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ORIGINAL ARTICLE

Neuroprotective Activity of *Datura inoxia* and *Turnera diffusa* Extracts in an *in vitro* Model of Neurotoxicity

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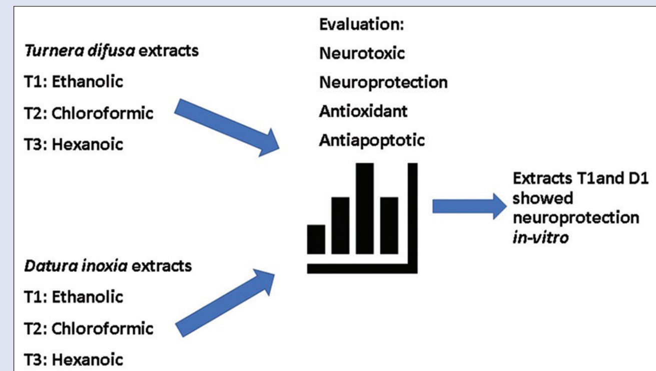
ABSTRACT

Background: Due to their therapeutic properties and easy access, in Mexican culture, plants are used to treat several diseases. This is the case of *Datura inoxia* and *Turnera diffusa***,** which affect their central nervous system with psychotropic, anticholinergic, analgesic, and stimulating properties. **Objectives:** In this research, extracts D1 (methanolic), D2 (chloroformic), and D3 (hexanoic) from *D. inoxia* and T1 (ethanolic), T2 (chloroformic), and T3 (hexanoic) from *T. diffusa* were evaluated to determine their neurotoxic and neuroprotective activity *in vitro*. **Materials and Methods:** Neurotoxic activity was evaluated in PC-12 cells exposed to the extracts at concentrations ranging from 3.12 to 200 μg/mL. The neuroprotective activity was evaluated by pretreating PC-12 cells with the extracts and subsequently exposing them to neurotoxic compound glutamate. In both assays, cell viability was evaluated with 4-(3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1). Antioxidant activity was studied with the 2,2-diphenyl-1-picrylhydrazyl (DPPH) assay, and antiapoptotic activity was evaluated with the caspase-3 fluorescent assay. **Results:** *T. diffusa* was more neurotoxic than *D. inoxia*, and both plants showed neuroprotective activity at low concentrations. *T. diffusa* had more antioxidant activity than *D. inoxia*. Neither plant had antiapoptotic activity. **Conclusion:** Extract D1 of *D. inoxia* and T1 of *T. diffusa* showed neuroprotective activity, and both extracts had the lowest neurotoxic effect *in vitro*.

Key words: Antioxidant, Damiana, DPPH, neuroprotective, neurotoxic, toloache

SUMMARY

- Three extracts of *D. inoxia* and three extracts of *T. diffusa* were evaluated *in vitro* for their potential neuroprotective activity. PC-12 cells exposed to glutamate were pretreated with the extracts. In addition, cytotoxic, antioxidant, and antiapoptotic activities were evaluated. *D. inoxia* methanolic extract and *T. diffusa* alcoholic extract showed neuroprotective activity.



Abbreviations used: DMSO = dimethylsulfoxide, DPPH = 2,2-diphenyl-1-picrylhydrazyl, WST-1 = 4-(3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate, GSH = glutathione, IC_{50} = half-maximal inhibitory concentration, LC_{50} = lethal concentration 50, RPMI-1640 = Roswell Park Memorial Institute Medium-1640

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INTRODUCTION

Neurotoxicity refers to the direct or indirect effect of chemicals that disrupt the nervous system. These chemicals may occur naturally, including endogenous, or arise by synthesis.[1] Chemicals can cause neuronal cell death by a variety of mechanisms, including disruption of the cytoskeleton, induction of oxidative stress, calcium overload, or by damaging mitochondria.[2] Neuronal death in adults leads to functional decline and underlies disease progression in neurodegenerative indications ranging from acute insults such as traumatic brain injury (TBI) and stroke to chronic conditions such as Alzheimer's disease (AD), Parkinson's disease (PD), and amyotrophic lateral sclerosis (ALS).[3]

Degenerative diseases of the nervous system impose substantial medical and public health burden on populations throughout the world. The prevalence and incidence of these diseases rise dramatically with age; thus, the number of cases is expected to increase for the foreseeable future as life spans in many countries continue to increase. AD is the

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most common form of dementia worldwide and makes up 60%–80% of all dementia cases, affecting an estimated 24 million people globally. The prevalence of PD is estimated to be 0.3% in the general population, ~1% in people older than age 60, and ~3% in people aged 80 years or older. The incidence rate of PD is 8–18 individuals per 100,000 person-years.[4] Huntington´s disease (HD) is rare, with a recent meta-analysis estimating the service-based worldwide prevalence of 2.7 cases per 100,000, with higher rates in Europe, North America, and Australia compared with Asia. The incidence was estimated to be 0.38 cases per 100,000 person-years.[5] Although neurodegenerative diseases are typically defined by specific protein accumulation and anatomic vulnerability, they share many fundamental processes associated with progressive neuronal dysfunction and death, such as proteotoxic stress and its attendant abnormalities in ubiquitin–proteasomal and autophagosomal/lysosomal systems, oxidative stress, programmed cell death, and neuroinflammation.[6] Due to this, there is no cure for neurodegenerative disease. The search for compounds that prevent or detent the neuronal damage associated with this disease or other insults is urgently needed. In this sense, plants constitute an important source of new molecules.

In Mexico, several plants have not been studied to validate their traditional use. Among these are *Datura inoxia* and *Turnera diffusa*. The former, also known as toloache, chamico, nacazul, etc., is a plant from the Solanaceae family. Several medicinal properties have been reported, such as psychotropic, anticholinergic, analgesic, anti-inflammatory, narcotic effects, and antispasmodic properties.[7,8] The main components found in this plant include alkaloids, amino acids, citric acid cycle intermediates, tannins, saponins, steroids, flavonoids, and terpenes.[9-11] *T. diffusa* has been used in traditional medicine to treat bronchitis, whooping cough, dysentery, dyspepsia, malaria, stomach and intestinal pain, and some types of paralysis. Their properties include stimulant, diuretic, aphrodisiac, anti-inflammatory, antiulcerative, and, recently, antioxidant properties,[12,13] the chemical content reported for this plant includes monoterpenes, alkaloids, and steroids.[13]

The objective of this work was to evaluate the neuroprotective activity of *T. diffusa* and *D. inoxia* extracts in an *in vitro* model of neurotoxicity.

MATERIALS AND METHODS

Plant collection and extract preparation

Both plants were collected in the state of Durango, Mexico, during March (voucher specimen for *D. inoxia* UAT-22755, voucher specimen for *T. diffusa* UAT-22756). Dried and ground leaves of *D. inoxia* (50 g) were extracted first with hexane (2 × 600 mL) and then with chloroform (3 × 600 mL) and finally with methanol (2 × 600 mL) by maceration for 72 h. Extracts were distilled *in vacuo* separately until dry. A total of 7.5 g of hexanoic, 6.7 g of chloroformic, and 6 g of methanolic extracts were obtained. The same procedure was done for *T. diffusa* (50 g of dried and ground leaves). Ethanol was used instead of methanol. A total of 7 g of hexanoic, 6 g of chloroformic, and 5.5 g of ethanolic extracts were obtained.

Reactants

For each extract, stock solutions were prepared at 10 mg/0.1 mL. From these stocks, work solutions at 400 μg/mL were prepared. Thereafter, 0.1 mL of work solution was added to a microtiter plate (1:1) to get a concentration of 200 μg/mL. Then, serial dilutions were made until 3.12 μg/mL was obtained. The PC-12 cell line used is part of the Universidad Autónoma de Nuevo León (UANL) Molecular Pharmacology and Biological Models laboratory catalog. Roswell Park Memorial Institute Medium-1640 (RPMI-1640) medium and EnzChek® caspase-3 assay kit #2 were purchased from Thermo Fisher Scientific. 2,2-Diphenyl-1-picrylhydrazyl (DPPH), glutamate monohydrate, ascorbic acid, dimethylsulfoxide (DMSO), and 4-(3-[4-iodophenyl]-2-[4-nitrophenyl]-2H-5-tetrazolio)-1,3-benzene disulfonate (WST-1) were purchased from Sigma-Aldrich (Toluca, Mexico). All compounds were of reagent grade.

Neurotoxic activity

Neuronal PC-12 cells were cultured in a microtiter plate at a concentration of 10,000 cells/well and incubated at 37°C in the presence of 5% CO_2 for 48 h. When the monolayer was formed, 0.1 mL of each extract was added. Each extract was dissolved in DMSO and subsequently in culture medium. Then, serial dilutions were made to achieve a concentration from 200 to 3.12 μg/mL. The microtiter plate was incubated for 24 h. After this exposure time, the medium was removed and WST-1 (Sigma-Aldrich) solution was added to each well (5% final concentration). Then, the plate was incubated at 37°C in the presence of 5% CO_2 for 2 h. After that, absorbance was measured at 450 nm.[15] Glutamate-free cells of 100% viability were used as a control. Each assay was performed in triplicate.

The following equation was used to calculate the viability percentage:

$$\text{Cell viability}(\%) = \frac{(\text{ABS}x - \text{ABS}o)\times 100}{(\text{ABSctl} - \text{ABS}o)}$$

where ABS*x* = test absorbance, ABS*o* = blank absorbance, and ABSctl = control absorbance.

Neuroprotective activity

To test the neuroprotection provided by the extracts, we used an *in vitro* model of neurotoxicity with glutamate as a neurotoxic agent. In this model, PC-12 cells, which express glutamate receptors in their membrane, are exposed to a toxic concentration of glutamate. Glutamate exerts its toxic effects on PC-12 in a dose- and time-dependent manner. The mechanism of cell death is through an increased intracellular Ca^{2+} concentration and glutathione (GSH) depletion.

After a PC-12 cell line monolayer was formed, RPMI-1640 medium was replaced with fresh medium. Then, the extracts were added to each well as pre-treatment. Each extract was dissolved in DMSO and subsequently in a culture medium. The range of concentrations added was 200–3.12 μg/mL. The microtiter was incubated at 37°C in the presence of 5% CO_2 for 4 h. After that, neurotoxicity was induced with the addition of 5 mmol/L of glutamate to each well.[14] The microtiter was then incubated for 16 h at 37°C in the presence of 5% CO_2. After this exposure, the medium was removed and WST-1 solution (5% per well) was added to each well. The microtiter plate was incubated at 37°C in the presence of 5% CO_2 for 2 h,[15] and at the end of the incubation period, absorbance was measured at 450 nm. The cell viability percentage was calculated with the same equation used in the neurotoxicity assay. Ascorbic acid 100 μmol/L (Sigma-Aldrich) was used as a positive control of neuroprotection; cells with 5 mmol/L of glutamate were used as a control of neurotoxicity (negative neuroprotection).[14,16] The assay was performed in triplicate.

Antioxidant activity

This assay was performed to reduce the free radical DPPH, which disappears in the presence of antioxidants.[17] The plant extracts were dissolved in methanol and placed in each well. The range of concentration was 200–3.12 μg/mL. After that, 0.1 mL of 100 μmol/L DPPH (Sigma-Aldrich) was added to the wells. The microtiter plate was incubated at room temperature in darkness, and after incubation, absorbance was measured at 517 nm. DPPH solution (100 μmol/L) was a negative control of antioxidant activity, and ascorbic acid was a positive control of antioxidant activity. A calibration curve of ascorbic acid was prepared at concentrations of 300, 200, 100, 80, 60, 40, and 20 μg/mL.

DPPH and ascorbic solutions were prepared with methanol as a solvent in the remaining well set with 0.1 mL of methanol.[18] The assay was performed in triplicate.

The result was expressed as an inhibition percentage of DPPH and was obtained with the following equation:

$$\text{Inhibition}(\%) = \frac{((\text{ABSctl} - \text{ABS}x) \times 100)}{\text{ABSctl}}$$

where ABSctl = control absorbance and ABSx = test absorbance.

Cell death assessment

During apoptosis, caspase-3 activity increased; therefore, measurement of this activity permitted preliminary evaluation of the type of cell death. Caspase-3 activity was measured using the EnzChek caspase-3 assay kit #2 (cat #: E-13184, Thermo Fisher Scientific), according to the manufacturer's instructions. Only extracts with the best neuroprotective results and lowest neurotoxicity were measured for caspase-3 activity; these were D1 and T1 at a concentration of 12.5 µg/mL.

PC-12 cells at a concentration of 1,000,000 cells/well were placed in a 96-well microtiter plate. When the monolayer was formed, the RPMI-1640 medium was replaced and the cells were exposed to the extracts as pre-treatment; each extract was dissolved in DMSO and subsequently in the culture medium. The microtiter plate was incubated at 37°C for 4 h. Neurotoxicity was induced by addition of 0.1 mL of 5 mM glutamate and the plates were incubated again for 12 h at 37°C. After exposure, the medium was removed and the cells were harvested and washed with phosphate-buffered saline (PBS). Then, cell lysis buffer was added and submitted to freeze–thaw cycles for 30 min. The lysed cells were placed in a microtube with a reaction buffer and centrifuged at 5000 rpm for 5 min. The supernatant with proteins was separated, and their concentration was determined with Bradford's methodology. The concentration was adjusted to 1.3 µg/mL and complemented with cell lysis buffer 1X to obtain a final volume of 50 µL. This volume was transferred to individual wells and they were incubated for 10 min at room temperature. After that, 50 µL of the caspase-3 substrate Z-DEVD-R110 was added to each test well and set in darkness for 30 min. Later, Z-DEVD-R110 was quantified; for this, a calibration curve in a concentration range of 0–25 µmol/L was prepared and fluorescence was measured at 496 nm/520 nm excitation and emission.

Ascorbic acid 100 µmol/L was used as a negative control of apoptosis. Cells without treatment were also considered as negative controls, while podophyllotoxin (12.2 µg/mL)- and glutamate (5 mmol/L)-treated cells were positive controls. The assay was performed in triplicate.

Statistical analysis

Analysis of variance (ANOVA) and Dunnett's *post hoc* test were performed to assess statistically significant differences between the controls and extracts. The GraphPad statistical software, Prism 5 and NCSS12, was used for all analyses.

RESULTS

Neurotoxic activity

D. inoxia: The three extracts decreased cell viability to values below 50% at concentrations higher than 25 µg/mL [Figure 1a]. The estimated half-maximal inhibitory concentration (IC_{50}) was 66.53, 76.73, and 30.23 µg/mL for the D1, D2, and D3 extracts, respectively. There was no statistically significant difference between the extracts ($P = 0.66$), but there was a significant difference between the concentrations studied.

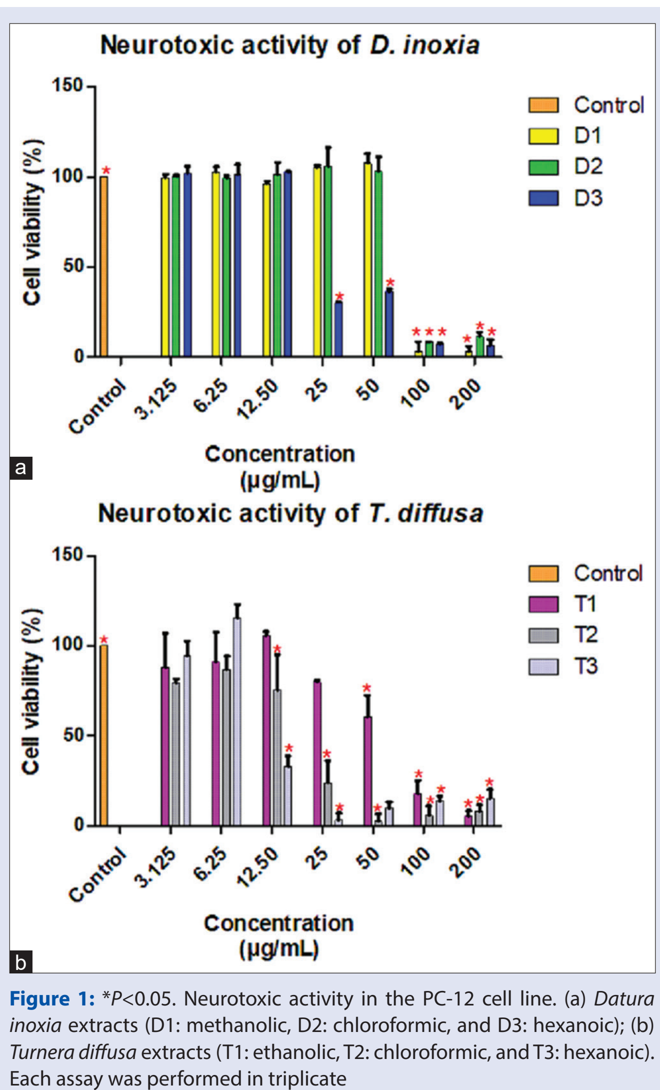

Figure 1: *$P<0.05$. Neurotoxic activity in the PC-12 cell line. (a) *Datura inoxia* extracts (D1: methanolic, D2: chloroformic, and D3: hexanoic); (b) *Turnera diffusa* extracts (T1: ethanolic, T2: chloroformic, and T3: hexanoic). Each assay was performed in triplicate

T. diffusa: Values lower than 50% cell viability [Figure 1b] were observed at 12.5 µg/mL. The estimated IC_{50} was 46.22, 16.44, and 16.95 µg/mL for the T1, T2, and T3 extracts, respectively. There was no significant difference between the extracts ($P = 0.47$), but there was a significant difference between the concentrations of each extract.

Neuroprotective activity

D. inoxia: The extracts at concentrations ranging from 3.12 to12.5 µg/mL increased the percentage of cell viability in cells exposed to glutamate. At these concentrations, the extracts were better than positive control of neuroprotection, 100 µmol/L of ascorbic acid (cell viability = 45.65%) ($P < 0.05$). The highest cell viability for D1 was at 6.25 µg/mL (77.0%), for extract D2 was at 12.5 µg/mL (76.6%), and for extract D3 was at 3.12 µg/mL (78.2%) [Figure 2a].

T. diffusa: The neuroprotective effect was also observed at low concentrations ($P < 0.01$) from 3.12 to 25 µg/mL. The extracts had a higher neuroprotective effect at these concentrations than the control group, 100 µmol/L of ascorbic acid (cell viability = 58.39%). For the three extracts, the highest cell viability was at the concentration of 3.12 µg/mL with a cell viability of 79.06%, 67.32%, and 62.11% for T1, T2, and T3, respectively [Figure 2b].

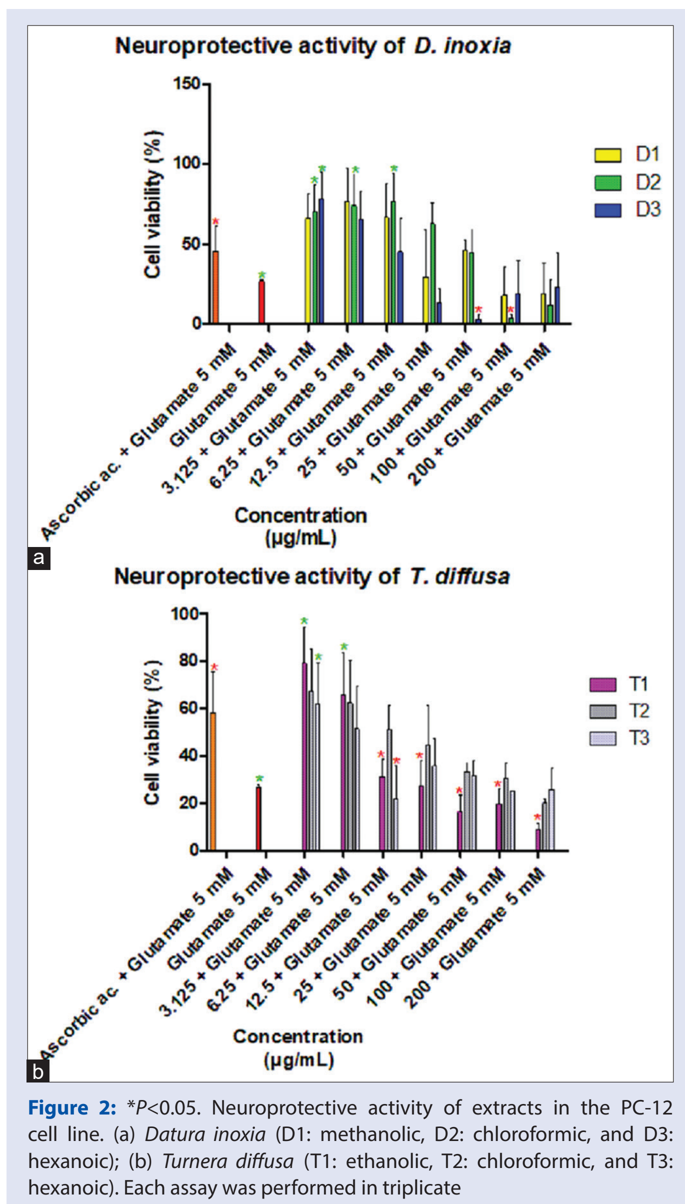

Figure 2: **P*<0.05. Neuroprotective activity of extracts in the PC-12 cell line. (a) *Datura inoxia* (D1: methanolic, D2: chloroformic, and D3: hexanoic); (b) *Turnera diffusa* (T1: ethanolic, T2: chloroformic, and T3: hexanoic). Each assay was performed in triplicate

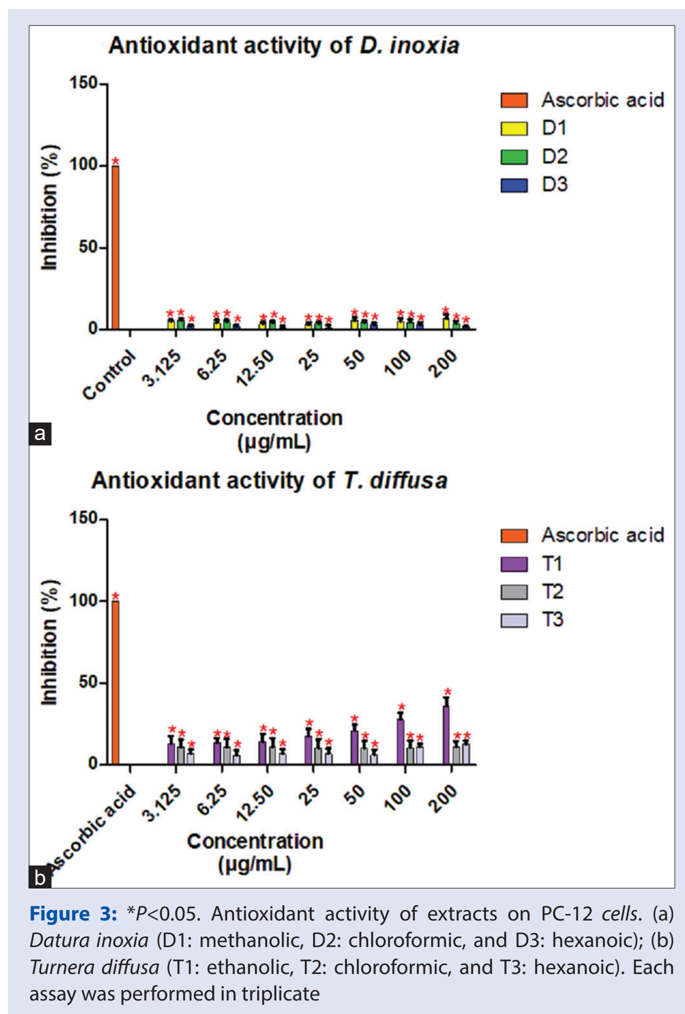

Figure 3: **P*<0.05. Antioxidant activity of extracts on PC-12 *cells*. (a) *Datura inoxia* (D1: methanolic, D2: chloroformic, and D3: hexanoic); (b) *Turnera diffusa* (T1: ethanolic, T2: chloroformic, and T3: hexanoic). Each assay was performed in triplicate

Antioxidant activity

D. inoxia: The extracts presented low antioxidant activity; none decreased the DPPH free radical percentage by 50% (IC_{50} >200 μg/mL). The most active extract was D1 with an inhibition percentage of 6.69% at 200 μg/mL [Figure 3a]. There was a statistically significant difference between the groups ($P < 0.001$).

T. diffusa: The extracts also presented low antioxidant activity. None decreased the percentage of DPPH free radicals to levels of 50% (IC_{50} >200 μg/mL). The extract with the highest antioxidant activity was T1 with an inhibition percentage of 36.23% at 200 μg/mL [Figure 3b]. There were statistically significant differences ($P < 0.01$).

Cell death assessment

During apoptosis, caspase-3 activity increased; therefore, this assay allowed a preliminary evaluation of the type of cell death. Our results showed that cells exposed to glutamate do not significantly differ in caspase-3 activity with respect to free cells. Similarly, pre-treatment of cells exposed to glutamate with D1 and T1 extracts did not show a significant difference in caspase-3 activity with regard to the cells exposed to glutamate, ascorbic acid (100 μmol/L), and free cells. Only cells exposed to podophyllotoxin, an apoptosis inducer, significantly increased caspase-3 activity ($P < 0.05$) [Figure 4].

DISCUSSION

Neurotoxic activity

This is the first study in which the extracts of *D. inoxia* were evaluated for their neurotoxic activity; however, several studies have evaluated its cytotoxicity in other non-neuronal cell lines or organisms. In a study on zebrafish (*Artemia* sp.) exposed to leaf, stem, and fruit extracts, a lethal concentration 50 (LC_{50}) ≤1000 μg/mL was found.[19] The aqueous and methanolic seed extracts of *Datura metel* presented an IC_{50} ranging from 3 to 7.5 mg/mL in VERO cells[20] and HEK-293 cells, respectively. The methanolic extract *D. inoxia* at a concentration ranging from 50 to 100 μg/mL did not show cytotoxic effect.[21] All these results suggest that toxicity of *D. inoxia* extracts is lesser in normal cells, and our results complement these studies since we included neuronal cells.

Regarding *T. diffusa,* previously, neurotoxicity was reported in primary cultures of rat cortex astrocytes using the hydroethanolic extract of the aerial part in concentrations ranging from 100 to 1000 μg/mL.[22] Their results are comparable to ours since we found a neurotoxic effect at 100 μg/mL. Another study observed a neurotoxic effect of aqueous extract in the SH-SY5Y cell line at higher concentrations of 0.500 mg/mL.[14]

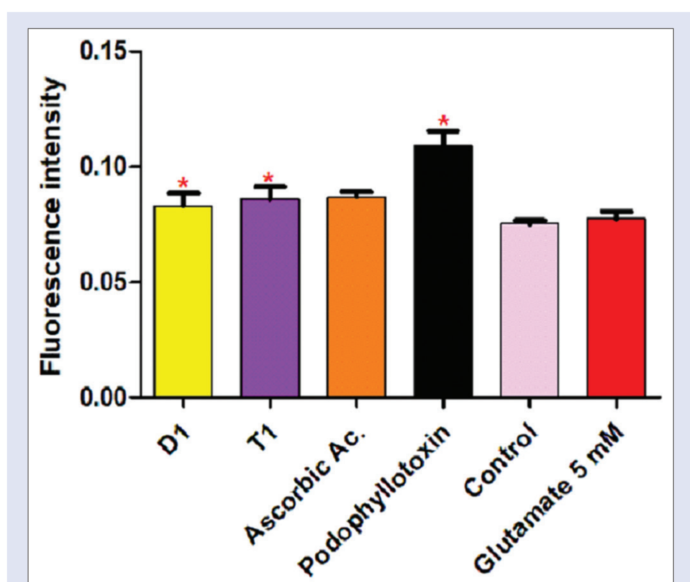

Figure 4: **P*<0.05. Evaluation of caspase-3 activity in cells exposed to glutamate and pretreated with *Datura inoxia* and *Turnera diffusa* extracts

Neuroprotective activity of *D. inoxia*

Our results showed that lower extract concentrations increase the survival of cells exposed to the neurotoxic agent glutamate. According to the literature, there have been no studies evaluating the neuroprotective effect of *D. inoxia* extracts on neuronal cell lines, so this would be the first study in this respect. The neuroprotective effect found in this study could be explained in part by the extracts' antioxidant activity, which has also been previously reported in this plant and other plants of the same genera. In a previous study, a decrease of radical DPPH by 50% by leaf methanolic extract at a value of 58.6 ± 0.5 µg/mL was reported.[18] In another study evaluating DPPH radical scavenging activity, an IC_{50} value of 22.83 µg/mL for the leaf aqueous extract and 383.31 µg/mL for *n*-hexane extract was reported.[19,23] Anant *et al.*[23] showed antioxidant activity in the hexane extract of *Datura stramonium* with an IC_{50} value of 81.34 ± 2.94 µg/mL. The considerable difference between our results and those reported by previous authors in the antioxidant activity could be due to different solvents used for the preparation of extracts. These results suggest that differences in antioxidant activity depend on the polarity of the solvent used.

The caspase-3 activity inhibition assay was performed to assess if apoptosis inhibition was another possible neuroprotective mechanism. Until now, there are no reports evaluating this activity in the literature. It was found that there was no significant difference in caspase-3 activity among cells exposed to glutamate, glutamate + D1 extract, and free cells. This finding indicates that glutamate, at the concentration used for neurotoxicity, did not induce cell death by apoptosis. With this in mind, it is not possible to conclude that neuroprotection by the D1 extract against glutamate is due to the inhibition of apoptosis. Also, with this assay, we cannot conclude that the D1 extract lacks antiapoptotic activity, so more assays are needed.

Neuroprotective activity of *T. diffusa*

The extracts showed neuroprotection similar to the positive control at low concentrations. Neuroprotective activity has been reported for this plant using the same model of this study. In this study, pre-treatment with *T. diffusa* aqueous extract delayed oxidative damage induced by glutamate excitotoxicity through reduction of reactive intracellular species.[14] Among the responsible components for this activity of *T. diffusa,* we found α-pinene and 1,8-cineole.[24] Porres-Martínez *et al.*[25] evaluated the neuroprotective potential of these compounds against oxidative stress induced by H_2O_2 in the PC-12 cell line and found protective effects on cell viability and morphology, inhibition of reactive oxygen species (ROS) production, attenuation of H_2O_2-induced changes in endogenous antioxidant status, and reduced caspase-3 activity.

The neuroprotective effect of *T. diffusa* may be due to the antioxidant activity. It was found that more polar the extracts of this plant are, more antioxidant activity is shown by them. Previous studies demonstrated antioxidant activity in the methanolic leaf extract at 1 mg/mL with values of 75.3% ± 5.8%.[13] The hydroalcoholic leaf extract at 1000 µg/mL also showed antioxidant activity of 82.4% ± 4.6%.[16] In addition, it has been found that one compound with high antioxidant activity, luteolin 8-C-β -[6-deoxy-2-O-(α-1-ramnopiranozil)-xilo-hexopyranos-3-uloside], is present in *T. diffusa*.[26]

The caspase-3 activity inhibition assay showed that cells exposed to glutamate and glutamate + T1 extract did not affect caspase-3 activity, since there was no significant difference when compared to free cells. As with the D1 extract of *D. inoxia*, there are no reports in the literature analyzing the activity. It is not possible to conclude that neuroprotection shown by the T1 extract is due to inhibition of apoptosis, since glutamate used at the concentration of 5 mmol/L does not induce cell death by apoptosis. Also, it is not possible to conclude with this assay that the T1 extract lacks antiapoptotic activity. On the other hand, it is worth mentioning that some studies have evaluated caspase-3 activity using pure compounds that are present in extracts of this plant. Porres-Martínez *et al.*[25] reported 70% inhibition of caspase-3 activity by 1.8-cineole at concentrations of 25 and 10 µmol/L. The α-pinene compound showed a reduction in caspase 3-activity of 50% at 25 µ mol/L and 30% at 10 µmol/L.

CONCLUSION

D. inoxia methanolic extract and *T. diffusa* alcoholic extract showed neuroprotective activity in PC-12 cells exposed to glutamate. Their antioxidant activity could contribute to the mechanism of action.

A main limitation of this study is the lack of a phytochemistry analysis of each extract; however, our results, although preliminary, are important since they provide new information regarding the potential neuroprotective activity of the plants studied. Future studies should consider this limitation and explore the neuroprotective activity in animal models.

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Conflicts of interest

There are no conflicts of interest.

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