Rapid Detection Of Residues Of Cardenolides Of Nerium Oleander (Linn.) By High-Performance Thin-layer Chromatography (HPTLC) In Autopsied Samples.

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INTRODUCTION

Oleander :

Nerium oleander (Common oleander)

Thevetia peruviana (Yellow oleander)

History-

- ✓ **Arrow poisons**
- ✓ **Emetics**
- ✓ **Diuretics**
- ✓ **Heart tonics**

Nerium oleander

Thevetia peruviana

Oleander and other species such as

Digitalis purpurea (Purple foxglove)

Digitalis lanta (Wooly foxglove)

Strophanthus gratus (Ouabain)

Convallaria majalis (Lily-of-the-valley)

contain toxic glycosides termed as

‘Cardiac glycosides’

Digitalis purpurea

Convallaria majalis

Strophanthus gratus

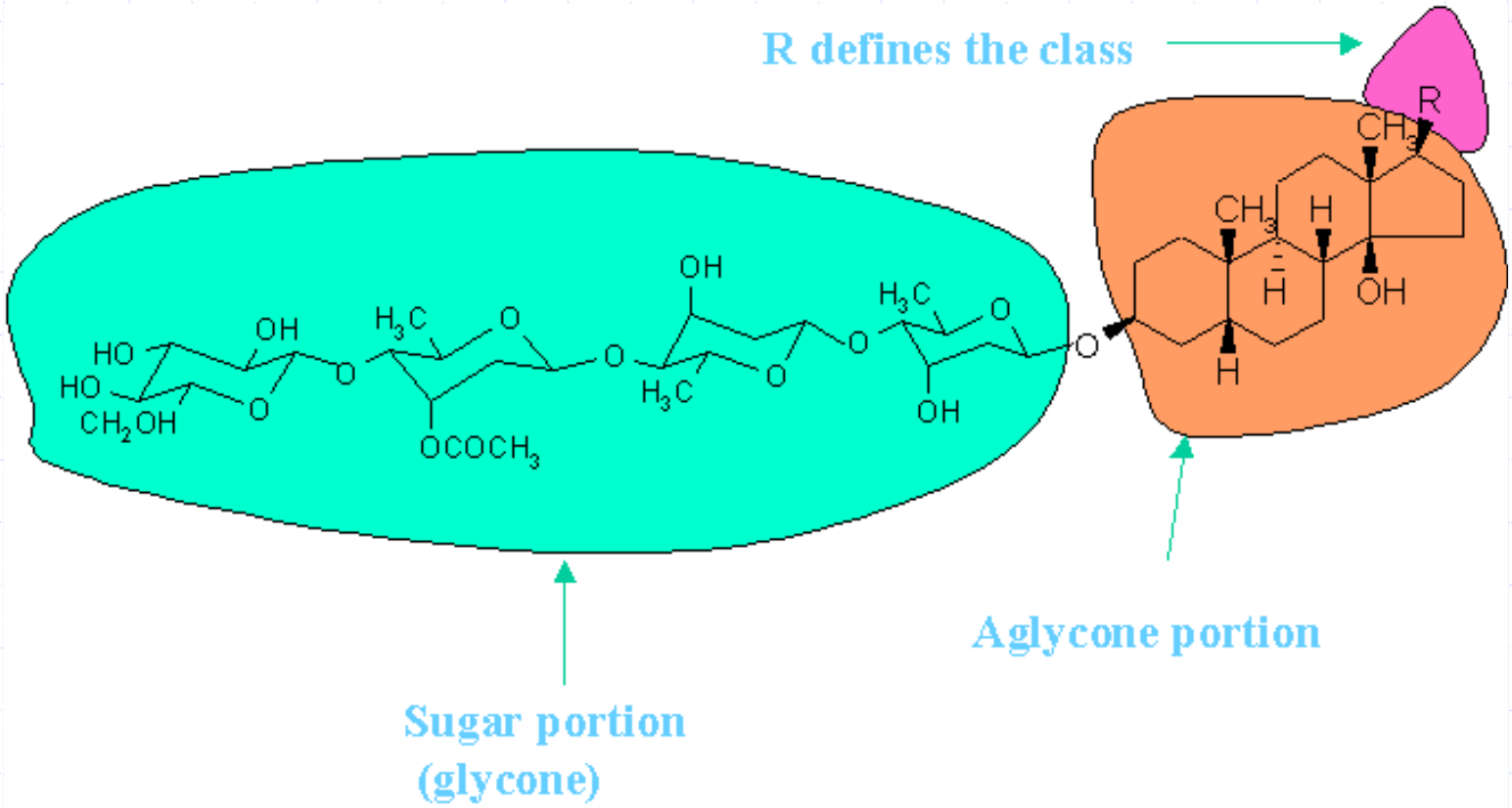
Cardiac glycosides

- Have positive inotropic activity i.e. increasing the force of contraction of the heart
- In plants, these glycosides serve several purposes

  - Defensive
  - Prevent decay of the damaged tissue

Cardiac glycosides : Structural features

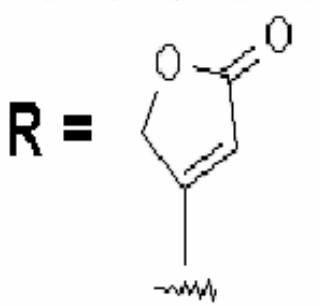
Composed of the sugar (glycone) and the non-sugar (aglycone - steroid) moieties.



Two classes of Cardiac glycosides observed in nature.

- **Cardenolides**

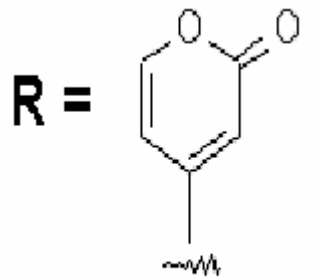
  Have an unsaturated butyrolactone ring



Cardenolides

- **Bufadienolides.**

  Have a-pyrone ring



Bufadienolides

GLYCONE MOIETY

- One to 4 sugars are found - attached to the 3β-OH group.
- The sugars most commonly used include L-rhamnose, D-glucose, D-digitoxose, D-digitalose, D-fructose.
- It possesses no biological activity.
- The sugar moiety appears to be important only for the partitioning and kinetics of action.

AGLYCONE MOIETY

- **Steroid nucleus has hydroxyls at 3- and 14-positions.**
- **The unsaturated lactone moiety at C-17 plays an important role in receptor binding.**
- **Lactones alone, when not attached to the steroid skeleton, are not active. Thus the activity rests in the steroid skeleton.**

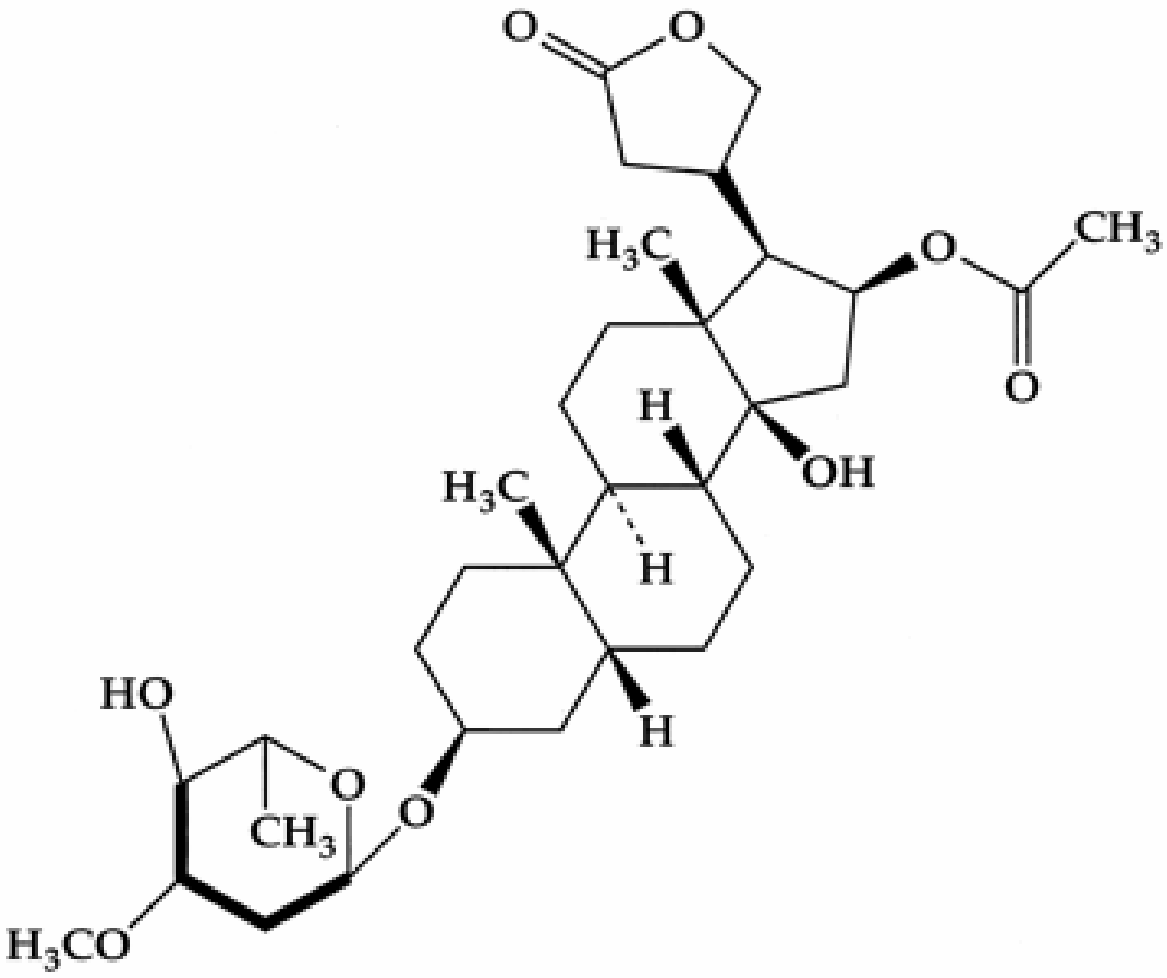
BIOCHEMICAL MECHANISM OF ACTION

- **Inhibit the membrane bound Na^+-K^+-ATPase pump responsible for Na^+-K^+ exchange.**

NERIUM OLEANDER POISONING

- **All parts of this plant including sap either fresh, dried or boiled are toxic.**
- **Main poisonous principles are cardiac glycosides.**
- **Contain at least 2% cardiac glycosides.**
- **Oleandrin, Odoroside, Neritaloside and aglycone Oleandrigenin**

Structure of Oleandrin



TOXICITY

- Cardiotonic properties of Oleander have been exploited therapeutically and as an instrument of suicide since antiquity.
- Significant toxicity usually is resultant of a suicide attempt.
- The data reviewed indicate that small children & domestic livestock are at increased risk of oleander poisoning.

CASE HISTORY

A 45-year-old male was suffering from paralysis since 3 months prior to his death as reported by his relatives. He could not recover completely despite treatment given in several hospitals and therefore was driven to the state of depression. It was therefore reported that he consumed crushed parts of *Nerium oleander*. He was declared dead after a day's treatment in the hospital. Autopsied samples of the deceased viz, stomach, small intestine, liver, kidney and blood samples were received in the laboratory for toxicological analysis.

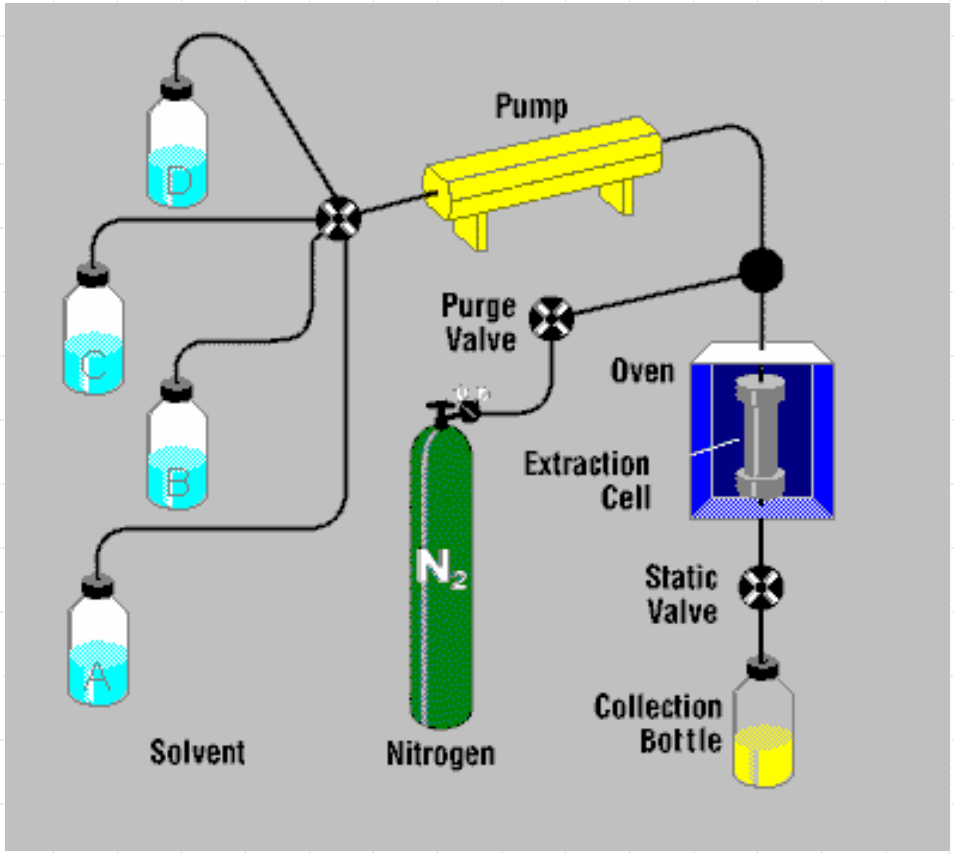
ANALYTICAL METHODS

Accelerated Solvent Extraction.

High-Performance Thin-Layer Chromatography (HPTLC).

Sample Preparation & Extraction

Accelerated Solvent Extractor - Flow-Chart



PLANT SPECIMENS

Nerium oleander plant was identified and samples of leaves, flowers and twigs (without leaves) were collected.

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 flasks protected from light and humidity.

AUTOPSIED SAMPLES

- Stomach tissue was finely minced and chemically dried using anhydrous sodium sulphate and diatomaceous earth (acid washed, approximately 95% SiO_2).
- Postmortem Blood with preservative sodium fluoride

Individual plant materials and autopsied tissue samples were packed in the extraction cell on a bed consists:

Aluminium oxide

(coloumn chromatography grade, particle size 100-125 mesh) and

Silica gel (10-40 microns particle size).

Accelerated Solvent Extraction (Dionex, ASE-300)

Extraction was carried out under the following conditions:

Extraction solvent	-	Chloroform
Oven temperature	-	Room Temperature
Pressure	-	1500 psi
Heat up time	-	5mins
Static time	-	10mins
Flush volume	-	60%
Purge time	-	100 sec.
static cycle	-	02

Residues from the whole blood sample were directly extracted using the same solvent after acid hydrolysis.

- **All the extracts were collected in 100ml glass vials and passed through the column containing activated charcoal, florisil and anhydrous sodium sulphate.**

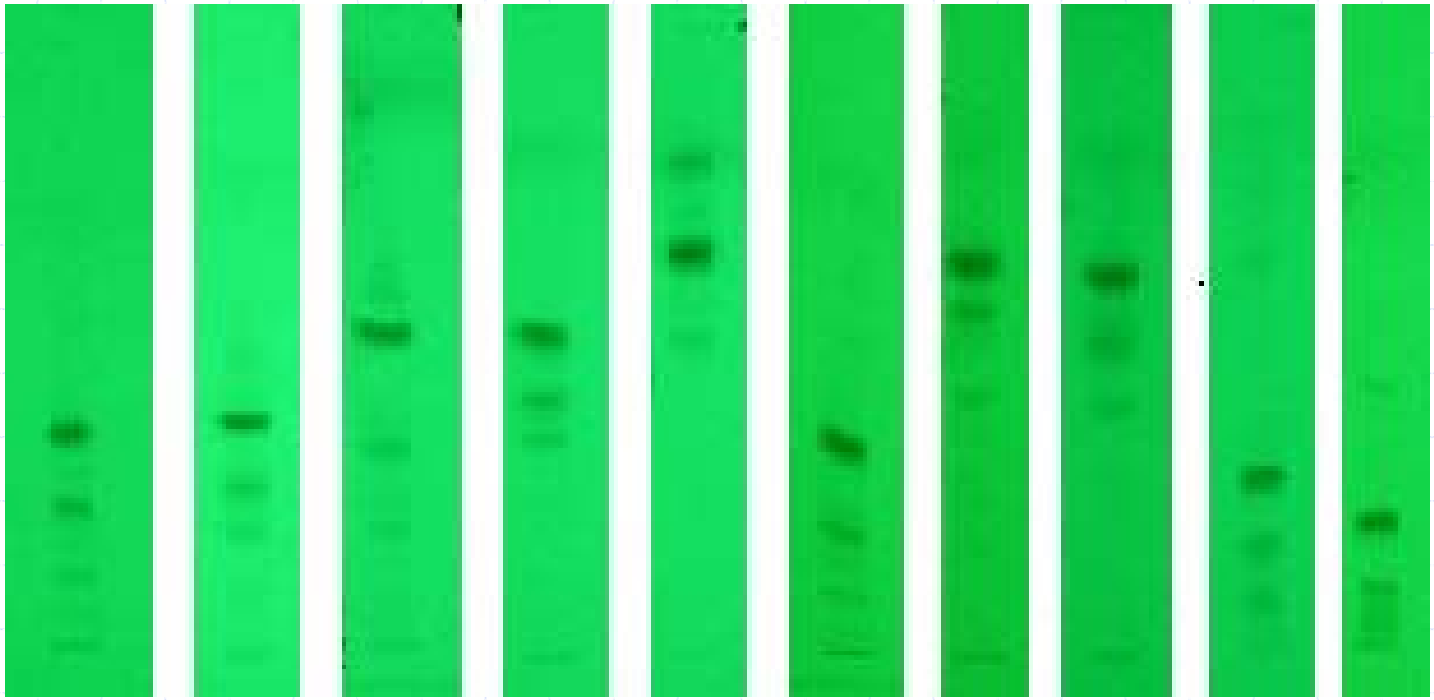
- **Plant material extracts, tissue extract and blood extracts were evaporated to dryness using N_2 gas and dissolved in minimum volume of methanol.**

High-Performance Thin-Layer Chromatography (HPTLC).

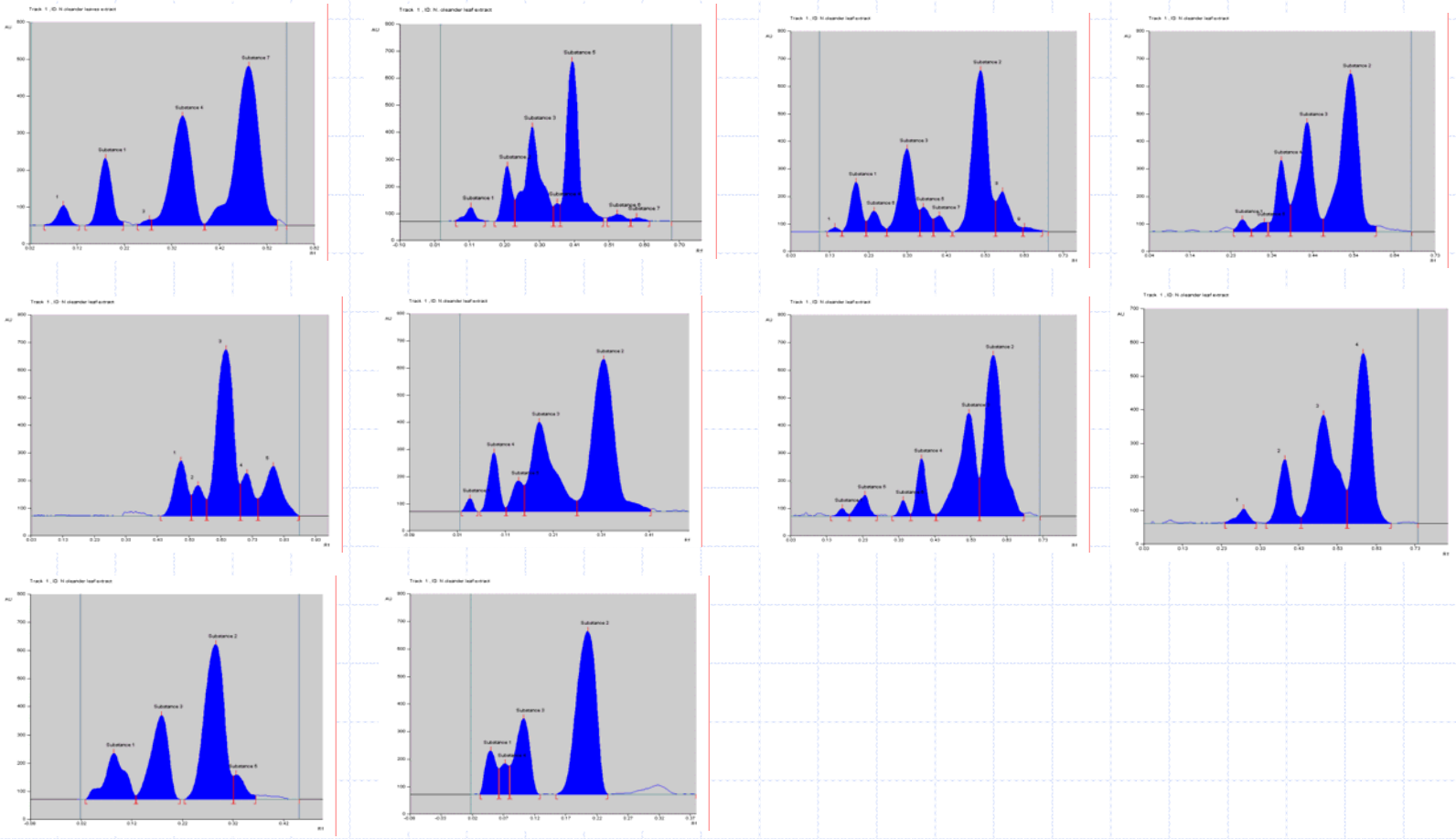
Optimization of Mobile phase

- Studies regarding the optimization of mobile phases were done on precoated silica gel glass plates (10x10cm) in Camag HPTLC Vario chamber.
- Twenty-five different mobile phases having binary, ternary and quaternary mixtures of different solvents with varying polarity were compared to assess their efficiency as mobile phases for the separation.

The study showed ten of the mobile phases were found to give good separation and compact spots.



Densitograms of N.oleander leaf extract at 275nm in ten different mobile phases.



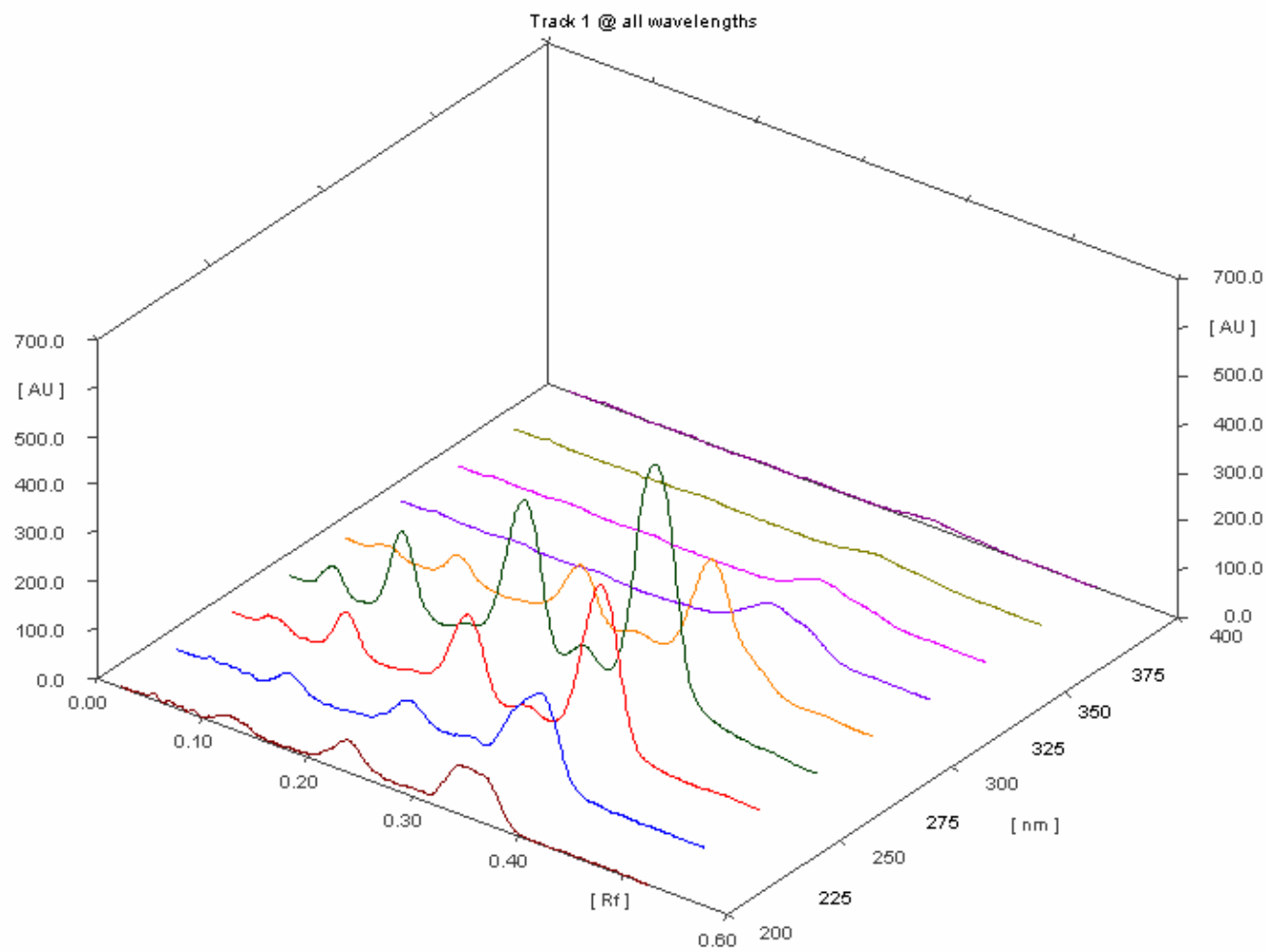
Rf values of N.oleander leaf extract in ten different solvent systems.

Sl. No	Mobile phase	Rf values
1	Benzene: Acetone (7:3)	0.14, 0.28, 0.41
2	Benzene: Ethanol (9:1)	0.20, 0.27, 0.38
3	Chloroform: Acetone: Acetic acid (8.5: 1: 0.5)	0.18, 0.31, 0.49
4	Dichloromethane: Methanol (9.5:0.5)	0.35, 0.42, 0.52
5	Ethyl acetate: Isopropanol: Water (7:2.5:0.5)	0.50, 0.63, 0.78
6	Chloroform: Acetone (8:2)	0.09, 0.19, 0.32
7	Ethyl acetate: Methanol: Ammonia (8.5: 1: 0.5)	0.39, 0.52, 0.59
8	Chloroform: Acetonitrile: Methanol (7:2.5:0.5)	0.38, 0.48, 0.59
9	Hexane: Ethyl acetate: Acetic acid (3:6.5:0.5)	0.07, 0.17, 0.28
10	Toluene: Ethyl acetate: Acetic acid (6:3.5:0.5)	0.04, 0.09, 0.19

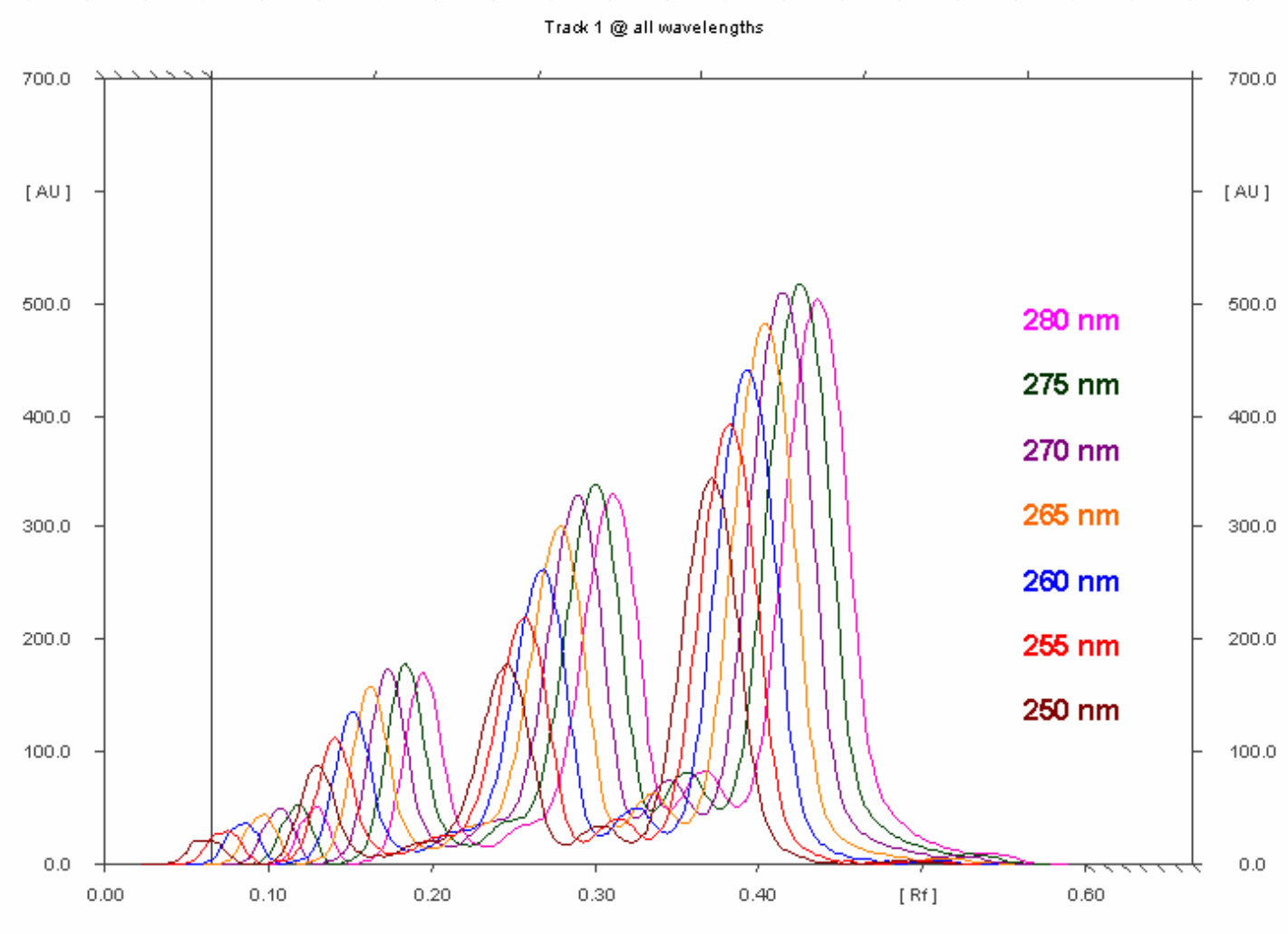
Optimization of Scanning Wavelength

- **Chromatogram developed in the mobile phase benzene: acetone (7:3) was subjected to multiwavelength scanning in the UV region 200-400nm.**
- **Maximum absorption was observed at 275nm, which also correlates with the *in situ* UV-spectra obtained, with peak having λmax 275nm**

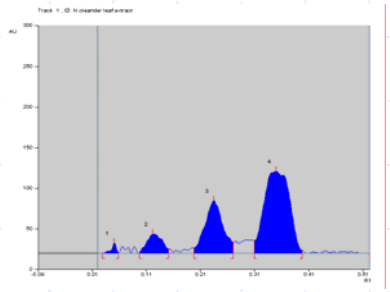
3D - display showing multiwavelength scan (200-400nm)



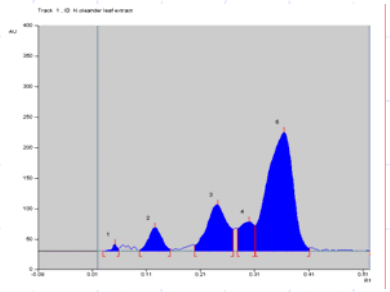
3D - display showing multiwavelength scan (250-280nm)



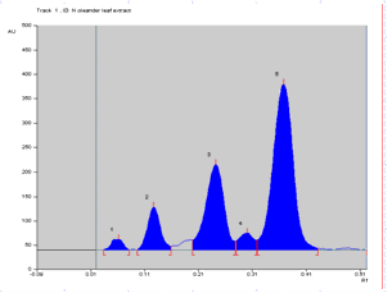
Densitograms of N.oleander leaf extract at nine different wavelengths (200-400nm).



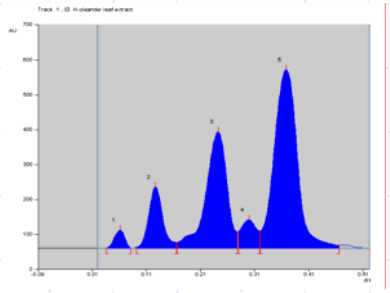
At 200 nm



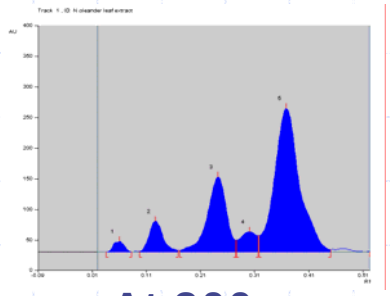
At 225 nm



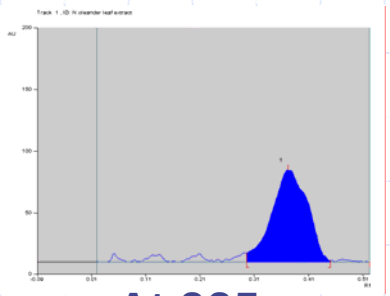
At 250 nm



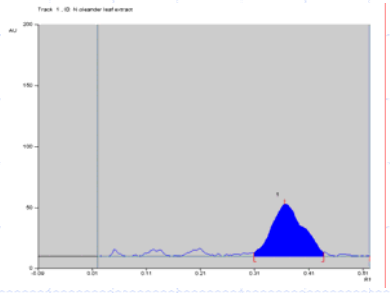
At 275 nm



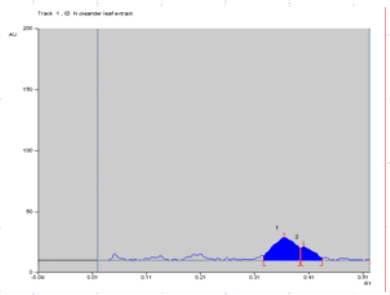
At 300 nm



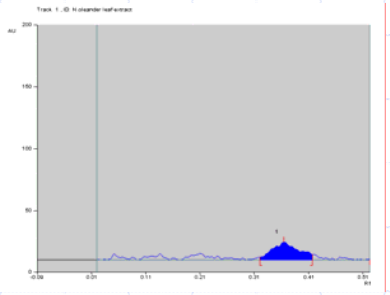
At 325 nm



At 350 nm



At 375 nm



At 400 nm

Analysis of Autopsied Samples

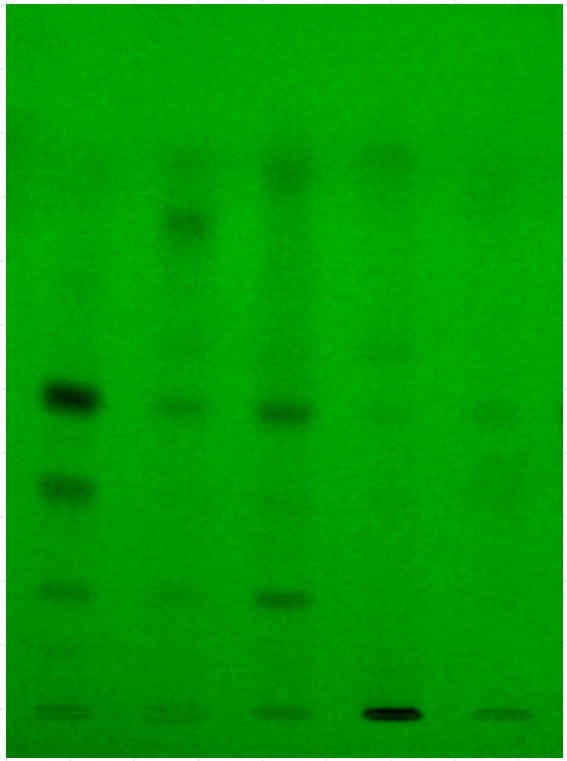
HPTLC

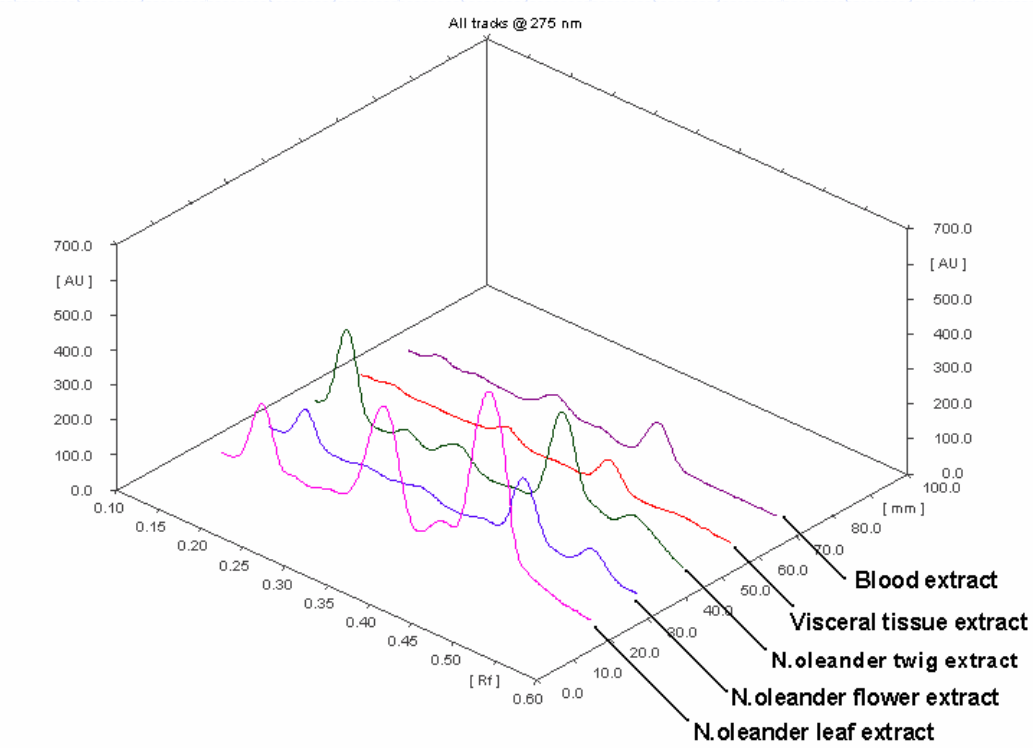
- Mobile phase benzene: acetone (7:3) was used for the separation of oleander leaf extract (OLE), oleander flower extract (OFE), oleander twig extract (OTE) and tissue (stomach tissue) and blood extracts of the deceased.

- Aliquots of samples were applied as 6mm narrow bands on the pre-coated HPTLC silica gel plates and the length of the chromatogram run was 8cm.

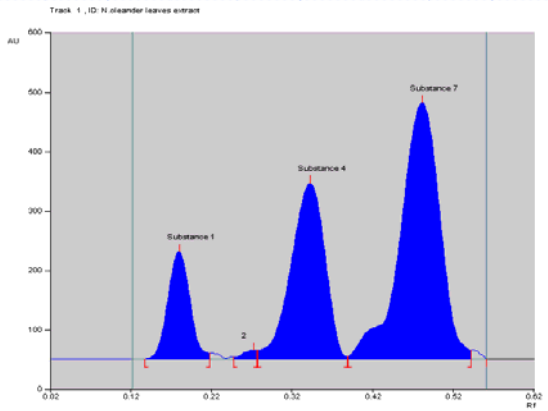
- **Chromatogram was subjected to densitometric scanning in absorbance mode at 275nm and spectrum scan in the UV region 200 to 400 nm.**
- **Rf value 0.42 obtained for the tissue and blood extracts and the peak with absorption maxima of 275nm were fully in conformity with the plant material extracts**

Chromatogram and 3D display of Densitometric Scanning

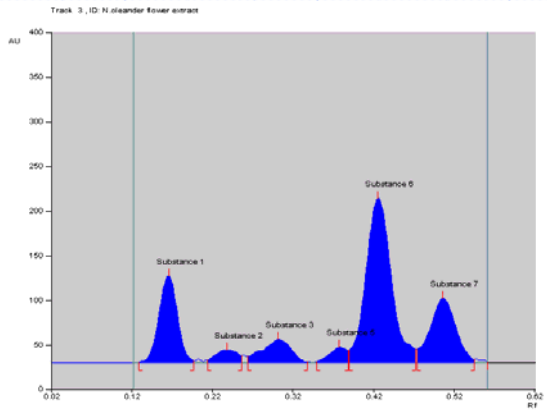




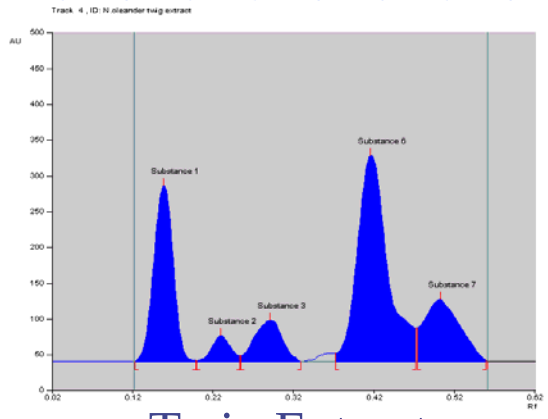
Densitograms of Plant Material Extracts with Autopsied Samples at 275nm.



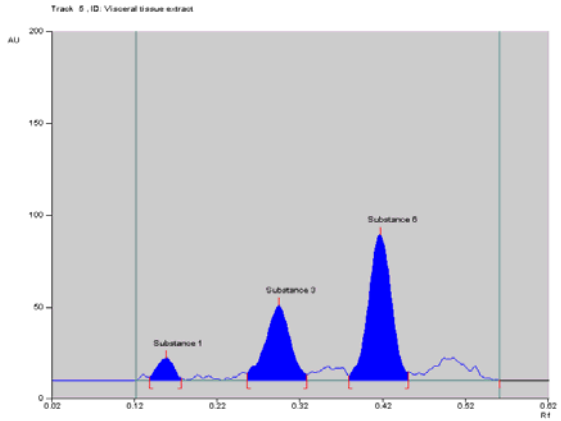
Leaf Extract



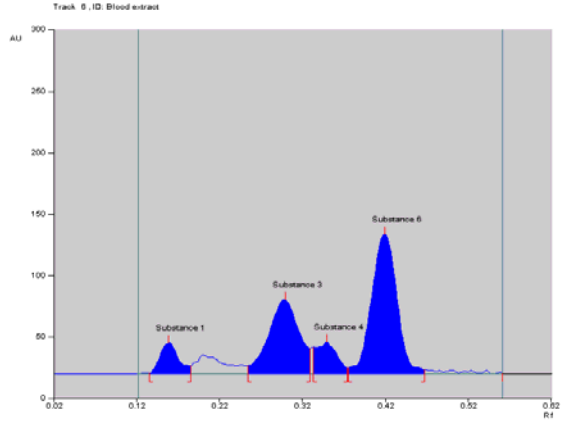
Flower Extract



Twig Extract



Tissue Extract

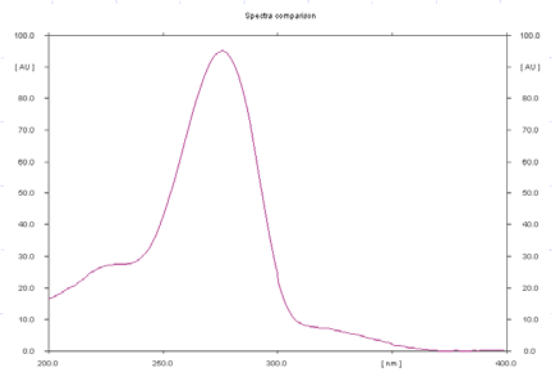


Blood Extract

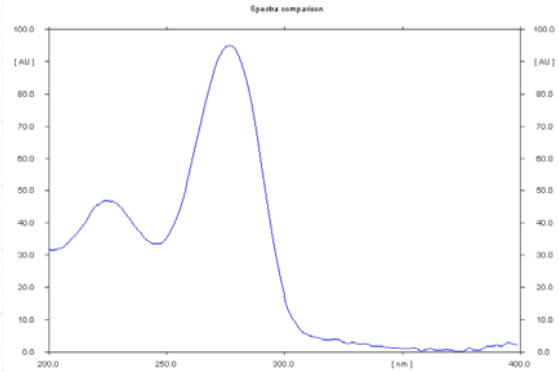
Mobile phase	Relative band speeds (Rf values)				
	OLE*	OFE*	OTE*	Visceral extract	Blood extract
Benzene: Acetone (7:3)	0.15, 0.29, 0.42	0.16, 0.42	0.16, 0.42	0.42	0.42

**OLE – oleander leaf extract, OFE – oleander flower extract, OTE – oleander twig extract*

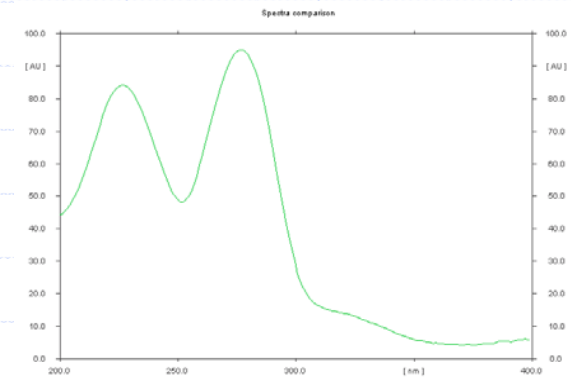
In situ UV spectra of Plant Material Extracts and Autopsied Samples.



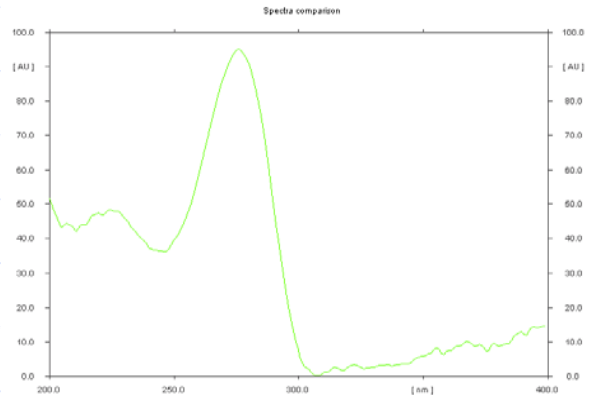
Leaf Extract



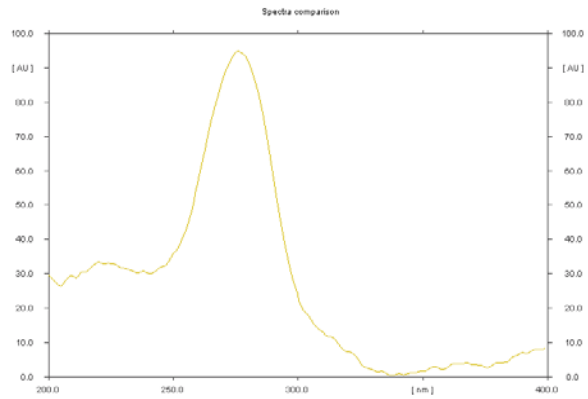
Flower Extract



Twig Extract

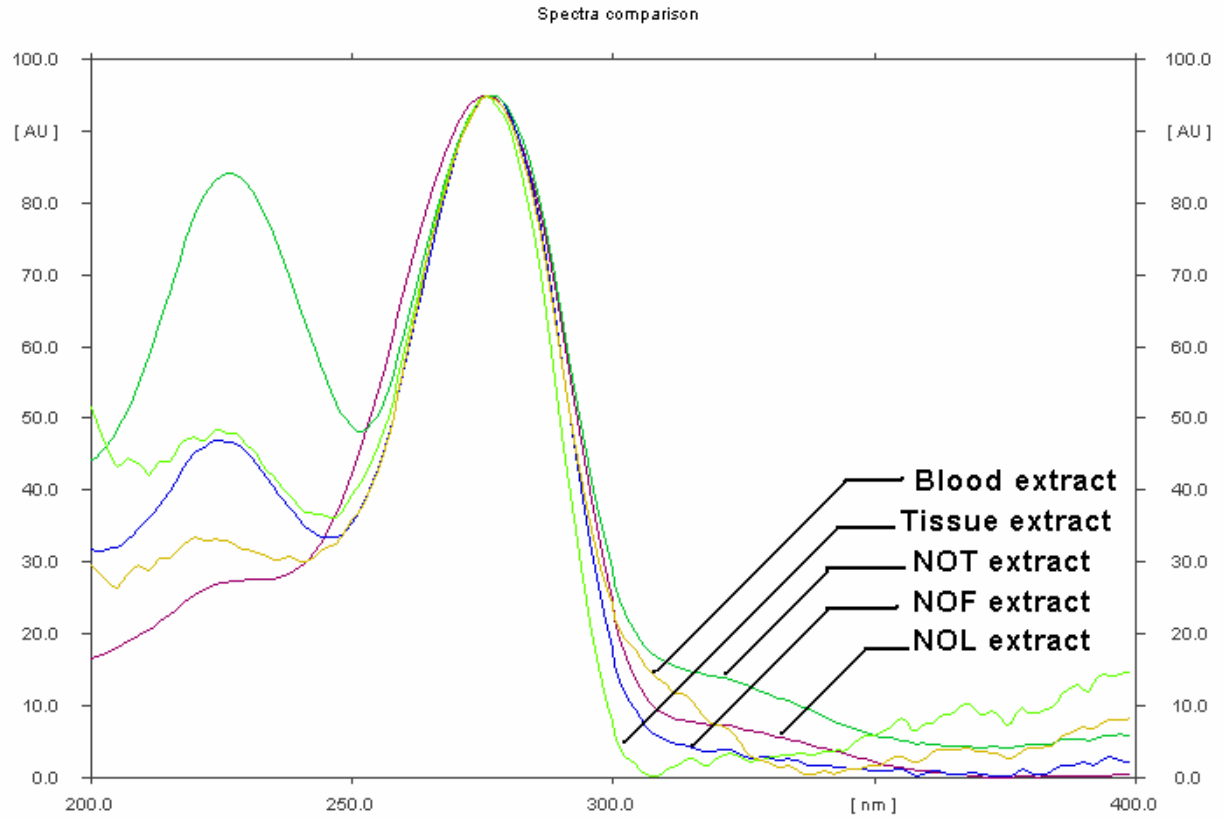


Tissue Extract



Blood Extract

Overlay spectra of plant material extracts and extracts of autopsied samples.



NOL – Nerium oleander leaf, NOF – Nerium oleander flower, NOT – Nerium oleander twig.

Post Chromatographic Derivatization

- Nine different chromogenic reagents were tested against the plates developed in each of the ten optimized mobile phases.
- The colour developed in the visible range, stability of the colour and the fluorescent characteristics were studied.

Responses of specific groups present in cardenolide (Oleandrin) with different spray reagents

Reagents	Colour	Stability (min)	Group (moiety) specificity
Keddle (7, 25)	Violet-red.	05.	Lactone
p-anisaldehyde (10)	Blue after heating the plate for 10 mins at 110^0C.	20-30.	Lactone
p-toluene sulfonic acid (26)	Yellow after heating the plate for 5 mins at 110^0C, blue fluorescence at 366 nm.	40-50.	Steroid
Aluminium(III)chloride	Yellow after heating the plate for 5-10 mins at 110^0C, blue fluorescence at 366 nm.	35-45.	Steroid
Antimony(III)chloride (26)	Yellow. Blue fluorescence at 366 nm.	35-45.	Steroid
Phosphoric acid	Yellow. Bright blue fluorescence at 366nm.	40-50.	Steroid
Orcinol	Yellow. Blue fluorescence at 366 nm.	40-50.	Glycone
Vanillin – Sulphuric acid (25)	Brown.	30-40.	Steroid

The components of each of the plant material extract were purified and their $_1H^1$-NMR is recorded. The structure of the component present in the spot is similar to those cardenolides present in *N. oleander*.

The presence of cardenolides of N.oleander in the autopsied samples was confirmed by comparing the similar Rf value, peak with λmax 275nm and the colour developed in presence of chromogenic reagents with those of plant material extracts.

Intensity of the color developed in presence of different chromogenic reagents,

the maximum absorption and peak obtained at 275 nm and

the densitogram obtained at the same migration distance

suggest that the component with Rf value 0.42 is present in much greater concentration and found to be important for the forensic analysis in cases of oleander poisoning.

The characteristic UV spectra with λmax 275nm,

colour developed due to the reaction of butyrolactone ring of cardenolides with 3, 5 – dinitrobenzoic acid in alkaline medium to give violet-red colour and

densitometric scanning in absorbance mode at 275nm

can be best utilized for the specific detection and quantification by HPTLC method.

THANK YOU

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BANGALORE, INDIA