spinous processes (Scale bars
499 4 µm, x 1000).

500 **Fig. 7** Transmission electron microscopy of glial cells. **A.** Electron micrograph of a control cell (Scale bars 1
501 µm). **B.** Cells treated with 250 µM of swainsonine, large vacuoles (V) and autophagic vacuoles (arrow) were
502 observed in the cytoplasm of glial cells (Scale bars 2 µm).

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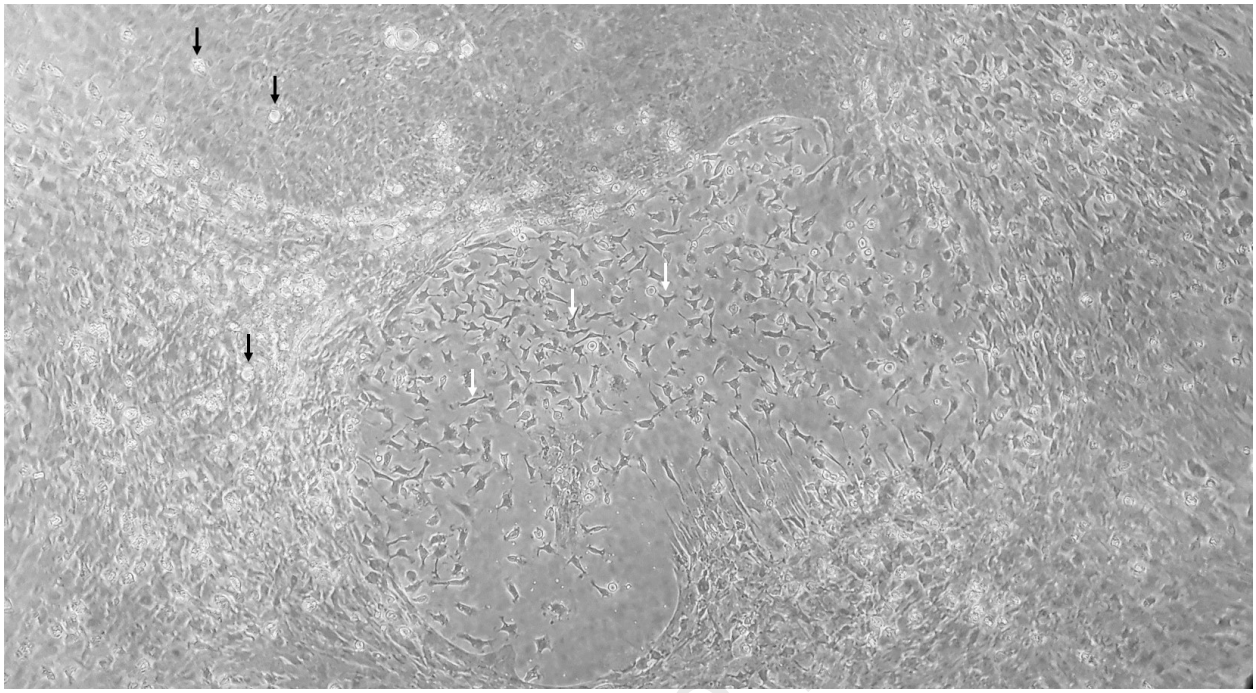

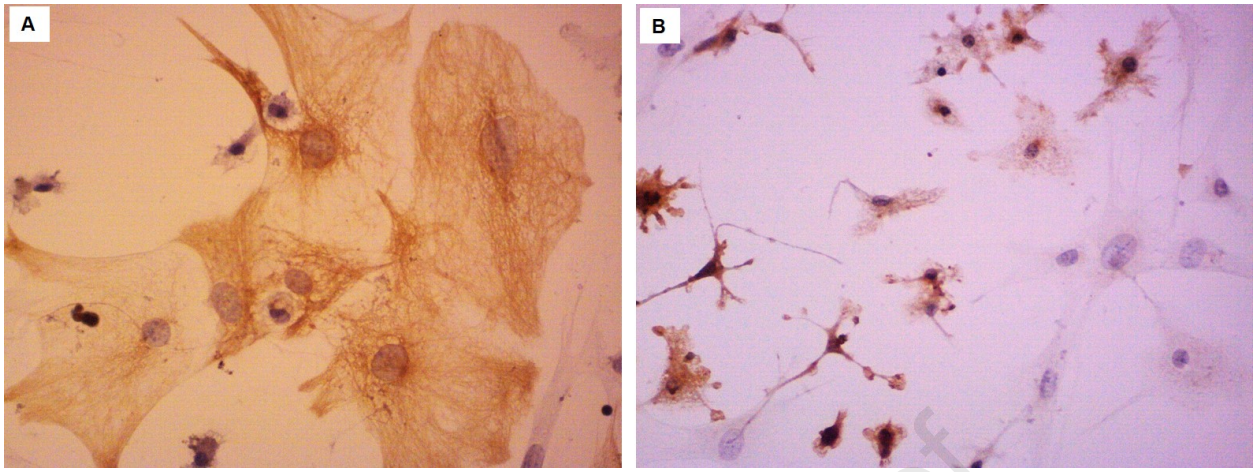

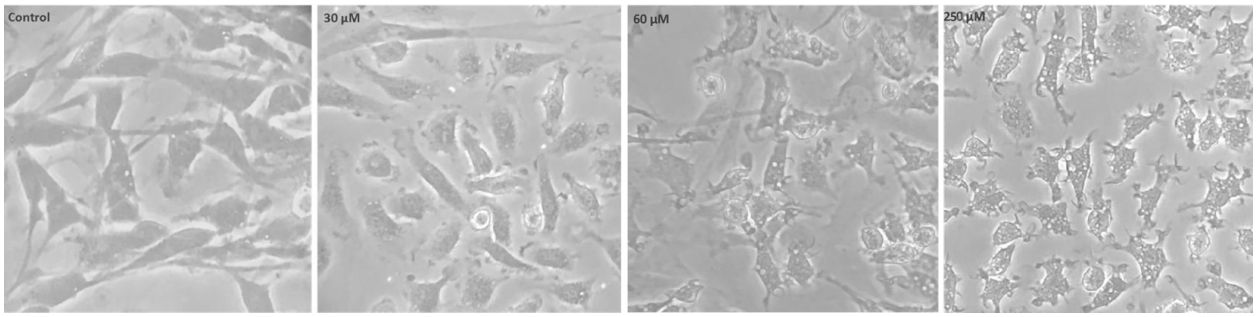

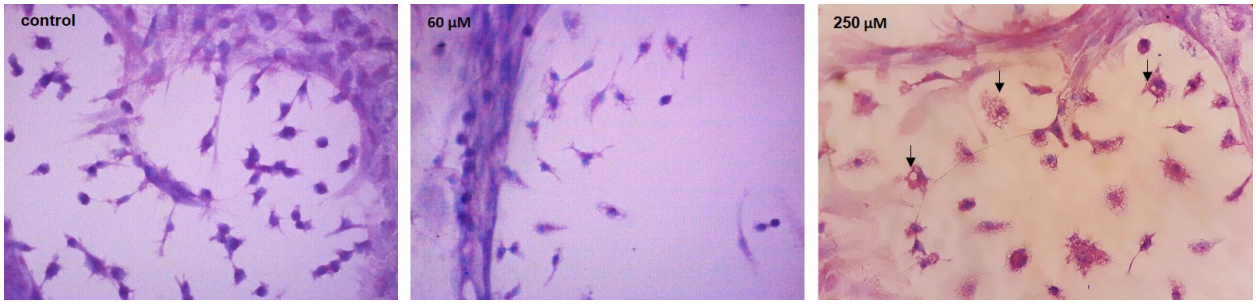

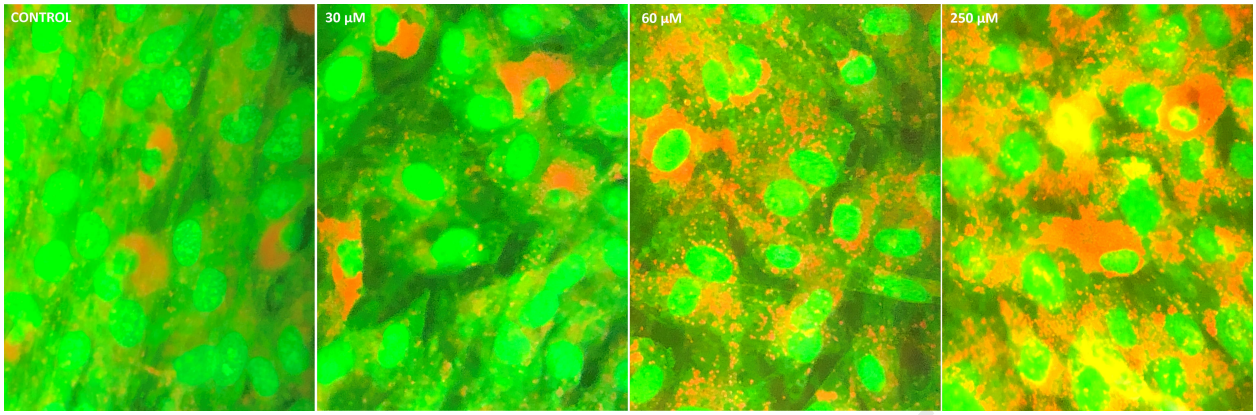

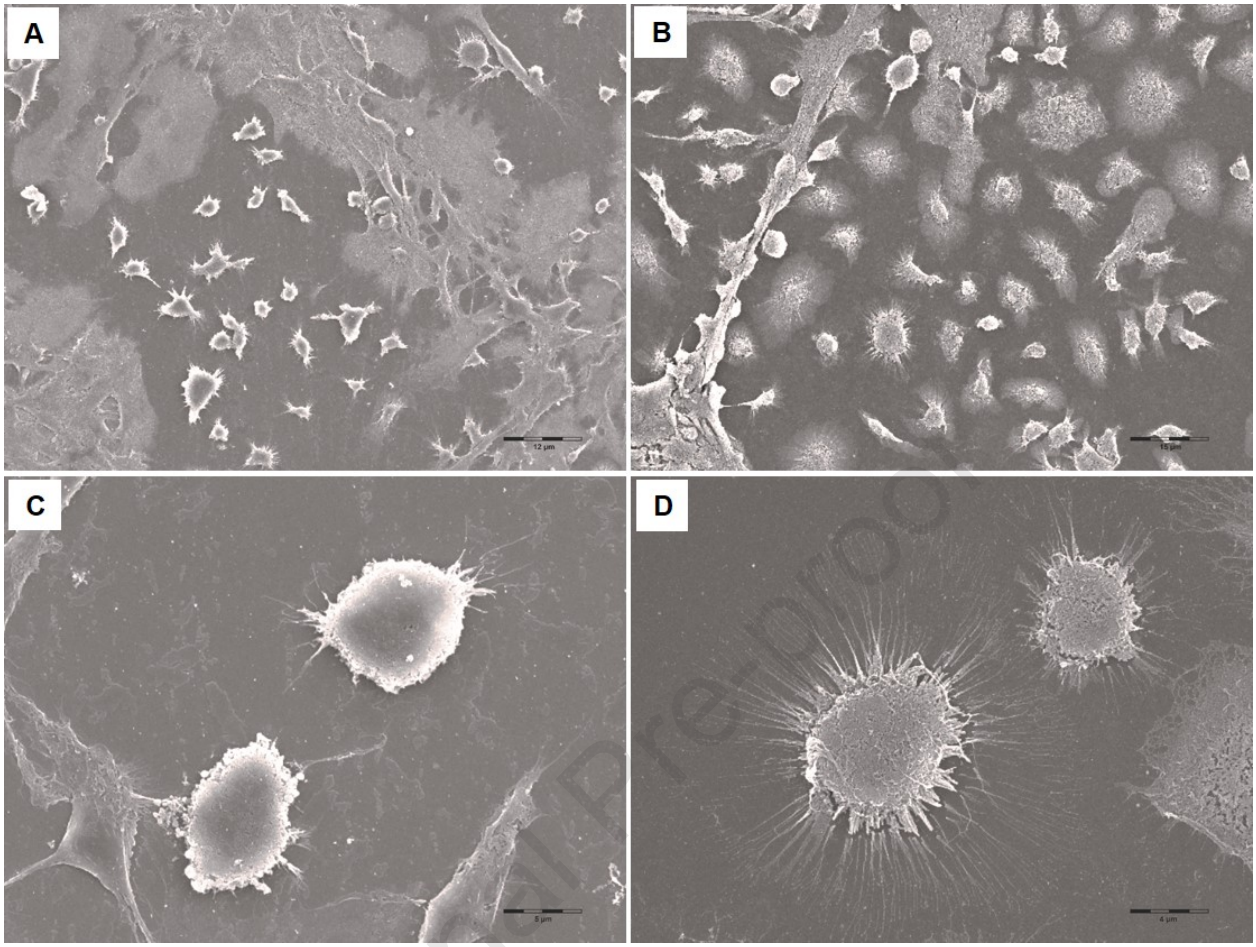

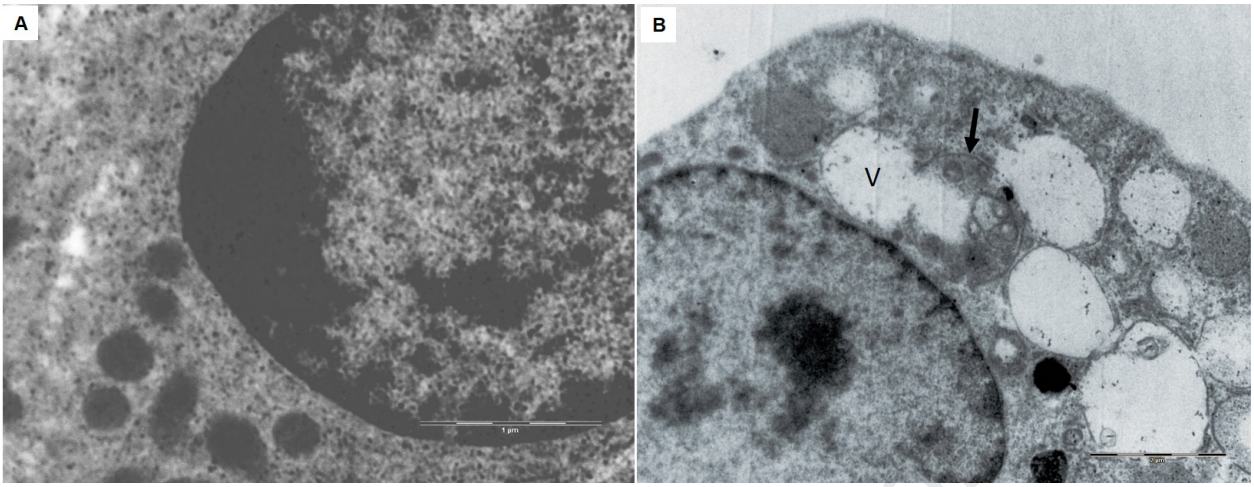

Highlights

- *Ipomoea carnea* alkaloid extract induced morphological and ultrastructural changes **in murine mixed glial primary cultures**
- Alkaloid extract induced cytoplasmic vacuolization and autophagy vacuoles formation due to possible lysosomal dysfunction
- Swainsonine **and calystegines are** mainly responsible **for** the activation of glial cells due to possible lysosomal dysfunction
- ***In vitro* cell model that does not require several weeks of animal intoxication to obtain similar neurotoxic effects**

Ethical Statement

The present study not includes plagiarism, forgery, use or presentation of other researcher's works as one's own, fabrication of data. The manuscript has been reviewed and approved by all authors.

PhD Luciana Andrea Cholich

Universidad Nacional del Nordeste.
Address: Sargento Cabral Nº 2139.
Tel: +54-03783-425753-146
e-mail: lucianaandreacholich@gmail.com

Declaration of interests

☒ The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

☐The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: