Revista de Biología Tropical, ISSN: 2215-2075, Vol. 73: e2025225, enero-diciembre 2025 (Publicado Nov. 03, 2025)

OTRO

REVISTA DE
Biología Tropical

https://doi.org/10.15517/3jcswj50

Alkaloid-enriched extracts from the tree *Neltuma flexuosa* (Fabaceae): chemical characterization, fractionation, and cytotoxic effects

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Received 07-VII-2025. Corrected 01-VIII-2025. Accepted 22-X-2025.

ABSTRACT

Introduction: *Neltuma flexuosa* is a key species in arid regions, offering nutritional benefits but also containing alkaloids that can pose toxicological risks.

Objective: To analyze the chemical composition and alkaloid content of *N. flexuosa* pods collected over four years (2019-2022) in Cafayate, Argentina, considering the influence of climatic variations, and to evaluate their potential cytotoxic effects on C6 glioma cells.

Methods: Dried and ground pods were analyzed for chemical composition using standard methods. Alkaloid-enriched extracts (AEE1-AEE4) were obtained through acid/base extraction and analyzed by HPLC-HRMS for juliprosine and juliprosopine quantification. AEE4 was fractionated using flash chromatography, yielding fractions (F1-F5), which were also analyzed for alkaloid content. Cytotoxicity on C6 glioma cell lines was evaluated using crystal violet staining assay, and apoptosis induction was confirmed in C6 cells treated with AEE4 using the TUNEL assay.

Results: Chemical analysis revealed interannual variations in pod composition, particularly in fiber and protein content. HPLC-HRMS identified juliprosine and juliprosopine, with the highest concentrations in AEE4. Fractionation of AEE4 by flash chromatography yielded an alkaloid-rich fraction (F4). Cytotoxicity assays demonstrated dose-dependent reductions in cell viability, with AEE4 exhibiting the lowest 50 % cytotoxic concentration (CC50) values. Furthermore, F4 displayed the highest cytotoxicity against C6 cells. TUNEL assays confirmed the induction of apoptosis by AEE4.

Conclusions: These findings provide valuable insights into the chemical composition, alkaloid content, and cytotoxic effects of *N. flexuosa* extracts, contributing to the development of guidelines for their safe and sustainable use as a feed resource.

Key words: cytotoxicity; food chemistry; piperidine alkaloids; rainfall; toxicology.

RESUMEN

Extractos enriquecidos con alcaloides del árbol *Neltuma flexuosa* (Fabaceae): caracterización química, fraccionamiento y efectos citotóxicos

Introducción: *Neltuma flexuosa* es una especie importante en regiones áridas, ofrece beneficios nutricionales, pero también contiene alcaloides que pueden presentar riesgos toxicológicos.
Objetivos: Analizar la composición química y el contenido de alcaloides de vainas de *N. flexuosa* recolectadas durante cuatro años (2019-2022) en Cafayate, Argentina, considerando la influencia de las variaciones climáticas y evaluar su potencial efecto citotóxico sobre células de glioma de la línea C6. Adicionalmente, evaluar los potenciales efectos citotóxicos de células de glioma C6.
Métodos: Las vainas secas y molidas, fueron analizadas para su composición química mediante métodos estándares. Los extractos enriquecidos en alcaloides (EEA1-EEA4) se obtuvieron mediante extracción ácido/base y se analizaron por HPLC-HRMS para cuantificar la juliprosina y la juliprosopina. El EEA4 se fraccionó mediante cromatografía flash, obteniéndose diferentes fracciones (F1-F5), que también se analizaron para determinar su contenido en alcaloides. Se evaluó la citotoxicidad de los extractos y fracciones a partir de la línea celular de glioma C6 mediante el ensayo de tinción con violeta cristal y se confirmó la inducción de apoptosis en las células C6 tratadas con EEA4 mediante el ensayo TUNEL.
Resultados: El análisis químico reveló variaciones interanuales en la composición de las vainas, particularmente en el contenido de fibra y proteína. El análisis por HPLC-HRMS identificó juliprosina y juliprosopina, con las mayores concentraciones en el EEA4. El fraccionamiento de EEA4 mediante cromatografía flash produjo una fracción rica en alcaloides (F4). Los ensayos de citotoxicidad demostraron disminución en la viabilidad celular dependiente de la dosis, exhibiendo, EEA4, los valores más bajos de concentración citotóxica del 50 % (CC50). Además, F4 mostró la mayor citotoxicidad contra las células C6. Los ensayos TUNEL confirmaron la inducción de apoptosis por EEA4.
Conclusiones: Estos resultados aportan información valiosa sobre la composición química, el contenido de alcaloides y los efectos citotóxicos de los extractos de *N. flexuosa*, contribuyendo al desarrollo de directrices para su uso seguro y sostenible como recurso alimenticio.

Palabras clave: citotoxicidad; composición química del alimento; alcaloides de piperidinicos; precipitación; toxicología.

INTRODUCTION

The genus *Neltuma* (*Prosopis*) belongs to the Fabaceae family and is well adapted to arid and semi-arid environments. In Argentina, *Neltuma flexuosa* plays a key ecological role in regions such as Chaco, Espinal Norte and Pampeana, where it is commonly known as “Algarrobos” (Hughes et al., 2022; Palacios & Brizuela, 2005). The pods of this genus serve as a valuable feed source for animals due to their nutritional content (Riet-Correa et al., 2012; Silva, 1981). They contain 9-17 % protein, 13-31 % sucrose, and 17-31 % crude fiber (González-Galán et al., 2008; Zolfaghari & Harden, 1985). Their protein content is reported to be comparable to that of major proteinaceous crops, such as cowpea, soybean, and pigeon pea (Abbiw, 1990; Sciammaro et al., 2015). However, they also contain antinutritional substances including oxalates, alkaloids, saponin and tannins (Anhwange et al., 2020).

In this sense, several studies have been conducted offering insights into their nutritional benefits and associated risks. In Africa, studies have highlighted the high protein content of *Neltuma africana* pods and their role in livestock diets, while also noting the presence of toxic compounds that pose risks to animal health (Okalebo et al., 2007). An Australian work has similarly assessed the impact of *Neltuma* spp. pods on the growth and health of grazing animals, showing that while they are a nutritious feed source, careful management of antinutritional factors is necessary (Kumar & Mathur, 2014). In Brazil, *Neltuma juliflora* has been extensively studied due to its widespread use and toxicity issues with documented cases of nervous disorders in cattle and other livestock (Lima et al., 2004; Silva et al., 2006; Tabosa et al., 2003). Various analytical methods, including spectroscopic techniques and biochemical assays, have been employed to assess toxic compound levels and their effects on animal health (Assis et al., 2009; Câmara et al., 2009). However, Riet-Correa et al. (2012) found no nervous signs or symptoms in animals consuming *N. juliflora* pods over an extended period, suggesting that toxicity may vary based on factors such as pod maturity, environmental conditions, and regional differences in plant chemistry.

In Argentina, outbreaks of nervous disease in cattle associated with the consumption of *Neltuma nigra* pods have been reported. Clinical symptoms include tongue protrusion, twitching and tremors of the masticatory muscles, weight loss, and lethargy (Micheloud et al., 2019). However, we later confirmed the presence of juliprosine and juliprosopine alkaloids in *N. flexuosa* but not in *N. nigra*, leading us to rule out *N. nigra* as the cause of spontaneous poisoning in animals (Cholich et al., 2021).

These issues underscore the need for a comprehensive understanding of the nutritional and toxicological properties of *N. flexuosa* pods. To address this critical gap, this study aims to analyze the chemical composition and alkaloid content of *N. flexuosa* pods collected over four consecutive years in Cafayate, Argentina, considering the influence of climatic variations, and to evaluate their potential cytotoxic and apoptotic effects on C6 glioma cells. This knowledge is crucial for optimizing the use of *N. flexuosa* pods as a complementary feed resource while mitigating potential health risks to livestock.

MATERIALS AND METHODS

Plant material: *N. flexuosa* subsp. *flexuosa* pods were collected from Cafayate, Salta Province, in Northwestern Argentina. A voucher herbarium specimen (CTES-1193) was identified by Lic. Walter Medina and deposited at the Instituto de Botánica, Facultad de Ciencias Agrarias (UNNE, CONICET) in Corrientes, Argentina. This region has an arid climate with a pronounced dry season. The average annual temperature is 17 °C, with summer highs reaching 35 °C and winter lows ranging between 5 °C and -15 °C. Rainfall is confined to the summer months, with an annual average of 207 mm occurring between November and March (Sampietro-Vattuone & Peña-Monné, 2016). The sampling area is located at an altitude of 1 683 m.a.s.l. (26°3’49.18” S, 65°56’52.99” W). Pods were collected over four years (2019, 2020, 2021 and 2022), and the annual rainfall and mean temperature for each year were recorded from the meteorological Station in Cafayate, National Institute for Agricultural Technology (INTA), Argentina (Table 1). The pods were then dried at 37 °C to a constant weight and pulverized in a mill.

Table 1
Annual precipitation and temperature for the study region (2019-2022). Climatic data were collected from a weather station in Cafayate, Salta.

Year	Mean temperature (°C)	Annual precipitation (mm)
2019	17.59	260.2
2020	17.65	264
2021	17.35	193
2022	17.7	130.4

Chemical analysis: The analytical determinations were carried out on pulverized pods of *N. flexuosa,* three independent samples were collected each year from representative locations within the study area. Dry matter (DM, method ID 934.01), ash (method ID 942.05), ether extract (EE, method ID 920.30) and crude protein (CP, method ID 984.13) were analyzed following AOAC methods (Cunniff, 1999).

Revista de Biología Tropical, ISSN: 2215-2075 Vol. 73: e2025225, enero-diciembre 2025 (Publicado Nov. 03, 2025)

Neutral detergent fiber (NDF), acid detergent fiber (ADF) and acid detergent lignin (ADL) were determined using an ANKOM fiber analyzer (Goering & Van-Soest, 1970). Total digestible nutrients (TND) were estimated using the equation: % TND = 92.51 – (% FDA X 0.7965) (Chalupa et al., 1996). Based on the TND results, digestible energy (DE) was estimated using the equation: DE (Mcal) = % TND × 4.409 Mcal / 100 (Ammar et al., 2004).

Alkaloid-enriched extracts (AEE) from *N. flexuosa*: Alkaloid-enriched extracts were obtained from 500 g of pulverized *N. flexuosa* pods using an acid/base extraction method, as described by Ott-Longoni et al. (1980). Briefly, dry pods were first defatted with hexane (1.5 l/Kg) for 10 days and then extracted with methanol (1.5 l/Kg) for seven days. The solution was filtered, and the methanolic extract was concentrated using a BÜCHI rotary evaporator under reduced pressure at 40 °C. The concentrate was then stirred with 0.2 N HCl (1:1 v/v) and kept at room temperature for 16 h. The resulting acidic solution was filtered, and non-basic impurities were removed by liquid-liquid extraction with chloroform. The aqueous phase was then adjusted to pH 11 with ammonium hydroxide and subsequently extracted with chloroform. This fraction was evaporated to dryness under reduced pressure, yielding a resinous, amber-colored alkaloid-enriched extract (AEE), which was stored at -20 °C. Extracts obtained from pods collected in 2019, 2020, 2021 and 2022 were designated as AEE1, AEE2, AEE3, and AEE4, respectively.

Identification and quantitation of alkaloids in *N. flexuosa* alkaloid-enriched extracts (AEE) and their fractions: The alkaloids present in each AEE, including fractions (F1-F5) obtained from AEE4, were identified and quantified by HPLC-HRMS, following the method described by Cholich et al. (2021). Analytical samples (1 mg) were dissolved in 100 µl of methanol. A 5 µl aliquot was then diluted with 995 µl of 50 % methanol containing 0.1 % formic acid. Juliprosine and juliprosopine were subsequently analyzed by HPLC-HRMS. Quantitation of these two alkaloids was determined by comparing the peak area ratios with those of a standard solution of juliprosine (m/z = 626.5619) and juliprosopine (m/z = 630.5929), prepared at a concentration of 1 µg/ml. The limits of detection for both alkaloids were 10 ng/ml in the final diluted solution analyzed. Juliprosine and juliprosopine (HPLC-MS purity > 92%) were kindly provided by Dr. Muhammad Ilias, National Center for Natural Products Research, University of Mississippi, USA. Identification was confirmed by matching retention times and exact m/z values with those of the analytical standards.

Fractionation of the alkaloid extract (AEE4) by flash chromatography: A total of 500 mg of AEE4, identified as the extract with the highest concentration of piperidine alkaloids, was dissolved in 96 % ethanol, mixed with silica gel, and dried under reduced pressure using a Büchi rotary evaporator to prepare the sample for fractionation. The dried sample was placed in a 24 × 400 mm column with a stopcock and ground glass stopper to maintain pressure. Flash chromatography was performed using silica gel flash 60 (0.04-0.063 mm, MN) with an elution flow rate of approximately 3.5 l/min, provided by a CX-1000 aquarium air pump. The extract was loaded onto the top of the column and sequentially eluted with 50 ml of chloroform/methanol mixtures, starting at a 48 : 2 ratio and gradually increasing the methanol content as follows: 46 : 4; 40 : 10; 35 : 15; 30 : 20; 25 : 25; 20 : 30; 15 : 35; 10 : 40; 5 : 45; 2 : 48). Elution was completed with 150 ml of pure methanol and 50 ml of methanol : ammonium hydroxide (40 : 10). Eluted fractions were collected continuously in 5 ml test tubes. Thin-layer chromatography (TLC) was performed on each tube using silica gel GF plates and chloroform/methanol (1 : 1) as the mobile phase. Plates were examined under UV light at 254 and 365 nm and visualized with iodine vapor. Fractions with similar chromatographic profiles were pooled as follows: F1: tubes 10-23, F2: tubes 24-31, F3: tubes 32-93, F4: tubes 94-97 and F5: tubes 98-100. These grouped fractions were dried under reduced pressure and reanalyzed by TLC followed by sequential spraying with Dragendorff's reagent and 10 % sodium nitrite for alkaloid detection. Selected fractions were subsequently analyzed by HPLC-HRMS to determine their alkaloid composition.

Cell culture: C6 glioma cells (ATCC: CCL-107™) were grown in 25 cm^2 flasks using Dulbecco's minimum essential medium (DMEM) (GIBCO-Invitrogen, Argentina) supplemented with 10 % fetal bovine serum (FBS Sigma Aldrich, USA), Penicillin- Streptomycin (1 %) (GIBCO-Invitrogen) in a humidified atmosphere with 5 % CO_2 at 37 °C. For cytotoxic assays, C6 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 3×10^4 cells per well, in growth medium (DMEM, 10 % FBS). When monolayers reached confluence, medium was replaced with fresh medium containing AEE1, AEE2, AEE3, and AEE4 (10, 20, 30, 40, 50 and 100 µg/ml) or AEE4 fractions (F1, F2, F3, F4 and F5; 2.5, 5, and 10 µg/ml) from *N. flexuosa*, previously dissolved in 0.1 % ethanol. Cells were incubated for 48 h at 37 °C, 5 % CO_2 and cell viability was determined by crystal violet staining method. 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 AEE, considered as 100 % viability) (Bustillo et al., 2012). The 50 % cytotoxic concentration (CC50) was defined as the concentration of AEE required to reduce viability by 50 %. The values of the percentages of cell viability were plotted against the logarithms of toxins concentrations, and CC50 was determined by linear regression analysis.

Determination of apoptosis by TUNEL: Apoptosis was assessed using the *in situ* Cell Death Detection Kit, AP (Roche Diagnostics). AEE4 (10-30 µg/ml) was selected for treatment due to its higher content of juliprosine and juliprosopine alkaloids. After a 48 h exposure, treated cells on coverslips were washed twice with PBS and fixed with 4 % paraformaldehyde. Cells were then washed with PBS, permeabilized with 0.1 % Triton X-100 in a 0.1 % sodium citrate solution, and incubated with the terminal deoxynucleotidyl transferase-mediated nick end labeling (TUNEL) reaction mixture for 60 minutes at 37 °C. Following three additional PBS washes, Converter-POD was applied for 30 minutes at 37 °C. Immunostaining was developed using DAB (3,3'-diaminobenzidine tetrahydrochloride), and the reaction was stopped by immersion in deionized water. The slides were then counterstained with hematoxylin. Apoptotic cells were quantified in 10 randomly selected representative fields at 40X magnification using a Primo Star Zeiss light microscope equipped with an AxiocamERc 5s Zeiss digital camera. Results were expressed as a percentage of total nuclei per field.

Statistical analysis: All values are presented as mean ± standard deviation (SD). Data represent the results of at least three independent experiments, each performed in triplicate. Statistical significance was assessed using two-way ANOVA, followed by Tukey test for multiple comparisons. Analyses were performed using Infostat software (Di Rienzo et al., 2019), with significance set at $p \leq 0.05$.

RESULTS

Chemical composition: The chemical composition of *N. flexuosa* pod samples for each year is presented in Table 2. All samples exhibited relatively similar contents of dry matter, humidity, energy digestibility (ED), ash, and ether extract contents, with no statistically

Table 2
Chemical composition of *N. flexuosa* pods by year.

	Year			
Components	2019	2020	2021	2022
Humidity (%)	6.13 ± 0.36	5.19 ± 0.42	6.03 ± 0.28	6.09 ± 0.21
Dry matter (%)	92.59 ± 0.42	94.80 ± 1.06	96.04 ± 0.57	93.90 ± 0.60
Crude Protein (%)	9.00 ± 0.40	7.60 ± 0.35*	9.56 ± 0.62	9.89 ± 0.89
Acid detergent fibre (%)	18.42 ± 0.28	19.58 ± 0.69	15.70 ± 0.60*	23.42 ± 0.71*
Neutral detergent fibre (%)	30.00 ± 0.31*	33.09 ± 0.17*	28.24 ± 0.30*	42.07 ± 0.32*
Hemicelulosa (%)	11.58 ± 0.38*	13.51 ± 0.30	12.54 ± 0.37	18.65 ± 0.20*
TDN (%)	77.84 ± 0.42	76.91 ± 0.40	80 ± 0.40*	73.85 ± 0.32*
ED (kcal/kg)	3.43 ± 0.27	3.39 ± 0.11	3.53 ± 0.20	3.62 ± 0.21
Ash (%)	3.81 ± 0.24	3.76 ± 0.20	3.73 ± 0.15	3.60 ± 0.14
Ether Extract (%)	1.76 ± 0.07	1.66 ± 0.09	1.65 ± 0.03	1.75 ± 0.08

* Significant differences among the four years analyzed ($p < 0.05$).

significant differences observed across the four years. However, crude protein content varied from 7.60 ± 0.35 to 9.89 ± 0.89 %, with the lowest values observed in 2020 ($p < 0.05$). Acid detergent fiber (ADF) and neutral detergent fiber (NDF) contents ranged from 15.70 ± 0.60 to 23.42 ± 0.71 % and from 28.24 ± 0.30 to 42.07 ± 0.32 %, respectively, with the lowest values recorded in 2021 and the highest in 2022 ($p < 0.05$). Hemicellulose content followed the same trend and showed significant variation, with the highest value in 2022 ($p < 0.05$). In addition, total digestible nutrients (TDN) also varied significantly over the years, ranging from 73.85 to 80.00 % ($p < 0.05$).

Identification and Quantification of Alkaloids in *N. flexuosa* Enriched Extracts: Samples of each *N. flexuosa* extract (AEE1, AEE2, AEE3 and AEE4) were analyzed by HPLC-HRMS detecting juliprosine and juliprosopine at different concentrations (Table 3).

Fractionation of AEE4 by flash chromatography and analysis of piperidinic alkaloids: Based on the identification and quantification of alkaloids in the AEEs, AEE4 was selected for fractionation. Analytical thin layer chromatography (TLC) analysis of the AEE4 fractions (F1-F5), using Dragendorff's reagent as developer, confirmed the presence of alkaloids in all samples. HPLC-HRMS analysis detected juliprosine and juliprosopine in all fractions at different concentrations, with the highest levels observed in F4 (Table 4).

Table 3
Alkaloid determination by HPLC-HRMS in *N. flexuosa* extracts over 4 years.

N. flexuosa	JPS (µg/mg)	JPSP (µg/mg)
AEE1 (2019)	128	19
AEE2 (2020)	109	21
AEE3 (2021)	51	7
AEE4 (2022)	222	42

JPS: Juliprosine; JPSP: Juliprosopine.

Table 4
Alkaloid quantification by HPLC-HRMS in *N. flexuosa* AEE4 Fractions.

AEE4 fractions	JPS (µg/mg)	JPSP (µg/mg)
F1	0.44	0.02
F2	2.05	0.58
F3	0.26	0.35
F4	33.5	104.1
F5	8.7	7.7

JPS: Juliprosine; JPSP: Juliprosopine.

Cytotoxic effects of Alkaloid-Enriched Extracts (AEEs) and fractions of AEE4: The results show a dose-dependent reduction in cell viability for all extracts, indicating a cytotoxic effect (Fig. 1). The corresponding CC50 values are presented in the inset table (Fig. 1), with AEE4 exhibiting the highest cytotoxicity, followed by AEE1, AEE2 and AEE3, which showed the lowest cytotoxic effects. These findings indicate that AEE4 has the highest cytotoxic activity on C6 cells compared to the other extracts.

On the other hand, the cytotoxicity of AEE4 fractions on C6 cells was also assessed using the crystal violet dye uptake assay. While F4 exhibited dose-dependent cytotoxicity, none of the assayed doses of F1, F2, F3 or F5 evidenced any significant effects on cell viability after 48 hours of incubation ($p < 0.05$) (Fig. 2).

Apoptosis in AEE4 of *N. flexuosa* by TUNEL assay: To determine whether the AEE4-induced reduction in cell viability was associated with apoptosis, C6 glioma cells were

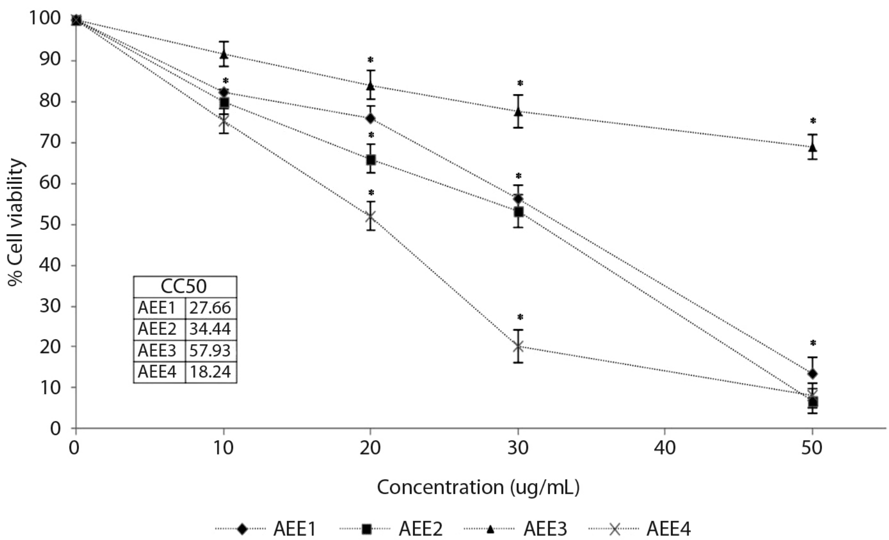

Fig. 1. Cytotoxic activity of AEEs from *Neltuma flexuosa* (0-50 μg/ml) on C6 glioma cells after 48 h assessed by crystal violet cell uptake assay in three independent experiments, *p < 0.05 differences compared to control. Inset: Cytotoxic concentration (CC50) values.

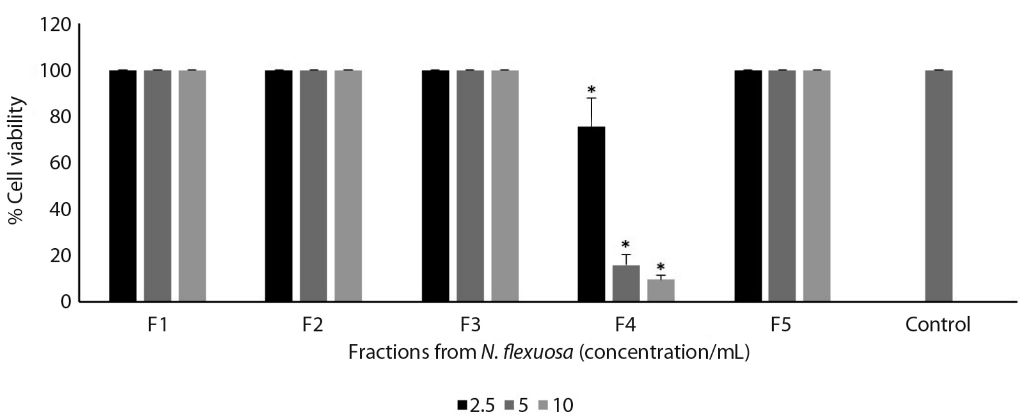

Fig. 2. Cytotoxic activity of fractions F1-F5 from AEE4 of *Neltuma flexuosa* (2.5, 5 and 10 μg/ml) on C6 glioma cells. Each column represents mean ± SD of triplicates of three independent experiments, *p < 0.05 differences compared to the control group.

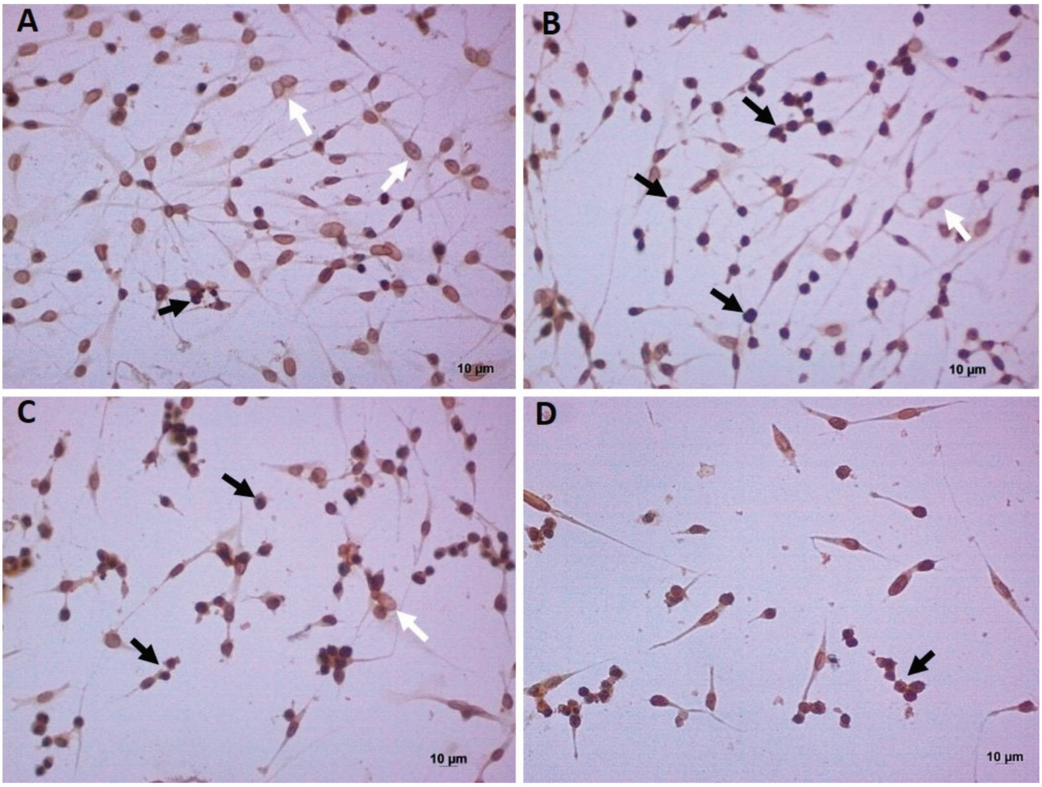

Fig. 3. TUNEL assay for apoptosis detection in C6 glioma cells. **A.** Control and AEE4-treated cells after 48 h: **B.** 10 µg/mL, **C.** 20 µg/mL, **D.** 30 µg/mL. Apoptotic nuclei are indicated by black arrows; non-apoptotic (normal) nuclei are indicated by white arrows. Scale bar = 10 µm.

treated with 10-30 µg/ml of AEE4 for 48 hours and analyzed by TUNEL staining. The extract exhibited a dose-dependent increase in the percentage of apoptotic cells. Specifically, treatment with 10, 20 and 30 µg/ml of AEE4 resulted in apoptosis rates of 35.1 ± 3.5 %, 48.12 ± 5.5 % and 63.6 ± 3.1 % respectively, whereas the control group displayed only 18.08 ± 2.1 % apoptotic cells (Fig. 3).

DISCUSSION

Species of the genus *Neltuma* are multipurpose trees that thrive in arid and semi-arid regions (Verga, 2009) and are valuable forage resources due to their nutritional composition (Riet-Correa et al., 2012). Their high tolerance to drought and salinity, along with notable growth capacity, reinforces their agronomic importance in regions with environmental constraints (Pasiecznik et al., 2001). In this context, the present study focused on evaluating the chemical composition, alkaloid content, and cytotoxic potential of *N. flexuosa* pods collected over four consecutive years (2019-2022) in Cafayate, a representative dryland region in Northwestern Argentina.

Our results revealed substantial year-to-year differences in pod composition, particularly in crude protein, neutral detergent fiber (NDF), and acid detergent fiber (ADF) content. For instance, NDF ranged from 28 to 42 %, reaching its highest level in 2022, while protein content was lowest in 2020. These fluctuations may be linked to environmental factors, such as the progressive decline in rainfall observed during the study period (from 260 mm in 2019 to 130 mm in 2022), despite relatively stable temperatures. Previous studies have shown that drought can promote fiber accumulation and affect protein content in legumes which supports this interpretation (Furlan et al., 2022; Ressaissi et al., 2023).

Notably, the highest NDF values observed in *N. flexuosa* pods were comparable to those reported for soft wheat bran (42.65 %, AACC), and exceeded those described for other *Neltuma* species, reinforcing their potential value as a fiber-rich feed. Protein content, although moderately variable (7.60-9.89 %), remained within acceptable ranges for ruminant supplementation, albeit lower than values reported for species like *N. alba* (up to 17 %) (Freire et al., 2003; Zolfaghari & Harden, 1985). Total digestible nutrients (TDN) ranged from 74 to 80 %, aligning with values for mature pods of *N. juliflora* and *N. laevigata* (Baraza et al., 2008; Sawal et al., 2004), confirming their potential as an energy source. These variations in nutritional composition may also be influenced by factors such as pod maturity at harvest, soil fertility, and environmental conditions during the growing seasons (Rostagno & Degorgue, 2011).

The adverse effects of antinutritional compounds, such as alkaloids, in *N. flexuosa* pods have been previously reported (Cholich et al., 2021). In this context, several studies have described toxic effects on the nervous system in goats (Assis et al., 2009; Lima et al. 2004; Tabosa et al., 2000). However, one study found that sheep and goats did not exhibit signs of toxicity after consuming *N. juliflora* pods (Riet-Correa et al., 2012). The authors proposed several explanations for this discrepancy, including variations in pod toxicity due to differences in storage conditions and chemical composition across harvests, which may reduce toxicity under specific circumstances. In the present study, *N. flexuosa* extracts obtained over a four-year period exhibited marked variability in the concentrations of the piperidine alkaloids juliprosine and juliprosopine, with juliprosine consistently representing the predominant compound.

The interannual variability observed in both the nutritional composition and alkaloid content of *N. flexuosa* pods appears to be closely associated with climatic conditions recorded during the study period. Notably, the year with the lowest rainfall (2022) corresponded to the highest concentrations of juliprosine and juliprosopine, as well as elevated NDF values. This pattern aligns with previous findings indicating that water deficit can induce physiological adjustments in legumes and woody species, promoting the accumulation of secondary metabolites and structural carbohydrates (Santos et al., 2022). Similar observations were reported by Honório et al. (2021), who demonstrated that drought stress in *Annona crassiflora* increased alkaloid biosynthesis. In *Prosopis juliflora*, Al-Soqeer et al. (2023) described significant differences in pod composition between agro-climatic regions with contrasting precipitation levels, highlighting the impact of environmental variability on nutritional and toxicological profiles. The relatively stable temperatures recorded across years suggest that precipitation was the primary climatic factor influencing pod composition in this study. Collectively, these results underscore the importance of monitoring environmental conditions when evaluating the suitability of *N. flexuosa* pods as a feed resource and assessing their potential risks.

In addition to these compositional variations, the cytotoxic effects of the extracts were also assessed. Previous studies have reported deleterious effects of *Neltuma* species, including *N. juliflora* leaves and *N. alpataco* pods, with low CC50 values of 31.07 µg/ml and 24.69 µg/ml, respectively (Cholich et al., 2024; Silva et al., 2013). Our findings align with these reports; however, this is the first study to link cytotoxicity to alkaloid content. Notably, the extract with the highest alkaloid concentration exhibited the lowest CC50.

In a related study, fractionation of a total extract from *N. juliflora* identified one fraction as the most cytotoxic (Silva et al., 2007), with further analysis revealing that it was a mixture of the alkaloids juliprosine (minor component) and juliprosopine (major component) (Silva et al., 2013). Our results also showed that only one fraction (F4) from the AEE4 extract of *N. flexuosa* exhibited a dose-dependent cytotoxic effect on the C6 cell line. In agreement with Silva et al. (2013), HPLC-HRMS analysis identified juliprosopine as the most abundant alkaloid in this fraction. The selective enrichment of alkaloids in F4 suggests that its chemical composition may facilitate the accumulation of specific piperidine alkaloids. These findings highlight the potential of fractionation to isolate and concentrate bioactive compounds, which can be useful for toxicological studies on the active alkaloids of *N. flexuosa*.

Our previous work demonstrated that *N. alpataco* extracts induced apoptosis followed by secondary necrosis in C6 cells, as observed using acridine orange/ethidium bromide fluorescence staining. Additionally, *N. juliflora* has been shown to induce apoptosis in neuron/glial cell co-cultures (Silva et al., 2017), as well as in various tumor cell lines, including MDA-MB-231, MCF-7, LS-174T, and HepG2 (Abbas et al., 2022; Utage et al., 2018). In line with these studies, the total extract of *N. juliflora* triggered cell death in primary glial cells through caspase-9 activation and nuclear condensation (Silva et al., 2017). In the present study, apoptotic markers increased in a dose-dependent manner over 48 hours, further supporting the role of apoptosis as the primary cytotoxic mechanism of *Neltuma* spp. extracts.

All together, these findings suggest that reduced precipitation may play a key role in modulating the chemical profile of *N. flexuosa* pods, particularly by promoting the accumulation of fiber and piperidine alkaloids such as juliprosine and juliprosopine. Although further studies are needed to elucidate the underlying mechanisms, our results highlight the importance of considering environmental variability as a key driver of both nutritional and toxicological changes in this species. Such insights could inform the development of guidelines for the safe use of *N. flexuosa* pods in animal feed, thereby helping to prevent poisoning events.

Ethical statement: The authors declare that they all agree with this publication and made significant contributions; that there is no conflict of interest of any kind; and that we followed all pertinent ethical and legal procedures and requirements. All financial sources are fully and clearly stated in the acknowledgments section. A signed document has been filed in the journal archives.

ACKNOWLEDGMENTS

This work was supported by the Secretaría General de Ciencia y Técnica, Universidad Nacional del Nordeste (UNNE; grant N° PI22B007) and by the Agencia Nacional de Promoción Científica y Tecnológica (PICT 2020/01484).

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Revista de Biología Tropical, ISSN: 2215-2075 Vol. 73: e2025225, enero-diciembre 2025 (Publicado Nov. 03, 2025)

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