



Improved method for diagnosis of *Nerium oleander* poisoning in necropsy tissues¹

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ABSTRACT.- Botelho A.F.M., Oliveira F.A.S., Fiúza A.T.L., Pedroza H.P., Branco S.E.M.T., Pierezan F., Melo M.M. & Soto-Blanco B. 2018. **Improved method for diagnosis of *Nerium oleander* poisoning in necropsy tissues.** *Pesquisa Veterinária Brasileira* 38(5):967-972. Departamento de Clínica e Cirurgia Veterinárias, Escola de Veterinária, Universidade Federal de Minas Gerais, Avenida Antônio Carlos 6627, Belo Horizonte, MG 30123-970, Brazil. E-mail: benito.blanco@pq.cnpq.br

Nerium oleander is an ornamental cardiotoxic plant found in tropical and subtropical areas of the World. Its toxicity is related to the content of cardioactive glycosides, mainly oleandrin, found throughout the plant. The present study aimed to describe a new and improved method for oleandrin detection in tissue samples. The determination of oleandrin was made after extraction with a modified QuEChERS technique and measurement by UFLC-MS/MS. A total of 36 guinea pigs (*Cavia porcellus*) were distributed into 3 groups (n=12): control group that received only water orally (CON), and two treated groups that received hydroalcoholic oleander extract at doses of 150mg.kg⁻¹ (OLE 150) and 300mg.kg⁻¹ (OLE 300) in single oral dose. After three hours, fragments of heart, kidneys, liver and brain were collected for determination of oleandrin levels. The extraction and chromatographic procedures were effective for oleandrin detection and quantification in tissues, with retention time of 1.2 min and detection limit of 0.001µg g⁻¹. The chromatographic analysis of treated guinea pigs indicated that oleandrin is distributed equally among the analyzed tissues. The developed methodology is a reliable, effective and rapid form of diagnosis of *N. oleander* poisoning based on necropsy tissue samples.

INDEX TERMS: Oleander, *Nerium oleander*, cardenolides, diagnostic testing, liquid chromatography, mass spectrometry, guinea pigs, toxicoses.

RESUMO.- [Método aprimorado para diagnóstico da intoxicação por *Nerium oleander* em tecidos de necropsia.]

Nerium oleander é uma planta cardiotóxica ornamental encontrada em áreas tropicais e subtropicais do mundo. Sua toxicidade é relacionada à presença de glicosídeos cardioativos, principalmente a oleandrina, encontrada em toda a planta. O presente estudo objetiva descrever um novo e aprimorado método para detecção da oleandrina em amostras

de tecido. A determinação da oleandrina foi feita após extração utilizando técnica modificada de QuEChERS e mensuração por UFLC-MS/MS. Um total de 36 cobaias (*Cavia porcellus*) foi distribuído em três grupos (n=12): grupo controle que recebeu apenas água por via oral (CON), e dois grupos tratados que receberam extrato hidroalcolólico de oleander nas doses de 150mg.kg⁻¹ (OLE 150) e 300mg.kg⁻¹ (OLE 300) em uma única dose oral. Após três horas, fragmentos do coração, rins, fígado e cérebro foram coletados para determinação dos níveis de oleandrina. A extração e procedimentos cromatográficos foram eficientes na detecção e quantificação da oleandrina nos tecidos, com tempo de retenção de 1,2min e limite de detecção de 0,001µg g⁻¹. A análise cromatográfica dos animais tratados indicou que a oleandrina é distribuída de forma equalizada pelos tecidos analisados. A metodologia desenvolvida representa uma forma de diagnóstica segura,

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efetiva e rápida da intoxicação por *N. oleander* a partir de amostras de tecidos de necropsia.

TERMOS DE INDEXAÇÃO: Oleander, *Nerium oleander*, cardenolídeos, teste diagnóstico, cromatografia líquida, espectrometria de massa, cobaias, toxicoses.

INTRODUCTION

Nerium oleander L. is an ornamental plant that belongs to the family Apocynaceae, native in Mediterranean regions of Europe and Asia and cultivated in tropical and subtropical areas of the world. All parts of the plant are poisonous, including flowers, fruits, leaves and roots (Langford & Boor 1996). Its ingestion can lead to acute heart lesions and death in humans and animals (Bandara et al. 2010, Caloni et al. 2012). Poisoning reports have been described in South and North America (Dorsey 1962), Europe (Caloni et al. 2012), Australia (Hughes et al. 2002) and Asia (Khan et al. 2010) and most cases are caused by accidental ingestion, attempted suicide and phytotherapeutic use of *N. oleander* (Bandara et al. 2010, Dorsey 1962). Veterinary reports are associated with times of drought, lack of forage and curiosity of the animals (Langford & Boor 1996, Soto-Blanco et al. 2006, Caloni et al. 2012) and have been reported in dogs (Caloni et al. 2012), cattle (Soto Blanco et al. 2006), horses (Hughes et al. 2002), sheep (Barbosa et al. 2008), and birds (Alfonso et al. 1994).

N. oleander toxicity is attributed to cardiac glycosides, including oleandrin, rosalenin, folinerin, nerlin and digitoxigenin (Begum et al. 1999).

These substances are structurally similar to digoxin and can potentially inhibit the Na⁺/K⁺ pump of the heart and cause severe clinical signs that may include nausea, vomiting, abdominal pain, diarrhea, drowsiness, paresthesia, dyspnea and cardiac arrhythmias (Soto-Blanco et al. 2006, Bandara et al. 2010).

Diagnosis of the poisoning is based on clinical signs, medical record, description of the plant and necropsy findings that are usually similar to other intoxications. Definitive diagnosis is only provided by laboratory testing (Bandara et al. 2010). Current available techniques for the detection of *N. oleander* toxic components include digoxin immunoassay, liquid chromatography, HPLC and spectrophotometric assay (Karawya et al. 1973, Dasgupta & Datta 2004, Tor et al. 2005).

The first description of cardiac glycoside identification was a colorimetric method based on Baljet's reagent (picric acid in alkaline medium) and produced initial data on *N. oleander* glycoside content (Karawya et al. 1973). Studies considering molecular similarities between digoxin and oleandrin suggested that radioimmunoassays could be helpful in diagnosing the poisoning. Several reports correlated serum levels of digoxin and oleandrin for presumptive diagnosis of *N. oleander* poisoning (Shumaik et al. 1988, Dasgupta et al. 2011), although important discrepancies between concentrations can be found (Blum & Rieders 1987).

Thin layer chromatography, fluorescence spectrometry, and immunoassays were also studied and determined to be insufficiently sensitive or specific to detect and quantify oleandrin in biological tissues (Blum & Rieders 1987). Especially in forensic situations, undeniable identification of oleandrin is required and liquid chromatography coupled to

mass spectrometry (LC-MS) is the most recommend method (Tor et al. 2005).

In these cases LC-MS is a rapid and precise methodology that confirms *N. oleander* exposure through its main component oleandrin (Tor et al. 2005). LC-MS methodologies require small volume of samples and solvents (Oliveira et al. 2014). Considering the significance of *N. oleander* poisonings, its severe toxicity and the importance of quick diagnosis, this study aimed to develop a simple, rapid and precise method for tissue extraction and quantification of oleandrin in necropsy samples.

The method is based on the QuEChERS (Quick, Easy, Cheap, Effective, Rugged and Safe) extraction, originally developed as a field of application for residue analysis of pesticides (Anastassiades et al. 2003). In recent years, however, the method has also been applied, with satisfactory results, to the analysis of other contaminants and residues, such as mycotoxins, veterinary drugs, steroids and plant toxins, in different matrices, such as milk, animal tissues, beverages, honey, egg and animal feed (Mol et al. 2008, Klinsunthorn et al. 2011, Tamura et al. 2011). Modified extraction technique and ultra-fast liquid chromatography/tandem mass spectrometry (UFLC-MS/MS) analysis, provides specific and faster diagnosis for *N. oleander* poisoning.

MATERIALS AND METHODS

Animals and groups. The research project was approved by the Ethical Committee for Animal Experimentation of Universidade Federal de Minas Gerais (CEUA/UFGM) under protocol number 216/2014. *Nerium oleander* L. (family Apocynaceae) leaves were harvested from a white flowered plant in Belo Horizonte/MG, Brazil. A voucher specimen was deposited in the BHC Herbarium at UFGM, Belo Horizonte/MG, Brazil, under number BHC 182685. Fresh leaves were cleaned and dried in a heater at 60°C for 48 hours. Leaves were grinded and extracted with hydroalcoholic (1:1) solution in ultrasonic bath for 20min (Karawya et al. 1973, Calderón-Montaña et al. 2013). Ethanol was eliminated with rotating evaporator and extract was diluted with water.

A total of 36 adult male guinea pigs, mean weight 700g (600-900g), were kept in cages maintained under appropriate conditions in the Laboratory of Animal Experimentation, Veterinary School, UFGM, and received commercial chow and water *ad libitum*. They were randomly distributed into three equal groups (n=12): CON control group that only received water, OLE 150 that received 150mg.kg⁻¹ of *N. oleander* extract and OLE 300 that received the same extract at 300mg.kg⁻¹. These administrations were performed once via oral gavage. They were clinically monitored and euthanized after 3 hours with 150mg.kg⁻¹ of thiopental, by intraperitoneal injection.

Tissue extraction. Immediately after necropsy exam was conducted, samples from the heart, liver, kidneys and cerebral cortex were collect, weighed and stored frozen in -20°C. Samples were homogenized for extraction with adapted QuEChERS methodology (Oliveira et al. 2014).

Ultrapure water, for preparation of the aqueous mobile phase, was obtained via a water purification system (Direct 3-Q UV, Millipore, Molsheim, France). Oleandrin standard (>95% purity; Sigma-Aldrich Chemical Co., St Louis, MO, USA) was used for control analysis. Tissue samples from control group, including brain, liver, kidney and heart were used as blank matrix.

Samples of 5g from each tissue (blank and sample) were weighed and 5mL of water added for homogenization in vortex mixer for 1min.

Extraction phase was conducted with 7mL of MeCN (acetonitrile MS grade, Tedia, Fairfield, OH, USA), 3mL of ethyl acetate PA (Tedia, Fairfield, OH, USA), 4g of MgSO_4 (magnesium sulphate; Sigma-Aldrich Chemical Co., St Louis, MO, USA) and 1g of CH_3COONa (anhydrous sodium acetate, purity $\geq 98\%$; Vetec, Rio de Janeiro, Brazil). Samples were centrifuged in 4000rpm for 11min and frozen overnight followed by another centrifugal process in 4000rpm for 5min. The supernatant (1.0mL) was transferred to a 1.5mL centrifuge microtube (Eppendorf, Hamburg, German) containing 150mg of MgSO_4 , 30mg of PSA (primary secondary amine; Varian, Harbor City, CA, USA) and 30mg of C18 (Sigma-Aldrich Chemical Co., St Louis, MO, USA). Samples were agitated for 1min in vortex mixer and centrifuge in 9000rpm for 12min for injection in UFLC-MS/MS system.

UFLC-MS/MS analyses. A stock solution of $1000\mu\text{g mL}^{-1}$ of oleandrin was made in acetonitrile. Subsequent dilutions of the stock solution were also made in acetonitrile. Six-point matrix-matched calibration curves were prepared 0.005, 0.01, 0.025, 0.05, 0.075 and $0.10\mu\text{g g}^{-1}$ by adding aliquots of oleandrin standard to dry, blank extracted samples.

Chromatographic analyses were performed using an ultra fast liquid chromatographic (UFLC) system (Shimadzu LC20ADXR, Shimadzu, Kyoto, Japan) equipped with a binary pump (Shimadzu LC20ADXR), an autosampler (Shimadzu SIL20ACXR), and a column oven (Shimadzu CTO20AC). Separations were achieved using an ODS column (Shimadzu Shimpack XR-ODSII column; $2.0 \times 100\text{mm}$, $2.2\mu\text{m}$ particle size). Isocratic separation was carried out with a mobile phase consisting of 20% ammonium acetate 10 mmol L^{-1} (anhydrous ammonium acetate, purity $\geq 98\%$; Vetec, Rio de Janeiro, Brazil) with A: 0.01% formic acid (PA, Tedia, Fairfield, USA), and B: 80% methanol (HPLC grade, Mallinckrodt, Phillipsburg, PA, USA) at a flow rate of 0.4 mL min^{-1} , with total run time of 3min. An injection volume of $10\mu\text{L}$ was used and the column temperature was set to 60°C .

Mass spectrometric analysis was carried out using a triple quadrupole mass spectrometer (5500 Triple Quad mass spectrometer,

Applied Biosystems/MDS SCIEX, Concord, Canada). The instrument was operated under electrospray ionization conditions (ESI) positive ion modes. Instrument settings, data acquisition, and processing were controlled by computer software (Analyst version 1.5.1, Applied Biosystems, Foster, CA, USA). Oleandrin was previously infused and optimized to the mass spectrometer. The source parameters optimized are as follows: ion spray voltage, 5.5 kV; curtain gas, 20psi; collision gas, 8psi; nebulizer and auxiliary gas, 30psi; ion source temperature, 500°C . The MS/MS conditions for oleandrin were: precursor ion- 577.2 [M+H]^+ ; quantification and confirmation ions- 433.1 and 113.0; declustering potential (DP) 201 V; whit energy potential (CE)- 13 and 21 V; collision exit potential (CXP)- 38 and 10 V respectively.

The chromatograms of the evaluated compounds were used as standards for comparison with the test samples, and spiked samples at 0.01 and $0.1\mu\text{g g}^{-1}$ were used with control of analytical quality.

Statistical analysis. Data are presented as mean \pm SEM (standard error of the mean). Statistical significance between groups were determined by T test. Significance level was set at $p < 0.05$. Data were analyzed using a computer software (GraphPad Prism v. 6.0, GraphPad, La Jolla, CA, USA).

RESULTS

Oleandrin was identified in the extract by retention time, mass weight and ion spectra and compared to the standard solution. Concentrations of oleandrin were determined by quantifying the peak area for the primary ion. HPLC-MS/MS detected oleandrin at retention time of 1.2min, with collision energy of 13, temperature of 520°C , cone exclusion potential of 28 and declustering potential of 201. Total run time was 3min and detection limit 0.001 ng g^{-1} . Concentration levels found in hydroalcoholic extract of *Nerium oleander* corresponded to 1mg of extract containing $4.2\mu\text{g}$ of oleandrin with linear correlation $R = 0.99$ (Fig.1).

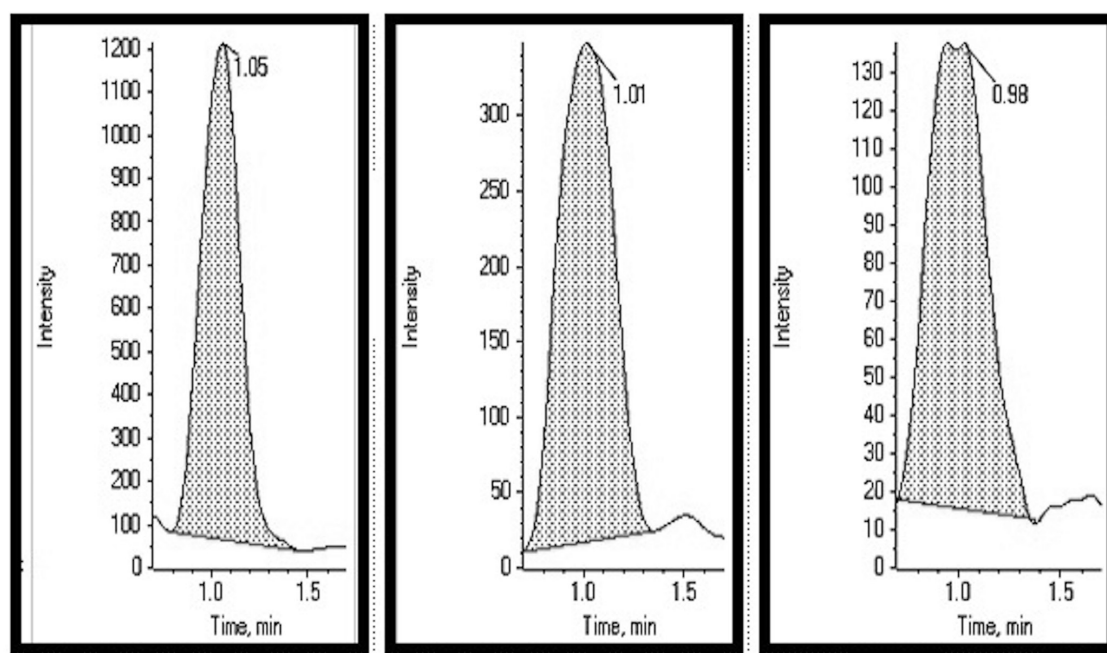


Fig.1. Chromatograms showing the retention time (in min) for oleandrin, obtained from total ion chromatogram from (A) control spiked sample of oleandrin. (B) Liver sample from guinea pig (*Cavia porcellus*) dosed with 300 mg g^{-1} of *Nerium oleander* extract. (C) Heart samples derived from the same individual.

As shown in Figure 2, oleandrin tissue concentration was significantly greater in cerebral cortex of OLE 300 group ($p < 0.05$) than in OLE 150, although no significant differences in levels from heart, kidney and liver were observed. Comparison among tissues also showed no significant differences in oleandrin content, although increasing values were found: cerebral cortex, heart, kidney and liver.

DISCUSSION

This appears to be the first study that evaluates tissue oleandrin detection and distribution in guinea pigs poisoned by *Nerium oleander*. It presents a new extraction technique

for oleandrin and a quick UFLC-MS identification and quantification method.

Several cases of *N. oleander* poisoning are reported as forensic cases, and the necropsy findings are usually mild and similar to those found in other poisonings, confounding the diagnosis. Plants that contain cardiac glycosides such as *Thevetia peruviana* and *Digitalis lanata*, and drug poisonings concerning digoxin and digitoxin intake, may cause the same clinical signs, cardiac arrhythmias and morphological lesions (Arao et al. 2002, Khan et al. 2010). In these cases, LC-MS/MS is the most recommended methodology to confirm the poisoning based on oleandrin content found in biological and plant tissues (Tor et al. 2005).

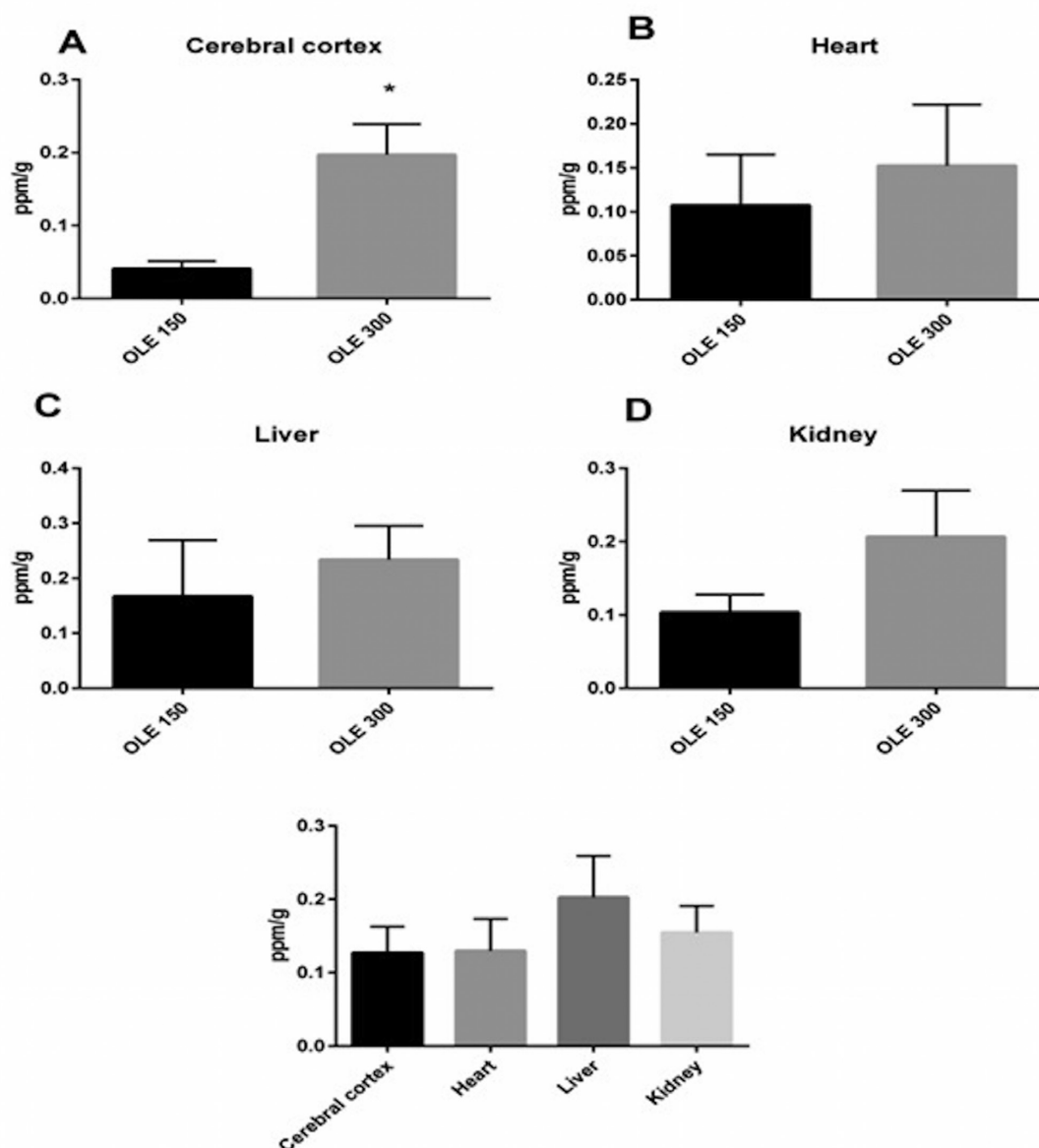


Fig.2. Mean and SEM of oleandrin concentration values (ppm) found in the organs of guinea pigs (*Cavia porcellus*) dosed with 150 and 300mg g⁻¹ de *Nerium oleander* detected by QuEChERS extraction and UFLC/MS (T test, $p > 0.05$). (A) Values found in the cerebral cortex with significant rise in the group that received 300mg g⁻¹ of *N. oleander* extract. (B) Values found in the heart of both groups, showing no significant differences. (C) Values found in the liver of both groups also showing no significant differences. (D) Values found in the kidney of both groups without statistical differences. (E) Oleandrin concentrations compared between organs of OLE 150 and OLE 300, showing no statistical difference.

Oleandrin is main cardiac glycoside component in *N. oleander*, responsible for the clinical signs reported (Begum et al. 1999). The extract also contains flavonoids, tannin and other steroids not accessed in this evaluation. Oleandrin concentrations in plant extracts were previously studied using HPLC, obtaining median values of oleandrin of 4.89mgg^{-1} , very similar to those found in the present study of 4.2mgg^{-1} , confirming the cytotoxic potential of the extract (Pedroza et al. 2014). No difference has been reported in oleandrin content between plant varieties, pink, red and white flowers (Pedroza et al. 2014), justifying present use of the white variety. Leaves were used in the production of the extract due to its accessibility, quantity and harvesting potential (Barbosa et al. 2008).

Previous work has detected oleandrin in the liver, heart, urine, cerebrospinal liquid, muscle, stomach content and serum of poisoned individuals with simple extraction techniques and different ionization methods (Galey et al. 1996). In order to establish a rapid, simple and robust technique of extraction and analysis of oleandrin this study was performed. QuEChERS methodology is frequently used for multiresidues analysis of pesticides in vegetable origin samples and has been modified for different matrixes analysis (Oliveira et al. 2014). It removes many polar matrix contaminants, such as polar pigments, sugars and organic acids that disrupt chromatography readings. It establishes a simple, inexpensive and fast process for determination of different substances in various matrixes with minimal amounts of solvents with high quality results (Anastassiades et al. 2003). This is a new extraction method for oleandrin identification that provides cleaner samples to enhance reliability and promote faster readings.

In this study, oleandrin retention time was 1.2min and total detection at medium 3min. Faster identification is associated with newer technologies in chromatography and mass spectrometry, and is directly related to cleaner extraction technique derived from QuEChERS method. Mass spectrometry previous analyses were conducted with positive ion electrospray ionization mode using a five point calibration curve and non-weighted linear regression. Retention time and spectrum of oleandrin in the heart muscle was identical to the liver (Tor et al. 2005). Detection limit determined by the same technique was 0.15ngml^{-1} in blood and cerebrospinal fluid samples (Arao et al. 2002).

Considering oleandrin toxicokinetics and metabolism, higher oleandrin levels are expected in the liver while kidney samples are expected to have lower levels (Ni et al. 2002). However, oleandrin levels found in the present study were similar in liver and kidneys, showing both tissues are valuable samples for this toxicological analysis. Myocardial tissue also showed a considerable amount of the glycoside, which partially explains cardiac toxicity and potential use of oleandrin in heart insufficiency therapy (Alfonso et al. 1994, Barbosa et al. 2008, Gayathri et al. 2011). Important ECG alterations are associated with the ingestion of *N. oleander* leaves, flower and pods, in humans and animals (Hughes et al. 2002, Barbosa et al. 2008), and the content of oleandrin in heart is especially important to determine the severity of poisoning in clinical and forensic reports (Arao et al. 2002).

It has been established that oleandrin can rapidly accumulate in the brain tissue of rats, crossing the blood barrier and cause important neurological depressant activity

in severe poisoning cases (Siddiqui et al. 1997, Ni et al. 2002). Clinical signs include disorientation, incoordination, convulsion and paresis (Siddiqui et al. 1997). In spite of these important signs, morphologic and histological alterations are usually nonspecific and include congestion and hemorrhage (Ada et al. 2001). Cerebral concentrations of oleandrin are dependent of other components of the extract, because these concentrations are higher after dosing the *N. oleander* extract than the pure oleandrin. However, the mechanism of this interaction remains to be clarified (Ni et al. 2002). This is the first report that oleandrin reaches the cerebrum in guinea pigs and represents an important finding, as the species is considered a model for cardiac toxicity evaluation and is significantly more sensitive to cardiac glycosides than rats and mice (Morissette et al. 2013).

CONCLUSIONS

The chemical study presents new conditions for diagnosis of *Nerium oleander* poisoning.

New techniques of extraction and oleandrin tissue identification provided detection time of only 1.2min and total run of 3min.

The procedure simplifies workup steps, leading to a cleaner sample for UFLC-MS/MS.

This is also the first report of oleandrin tissue distribution in guinea pigs and the first to identify the glycoside in brain tissue of this species.

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