Detection of Residues of Cardenolides of *Nerium oleander* by High-Performance Thin-Layer Chromatography in Autopsy Samples

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ABSTRACT

Background*: Nerium oleander* is an evergreen shrub of Apocynaceae family cultivated worldwide as an ornamental plant. All parts of the plant are toxic and contain a mixture of very toxic cardiac glycosides of cardenolides. A number of techniques were used to determine the cardenolides of *N.oleander* in various biological matrices. A survey of literature has revealed that the use of high-performance thin-layer chromatography (HPTLC) for the detection of oleander glycosides is very scanty.

Method: A simple and rapid HPTLC method for separation and identification of cardenolides of *N.oleander* is reported. The cardenolides present in the aerial parts of the plant and residues available in the autopsy samples sent in cases of poisoning; were extracted with chloroform by using accelerated solvent extractor (ASE).

Results: Separation of cardenolides was achieved on precoated silica gel $60F_{254}$ HPTLC plates with chloroform-acetone-acetic acid 8.5:1:0.5 (v/v) as mobile phase and densitometric analysis was carried out at 275 nm. A comprehensive study for the separation and detection of cardenolides in general and oleandrin in particular were studied by new mobile phases and spray reagents. The ^{1}H-NMR spectra were recorded for the separated components and the component corresponding to oleandrin was identified.

Conclusion: The method has specific advantages that it is simple, rapid and has higher resolution of separation achieved so as to be free from interferences from the plant and forensic matrices.

►*Implication for health policy/practice/research/medical education:*
Residues of Cardenolides of *Nerium oleander*

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1. Introduction:

Nerium oleander (common oleander) is an evergreen shrub belongs to the family of

Apocynaceae. It originates from the Mediterranean region and widely cultivated as an ornamental plant in many parts of the world, particularly in warm temperate and subtropical regions. This plant has long been known to be poisonous to animals and human beings (1). All parts of the plant are toxic and dry leaves are about as toxic as green ones (2). The most frequent source for oleander exposure is plant clippings especially leaves. Parts of the plant can be ingested intentionally in suicidal attempts or accidentally. Following the ingestion of oleander, the clinical picture usually starts with gastrointestinal signs: nausea, vomiting, abdominal pain and diarrhea. Later cardiovascular and neurological symptoms can occur. Sinus bradycardia or different degrees of atrioventricular (AV) block are the most frequent cardiac features. The main neurological symptoms are tremor, drowsiness and ataxia (3, 4).

Oleander contains a mixture of cardenolides (5), the most important of which are oleandrin and oleandrigenin (3, 5, 6). Oleandrin constitutes approximately 0.08% of the total content of cardenolides present in *N.oleander* (3). The oleandrin cause poisoning by inhibiting plasmalemmal Na^+/K^+-ATPase. The inhibition of membrane bound Na^+ K^+-ATPase is the principal mechanism of action for sodium and potassium ion exchange resulting in positive inotropic effect. This effect is similar to that of digitalis glycosides (7). Oleandrin is more lipophilic in character and is absorbed faster resulting in slow urinary excretion rate and longer duration of action as cardiotoxic. It is reported that the specific orientation of unsaturated lactone ring at -17 positions and a hydroxyl group at -14 position of oleandrin (Figure 1) have direct bearing on the efficacies of inhibition of Na^+ K^+- ATPase (8).

Many authors in India, Sri Lanka, Australia and United States have reported reports of toxicity and deaths in children, adults and domestic animals. Generally, children younger than six years are exposed to this plant accidentally and without significant toxicity. More serious toxicity is usually resultant of suicidal attempt by the adults. Blum & Rieders has described a fatal case of poisoning following rectal and oral administration of *N.oleander* extract (9). In cases of deaths by the ingestion of *N.oleander*, although various other pathological markers, K^+ level in serum are available as indicators of poisoning by cardenolides, it is necessary to determine oleandrin from the autopsy samples in order that the precise cause of death is evolved.

A significant number of deaths due to consumption of oleander extract in a suicidal intention have been reported in our laboratory. The available techniques reported for the detection of toxic components of N.oleander in various biological matrices include spectrophotometric assay (3), TLC (9, 10), HPLC (11, 12), HPLC/MS (13), LC/MS/MS (4, 14, 15) and digoxin immunoassay (16, 17). To our knowledge, there is absolute paucity on the use of high-performance thin-layer chromate-graphy (HPTLC) for the detection of cardenolides of *N.oleander*. HPTLC coupled with densitometry is simple, rapid, and of high analytical precision. It has advantages over other techniques because several samples (10-20 samples) can be separated in one run and small quantities of solvents are used, this reduces the time and cost of analysis. For this purpose, we developed a simple and rapid method for the detection of toxic components of *N.oleander* in different parts of the plant and in the autopsy samples. The method is based on the extraction of toxic components of *N.oleander* by a new accelerated solvent extraction (ASE) procedure and detection of the extracted residues by HPTLC and characterization of the band corresponding to oleandrin by ^{1}H-NMR.

2. Materials and Methods:

Reagents and Chemicals

All chemicals and solvents used were of analytical grade (Merck) and used without further purification. The spray reagents were prepared as reported.

Nerium oleander plant was identified and samples of leaves, flowers and twigs (without leaves) were collected. All the samples were air-dried at room temperature and grinded to a particle size of 2-3 mm. The powdered plant material was stored in glass vials protected from light and humidity.

Extraction Procedure

Extraction was carried out in accelerated solvent extractor (ASE 300, Dionex) by use of chloroform as extraction solvent at ambient temperature; pressure: 1500 psi; heat-up time: 5 min; static time: 10 min; flush volume: 60%; purge time: 100 seconds; and static cycle: 2.

Finely minced tissue was chemically dried using anhydrous sodium sulphate and diatomaceous earth (acid washed, approximately 95% SiO_2). Dried sample of the tissue and the plant materials were packed in the 66 mL stainless steel extraction cells on a bed consisting of cellulose filter disc, aluminium oxide (column chromatography grade, particle size 100-125 mesh) and silica gel of particle size 10-40 microns. The extract was collected in 100 mL glass vials. 5 mL of the postmortem blood was protein precipitated, centrifuged for 10 min at 3000 rpm and the supernatant was vortex mixed with chloroform for 5 min. The organic phase was separated by centrifugation at 3000 rpm for 10 min and was evaporated to dryness using N_2 gas and reconstituted with 1 mL of methanol.

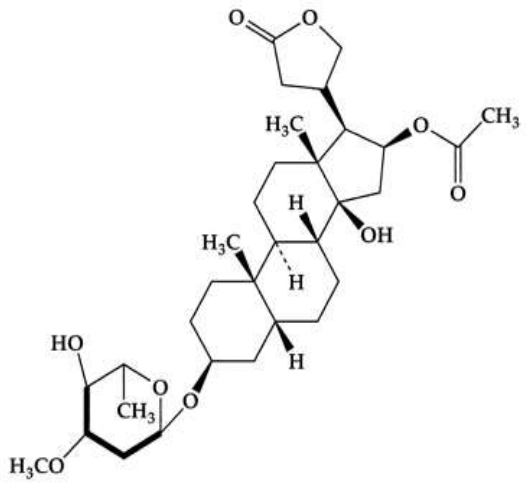

Fig. 1. Structure of oleandrin.

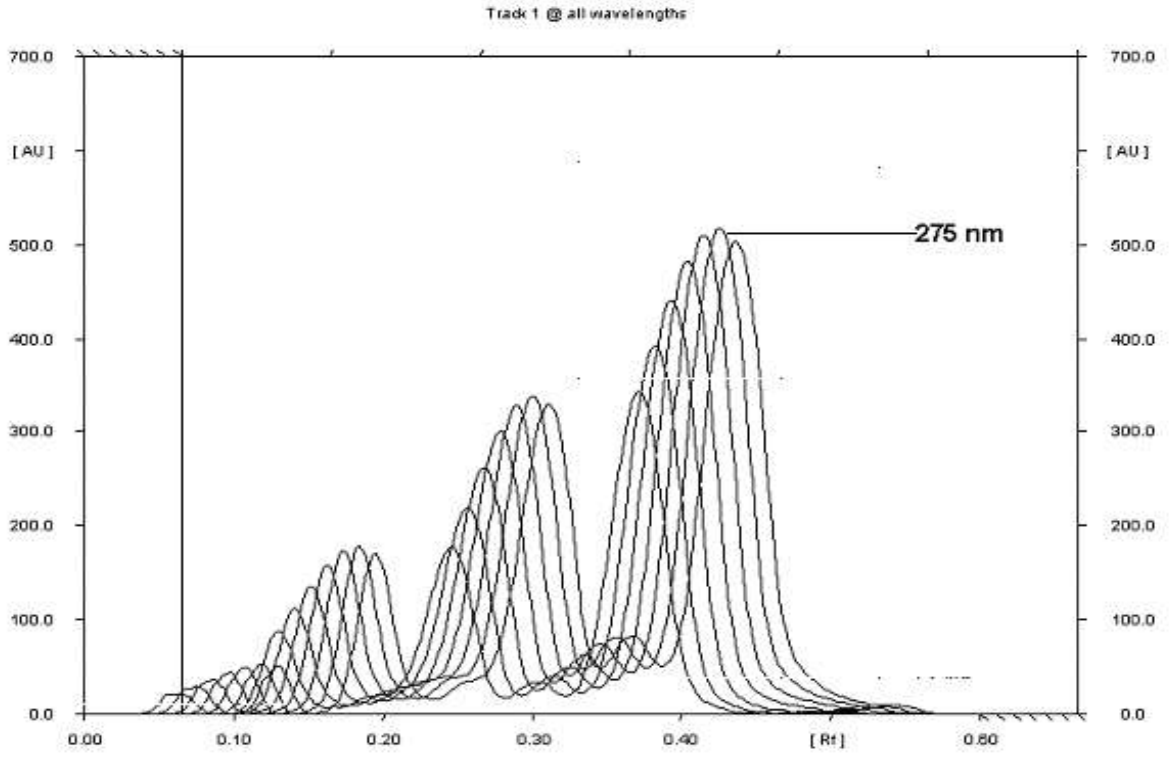

Fig. 2. Multiwavelength scan of cardenolides showing UV apex of absorbance at 275 nm.

Table 1: Retardation factors (R_F) of cardenolides present in the leaf extract of Nerium oleander.

Mobile phase	R_F
Benzene-Acetone 7:3 (v/v)	0.14, 0.28, 0.42
Benzene-Ethanol 9:1 (v/v)	0.20, 0.27, 0.38
Chloroform-Acetone-Acetic acid 8.5: 1: 0.5 (v/v)	0.18, 0.31, 0.49
Dichloromethane-Methanol 9.5:0.5 (v/v)	0.35, 0.42, 0.52
Ethyl acetate-Isopropanol-Water 7:2.5:0.5 (v/v)	0.50, 0.63, 0.78
Chloroform-Acetone 8:2 (v/v)	0.09, 0.19, 0.32
Ethyl acetate-Methanol-Ammonia 8.5: 1: 0.5 (v/v)	0.39, 0.52, 0.59
Chloroform-Acetonitrile-Methanol 7:2.5:0.5 (v/v)	0.38, 0.48, 0.59
Hexane-Ethyl acetate-Acetic acid 3:6.5:0.5 (v/v)	0.07, 0.17, 0.28
Toluene-Ethyl acetate-Acetic acid 6:3.5:0.5 (v/v)	0.04, 0.09, 0.19

Chromatography

Chromatography was performed on 20 cm × 10 cm aluminum foil HPTLC plates coated with silica gel $60F_{254}$ (Merck). Samples and standard solutions were applied to the plates as 6 mm bands, 15 mm apart, 10 mm from the edges and bottoms of the plates, by use of a CAMAG (Muttenz, Switzerland) Linomat 5 automatic sample applicator equipped with a 100-µL syringe and N_2 flow. The volume applied was 2-50 µL with a sample-delivery speed of 100 nL s^{-1}. The plates were developed in a CAMAG glass twin-trough chamber, with chloroform-acetone-acetic acid 8.5:1:0.5 (v/v), as mobile phase. The development distance was 8 cm from the lower edge of the plate.

Photo documentation was by illumination of the plate at 254 nm and 366 nm and use of a CAMAG Reprostar 3 digital documentation system with built in high-resolution 12-bit CCD camera.

In-situ densitometric scanning was performed with a CAMAG TLC Scanner 3 equipped with CATS 3 software, in absorption mode 275 nm with slit dimensions 6.00 mm × 0.45 mm, using the deuterium light source.

3. Results:

Accelerated solvent extraction using chloroform as extraction solvent yielded clean extracts of the samples. The efficacies of separation of *N.oleander* components were high in the mobile phase consisted of chloroform-acetone-acetic

Table 2: Responses of specific groups present in cardenolide (Oleandrin) with different spray reagents.

Reagents	Color	Stability (min)	Group (moiety) specificity
Keddle (3, 19)	Violet-red.	5.0	Lactone
p-anisaldehyde (9)	Blue after heating the plate for 10min at 110^0C.	20-30	Lactone
p-toluene sulfonic acid (20)	Yellow after heating the plate for 5min at 110^0C, blue fluorescence at366 nm.	40-50	Steroid
Aluminium (III) chloride	Yellow after heating the plate for 5-10 min at 110^0C, blue fluorescence at 366 nm.	35-45	Steroid
Antimony (III) chloride (20)	Yellow. Blue fluorescence at 366 nm.	35-45	Steroid
Phosphoric acid	Yellow. Bright blue fluorescence at366nm.	40-50	Steroid
Orcinol	Yellow. Blue fluorescence at 366 nm.	40-50	Glycone
Vanillin – Sulphuric acid (19)	Brown.	30-40	Steroid

The references are indicated in parenthesis.

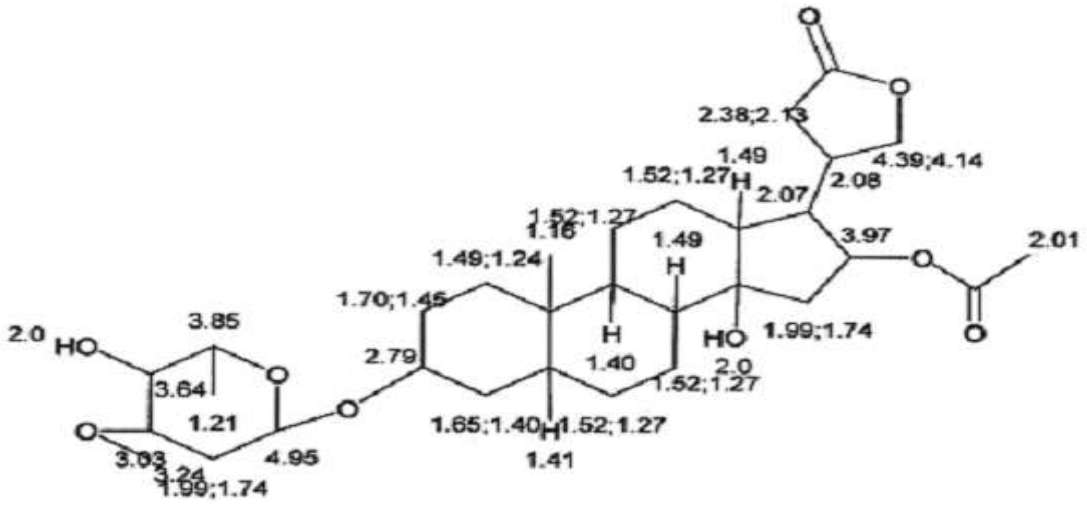

Fig. 3. Structure of oleandrin (^{1}H NMR at 400 MHz).

acid 8.5:1:0.5 (v/v). The components were separated into three bands with R_F values 0.18, 0.31 and 0.49. The wavelength of maximum absorption was determined to be

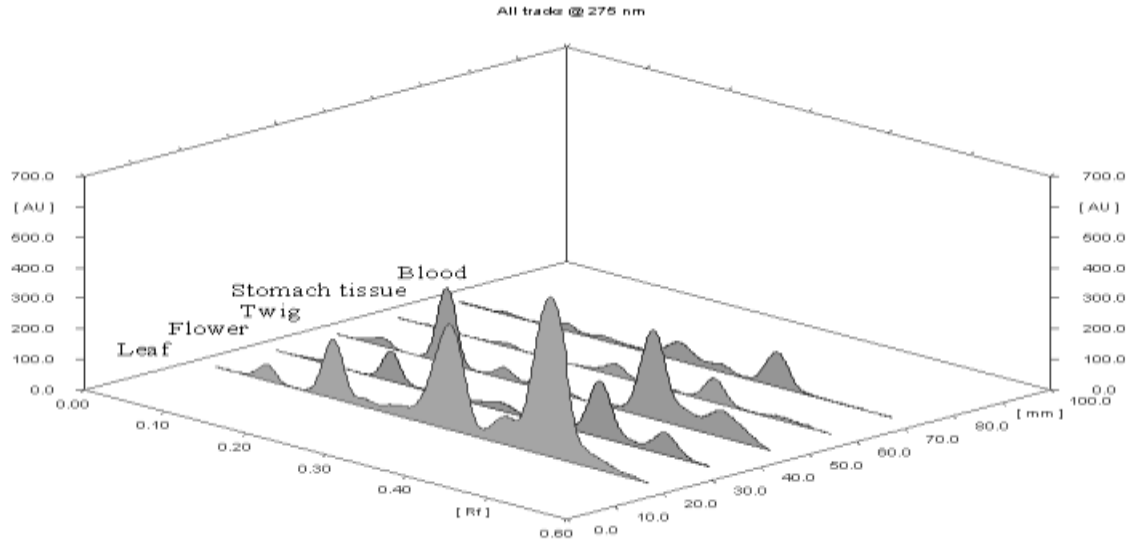

Fig. 4. Densitogram of cardenolides present in the plant parts and autopsy samples.

275 nm. The obtained densitometric profile, use of different spray reagents in succession and characterization of the separated components by ^{1}H-NMR has confirmed the presence of oleandrin in the plant and autopsy samples.

4. Discussion:

The extraction procedure adapted in this study has the advantages of requiring minimum quantity of solvent, prevents exposure to hazardous solvent vapors and reduces the extraction time, emulsion formation and excess clean-up procedure. The use of ASE eliminates the filtration step since the extracts pass through the cellulose filter disc and 10-μm frits as they exit the extraction cell. Drying of the samples using anhydrous sodium sulphate and diatomaceous earth makes the extracts free of troublesome co-extractables because the water typically found in the tissues can act as a polar solvent and produce co-extractables, which complicate extract clean-up.

For the separation and resolution of *N.oleander* components, mobile phase composition was optimized using the extracted residue obtained from the leaves. A total of twenty-five solvent systems were screened for their function as mobile phase by using CAMAG HPTLC vario system in which six mobile phases can be tested in a single run. The visible and perceptible differences in separation were seen in ten mobile phases which are documented at 254 nm and 366 nm. The efficacies of separation was high in the mobile phase consisted of chloroform-acetone-acetic acid 8.5:1:0.5 (v/v), although the separation was found to be satisfactory in the other mobile phases (Table 1). In each of the mobile phase, the components of the *N.oleander* were separated into three bands. Densitometric profile and in-situ UV spectrum was recorded for the separated components in each of the mobile phase. The wavelength of maximum absorption was determined to be 275 nm by measurement of the *in-situ* UV absorption spectrum and by the multiwavelength scan between 200-400 nm (Figure 2). The densitometric profile in each of the mobile phase revealed that the separation was excellent in the mobile phase consisted of chloroform-acetone-acetic acid 8.5:1:0.5 (v/v). Increasing the polarity of mobile phases resulted not only in crowding of the cardenolides but also in the co-elution of excipients from the plant matrix. The lower polarity of the solvent and better efficiency of separation appears to draw support from the structure of cardenolides.

The suitability of few chromogenic spray reagents was examined (Table 2). It is observed that the use of Keddle reagent and p-anisaldehyde reagent indicated the presence of lactone moiety while orcinol reagent indicated the presence of glycone moiety. The other spray reagents responded for the presence of steroid moiety. The orcinol and phosphoric acid reagents give yellow color spots immediately and the stabilities range from 40-50 min. The increased stability is believed to be due to specific group reactivity of glycone and steroid moieties present in oleandrin. The use of spray reagents (listed in the table) in succession has confirmed the presence of lactone, steroid and glycone moieties present in oleandrin.

The densitometric profile obtained has revealed that the band with R_F value 0.49 has larger peak area and peak height when compared to the other two bands. The characteristic color obtained by use of Keddle and p-anisaldehyde reagents for the band with the R_F value 0.49 has indicated the presence of oleandrin. This was further confirmed by recording ^{1}H NMR spectra for each of the separated bands. Each of the separated bands were isolated into methanol and evaporated to dryness. The residue was reconstituted with $CDCl_3$ and ^{1}H NMR was obtained at 400 MHz. The results of the ^{1}H NMR have confirmed the molecular structure of the constituent present in the band with R_F value 0.49 was that of oleandrin. The results of NMR were comparable to that of those obtained at 300 MHz (18). The structure is presented in Figure 3. The band thus confirmed as oleandrin was used as standard for identification of oleandrin in the extracts of the autopsy samples obtained from a forensic case. The deceased was a 45-year-old male suffering from paralysis. Despite treatment given in several hospitals, he could not recover completely and he consumed crushed parts of *Nerium oleander* under the state of depression. He was declared dead after a day's treatment in the hospital. Autopsy samples of the deceased viz, stomach, small intestine, liver, kidney and blood samples were received in the laboratory for toxicological analysis. The results have indicated the presence of higher concentration of oleandrin in the tissue extracts than other cardenolides (Figure 4). This could be the unmetabolized oleandrin and possibly have originated from excessive ingestion of cardiac glycosides, which most commonly occurs in cases of suicides.

5. Conclusion:

The unique finding of oleandrin in autopsy samples in the absence of standard would be of paramount interest in forensic science particularly in cases of deaths by cardiac glycosides. The HPTLC method developed is simple, rapid and efficient for the separation and residual determination of oleandrin from *Nerium oleander* and autopsy samples. The method could be applied routinely for the determination of oleandrin from a complex forensic matrix and postmortem samples, without any interference from the components of matrix; saving time and quantum of solvents and with higher accuracies of determination.

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References

1. Frohn D, Pfander HJ. A Color Atlas of Poisonous Plants, (Wolf Science, London). 1983.
2. Galey FD. Oleander (Nerium oleander) Toxicosis. In: J.L. Howard (ed.), Current Veterinary Theraphy 4, Food Animal Practice, (W.B. Saunders, Philadelphia). 1999;275-276.
3. Nerium oleander: International Programme on Chemical Safety Poisons Information Monograph 366.

4. Langford SD, Boor PJ. Oleander toxicity: an examination of human and animal toxic exposures. Toxicology. 1996;109(1):1-13.
5. Khare CP (Ed). Encyclopedia of Indian Medicinal Plants, Rational Western Therapy Ayurvedic & Other Traditional Usage & Botany. Springer-verleg, Germany. 2004;328-330.
6. Gossel TA, Bricker JD. Principles of Clinical Toxicology. 3rd Edn. Raven press, New York. 1994; Chapter 11 & 18.
7. Kingsbury JM. Poisonous Plants of the United States and Canada. Printice-Hall, Inc, Englewood Cliffs, Newjersey, 1964;626.
8. Blum LM, Rieders F. Oleandrin distribution in a fatality from rectal and oral Nerium oleander extract administration. J Anal Toxicol. 1987;11(5):219-221.
9. Wagner H, Bladt S. Plant Drug Analysis, 2nd ed. Berlin, Springer. 1995.
10. Hamad K, Iwamoto A, Miyazaki S, Yamanaka N, Guruge KS. Determination of bovine blood oleandrin by high-performance liquid chromatography and postcolumn derivatization. J Chromatogr Sci. 2002;40(9):515-518.
11. Namera A, Yashiki M, Okada K, Iwasaki Y, Kojima T. Rapid quantitative analysis of oleandrin in human blood by high – performance liquid chromatography. Nippon Hoigaku Zasshi. 1997;51(4):315-318.
12. Tracqui A, Kintz P, Branche F, Ludes B. Confirmation of oleander poisoning by HPLC/MS. Int J Legal Med. 1998; 111(1):32-34.
13. Pietsch J, Oertel R, Trautmann S, Schulz K, Kop B, Dressler J. A non-fatal oleander poisoning. Int J Legal Med. 2005;119:236-240.
14. Tor ER, Filigenzi MS, Puschner B. Determination of oleandrin in tissues and biological fluids by liquid chromatography-electrospray tandem mass spectrometry. J Agric Food Chem. 2005;53(11):4322-4325.
15. Wang X, PlomLey JB, Newman RA, Cisneros A. LC/MS/MS analyses of an oleander extract for cancer treatment. Anal Chem. 2000;72(15):3547-3552.
16. Dasgupta A, Datta P. Rapid detection of oleander poisoning using digoxin immunoassays: comparison of five assays. Ther Drug Monit. 2004;26(6):658-663.
17. Jortani SA, Helm RA, Valdes R Jr. Inhibition of Na, K-ATPase by oleandrin and oleandrigenin, and their detection by digoxin immuno assays. Clin Chem. 1996;42(10):1654-1658.
18. Subashini HD, Malarvannan S, Pillai RR. Dodnaea angustifolia-a potential biopesticide against Helicoverpa armigera. Current Science. 2004;86 (1):26-28.
19. Junior P, Krueger D, Winkler C. Adonis aleppica boiss. Phytochemical investigation, isolation and structural clearing-up of cardenolides. Dtsch. Apoth. Ztg (From Camag Bibliography Service). 1985;125:1945-1949.
20. Boisio ML, Esposito M, Merlo F. Separation and identifying features of the cardiac aglycons and glycosides of Nerium oleander L. flowers by thin-layer chromatography. Minerva Med. 1993;84(11):627-632.