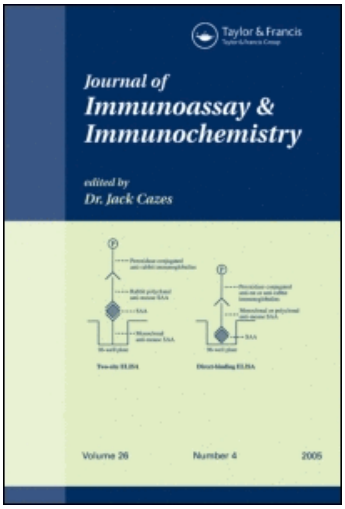

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ENZYME - LINKED IMMUNOSORBENT ASSAY FOR THE PHYTOTOXIN THEVETIN

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ABSTRACT

An enzyme - linked immunosorbent assay is reported for monitoring thevetin, an active constituent of the highly poisonous plant Thevetia nerifolia. A thevetin - BSA conjugate was employed as the immunogen and the antibodies raised in rabbits were used for the development of an ELISA. Penicillinase served as the marker enzyme and its conjugation to thevetin by the periodate method is reported for the first time. The present ELISA method could detect 2 ng/ml of thevetin. Cross-reactivity studies with structural analogues and other phytotoxins and drugs of common occurrence in clinical and forensic toxicology established the superiority of the ELISA over the existing analytical methods for determining thevetin in various biospecimens.

(KEY WORDS : Thevetin, T.nerifolia, Oleander, ELISA, Penicillinase, Phytotoxin)

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INTRODUCTION

Thevetia nerifolia popularly known as yellow oleander is an ornamental plant and is abundant in India. The plant belongs to the family Apocynaceae. The plant is highly toxic to humans and animals (1,2). Several cases of death due to yellow oleander poisoning have been documented in the literature (3-8). Seeds are mainly used for suicidal, homicidal and cattle poisoning and for procuring criminal abortion (2). The plant is also known for its therapeutic efficacy in cardiac disorders. The therapeutic and toxic properties of the plant have been attributed to the presence of certain cardiac glycosides like thevetin (which is a mixture of thevetin-A and thevetin-B), thevetoxin, cerberin, peruvoside, ruvoside and nerifolin (5,9).

Analysis of the different parts of the plant showed the seeds (kernel) to be the major source of cardiac glycosides, the contribution from thevetin being significant (1, 3, 5). Few methods are available for the identification of the glycosides of T.nerifolia (10-12). Immunoassay employing digoxin specific antibodies has been suggested for the detection of T.nerifolia (13, 14). However these methods all suffer from disadvantages like elaborate pretreatment of samples and lack of specificity and sensitivity.

The increasing incidence of yellow oleander poisoning is of significant concern to clinicians and toxicologists and calls for a specific yet simple identification technique.

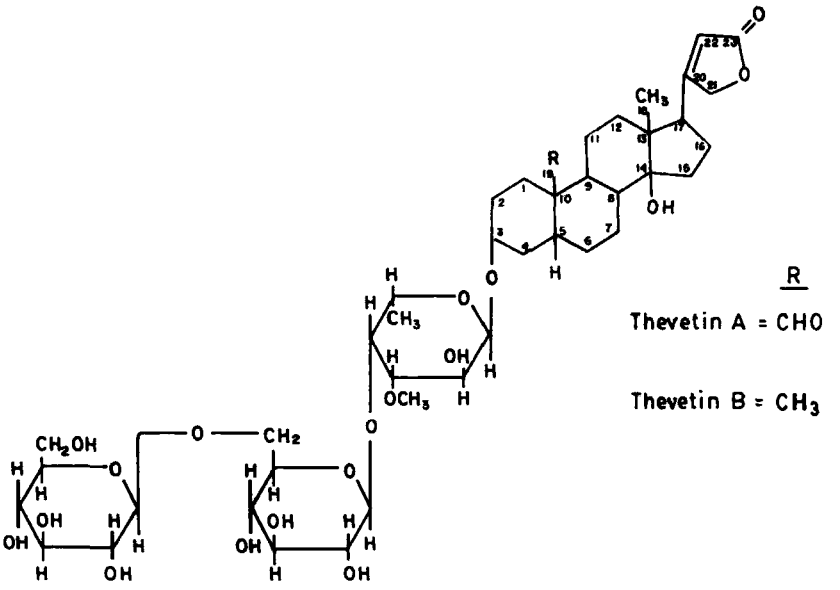

FIGURE 1 Structure of thevetin.

The objective of the present investigation has been to develop an ELISA for the glycosides of yellow oleander. For this purpose, thevetin was chosen as a target (Figure 1) since it is the most active and highly stable principle (2). Thevetin has been reported to be a mixture of thevetin-A and thevetin-B, the former having an aldehyde group instead of a methyl group at position C-19 (Figure 1) (9). In the present study thevetin isolated from seeds was used for the development of an immunoassay. Thevetin was coupled to a carrier protein and the resulting immunogen used for the production of antibodies in rabbits. The antibodies were purified and employed in conjunction with a preparation of penicillinase-labelled thevetin to develop an ELISA.

MATERIALS AND METHODS

Thevetin was isolated from the seeds of T.nerifolia according to the method of Abe and Yamauchi (15) with slight modification. Purification of thevetin was done by preparative thin-layer chromatography and its purity checked through melting point analysis and spectral data by comparison with an authentic sample obtained from Andhra University, Waltair (India) (9).

All chemicals and solvents were of analytical grade: bovine serum albumin (BSA) (A 4503), human serum albumin (HSA) (A 1653), ovalbumin (A 5530), keyhole limpet hemocyanin (KLH) (H 2133) sodium borohydride (S 9125), sodium metaperiodate (S 1873) and CNBr-activated Sepharose 4B (C 9142) were obtained from Sigma, U.S.A ; ethylene glycol was from E.Merck, India; complete and incomplete Freund's adjuvants were from Difco Laboratories, U.S.A.; agarose was from SISCO Research Laboratories, India; and, penicillinase and penicillin V were from Hindustan Antibiotics Limited, India. Buffer salts of reagent grade quality were used.

Preparation of thevetin - protein (BSA) immunogen

Thevetin - BSA, thevetin - HSA and thevetin - ovalbumin conjugates were prepared according to the method developed earlier by us (16). Thevetin-BSA was utilized for raising antibodies and all three conjugates were used in the double immunodiffusion experiment.

Enzyme labelling of thevetin

Thevetin was conjugated with the marker enzyme penicillinase in a modification of the procedure reported for digoxin (17).

To 1.3 mg of thevetin, 500 µl of triple distilled water was added and the solution warmed till thevetin dissolved completely. To this solution, 100 µl of 0.1M sodium metaperiodate was added dropwise with stirring. The reaction mixture was kept in the dark for 30 min at room temperature with occasional shaking. At the end of 30 min, 50 µl of ethylene glycol (0.16M) was added; the reaction mixture was allowed to stand at room temperature for 5 min with occasional shaking and then added to a solution of penicillinase (3.3 mg) in 500 µl phosphate buffer (PB), pH 7.4, 200 m mol/L, followed by gentle stirring for 45 min at room temperature maintaining the pH at 7.0 - 7.5 with the addition of PB. To the above mixture, 200 µl of sodium borohydride was added and incubated at 37°C for 3h. The solution was then dialyzed against phosphate buffered saline (PBS), pH 7.4, 10 m mol/L, at 4°C for 48h with repeated changes. To the dialyzed sample of enzyme conjugate , BSA (10 g/L) and sodium azide (1 g/L) were added, mixed, aliquoted and stored at refrigerated condition.

Evaluation of molar participation of hapten in thevetin-BSA and thevetin-penicillinase conjugates

Determination of the number of haptenic thevetin residues per protein or enzyme molecule was carried out by

spectrometric analysis (18). The protein content before and after conjugation was assayed by the method of Lowry et al (19) and the enzyme activity as described by Joshi (20).

Preparation of antiserum to thevetin

New Zealand white rabbits (weighing 1.5 to 2.0 kg) were injected intradermally at multiple sites with 1 ml of thevetin - BSA conjugate (corresponding to a concentration of 100 μg of bound thevetin) dissolved in PBS and emulsified in Freund's complete adjuvant (1 ml). After 5 weeks, a booster injection of the immunogen (40 μg) in Freund's incomplete adjuvant was given. The antiserum obtained after seven days of the booster dose was tested for the presence of anti-thevetin antibodies by double immunodiffusion and direct ELISA techniques. The antiserum titres for different rabbits were also determined by direct ELISA. The anti-thevetin antibodies were purified by ammonium sulfate precipitation followed by affinity chromatography using a thevetin-KLH-Sepharose 4B affinity column. They were then aliquoted and stored at -90°C until use.

Development of competitive ELISA for thevetin

Optimal combinations of the dilutions of the antibody and enzyme labelled analyte for use in ELISA were ascertained as described in 'Results'. Accordingly 200 μl of anti-thevetin antibody diluted with coating buffer (bicarbonate buffer; pH 9.6, 50 m mol/L) (1:4000) was added to the wells of microtitre plates and incubated for 3h at 37°C or

overnight in a refrigerator. After washing the plates three times with wash buffer (PBS-containing 0.5 g/L tween 20), the empty sites were blocked with a 20 g/L solution of gelatin in PBS. The plates were then incubated for 2h at 37°C and washed again with wash buffer. This was followed by the addition of known amount of thevetin (100 µl) or analyte samples (100 µl), and 100 µl of thevetin - penicillinase conjugate diluted 1:4000. The plates were incubated at 37°C for 2h. Subsequently the plates were washed with wash buffer and 200 µl of the substrate solution was added to each well. The substrate solution was starch-iodine-penicillin (SIP) reagent freshly prepared by mixing 0.2 ml of iodine reagent (potassium iodide (5.32 g) and iodine (200 mg) in 10 ml of distilled water), and 20 ml of 20 g/L starch solution with 100 ml of penicillin V (15.2 mg) solution in PB. The plates were left at ambient temperature for 20 min following which absorbance measurements were made at 620 nm with an ELISA Reader (Biotek, EL 310 model). Figure 2 represents the entire protocol.

Evaluation of ELISA for matrix effect

In order to check the effect of variable matrices on the performance of ELISA, calibration curves were prepared from thevetin standards in PBS-BSA, pooled urine, pooled serum, liver homogenate and brain homogenate.

Evaluation of ELISA for specificity to thevetin

Thevetin analogues present in T.nerifolia, other phytotoxins, hormones and drugs of common occurrence in

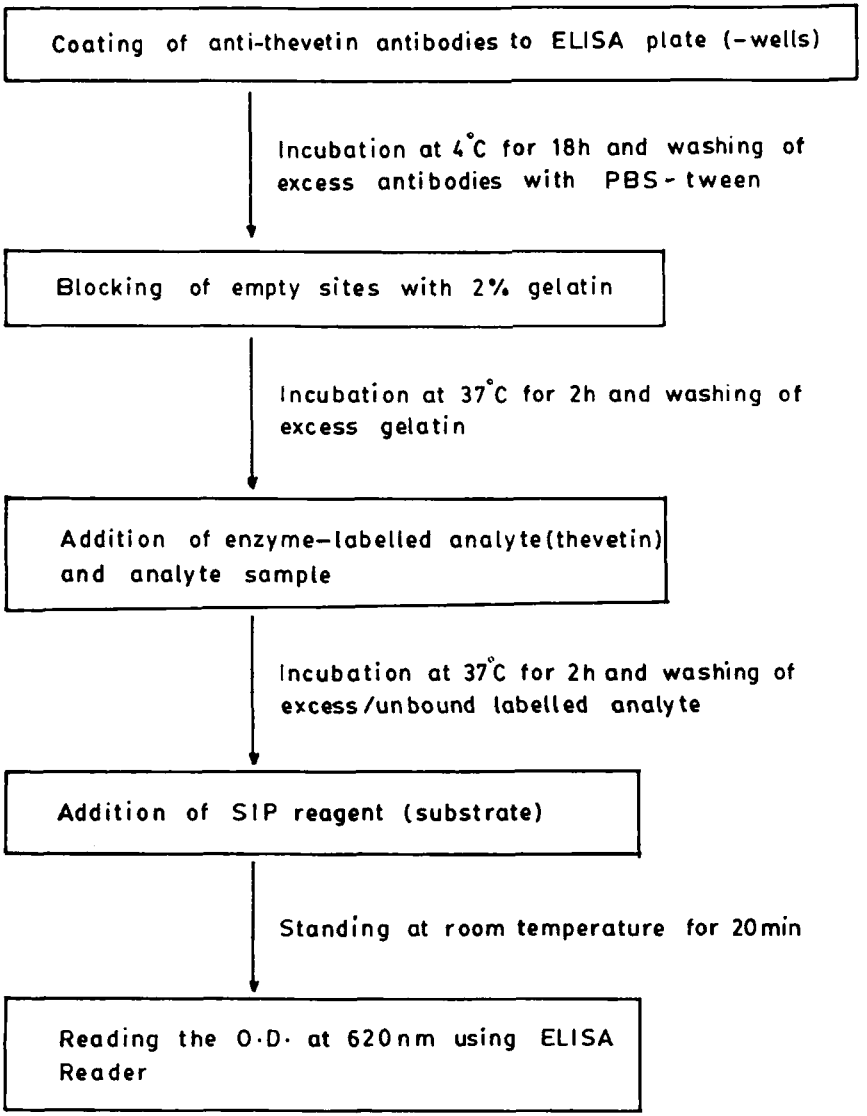

FIGURE 2 Competitive ELISA protocol for thevetin.

clinical and forensic specimens were assayed in the ELISA . The cross-reactivity and the percent binding with various concentrations of the cross reacting compounds (CRC) were calculated as described in our earlier publication (21).

RESULTS

Evaluation of the molar participation of the haptenic molecule showed 18 molecules of thevetin per molecule of BSA while the penicillinase tagged conjugate retained on an average 10 haptens per mole. Determination of penicillinase activity in the enzyme conjugate showed retention of more than 82% of the original activity.

Optimal dilutions of anti-thevetin antibody and thevetin - penicillinase were found to be 4000 for both the antibody and the enzyme conjugate.

A calibration graph showed linearity in the range 4-128 ng/ml (Figure 3). The detection limit of the ELISA developed for thevetin was 2 ng/ml. The recovery of thevetin in spiked urine samples is shown in Table 1, together with precision data.

To assess matrix effects, PBS - BSA, serum (1:50), urine (1:50), liver homogenate (1:100) and brain homogenate (1:100) were spiked with known quantity of thevetin and assayed. The curves obtained are shown in Figure 4. Matrix effects were negligible.

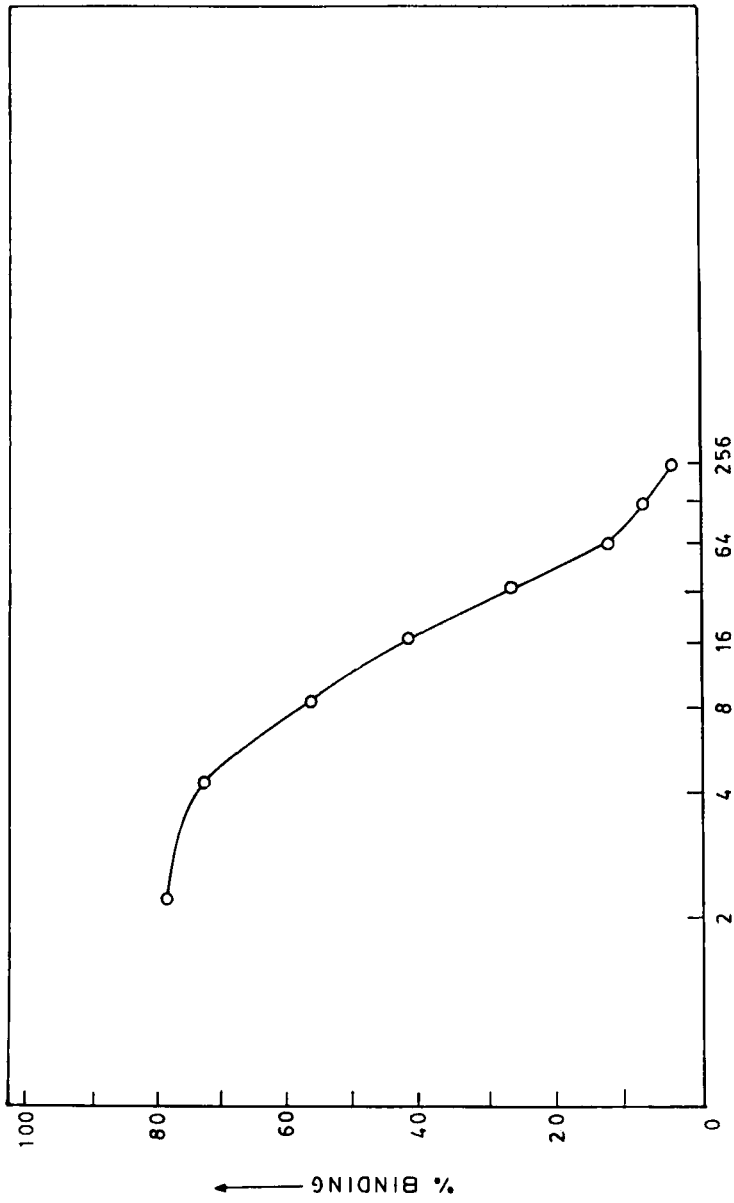

FIGURE 3 Calibration curve for thevetin.

TABLE 1

Reproducibility of ELISA for the estimation of thevetin

Added (ng/ml)	Estimated recovery*							
	Intra-assay				Inter-assay			
	ng/ml	SD	%	CV%	ng/ml	SD	%	CV%
4	3.92	0.237	98.00	6.046	4.00	0.172	100.00	9.300
8	7.90	0.312	98.75	3.950	7.70	0.624	96.25	8.104
16	15.29	0.863	95.56	5.644	15.25	0.925	95.31	6.070
32	31.14	2.950	97.31	9.473	31.49	3.099	98.41	9.840
64	65.00	5.542	101.56	8.526	62.14	2.860	97.09	4.603
128	126.00	4.950	98.44	3.93	129.10	4.008	100.26	3.105

* Each sample (spiked pooled urine) was assayed in quadruplicate

The cross-reactivity of anti-thevetin antibody with other structurally similar glycosides is shown in Table 2. Table 3 lists non - T.nerifolia phytotoxins, hormones and drugs of common occurrence in forensic and clinical toxicology that were tested in the ELISA for cross-reactivity and ruled out therefor.

DISCUSSION

Various methods are available to conjugate haptens with proteins (18,22,23). The choice depends on the nature of the compound, the functional groups and the purpose for which the conjugate is required (24). Thevetin (MW 872), as shown

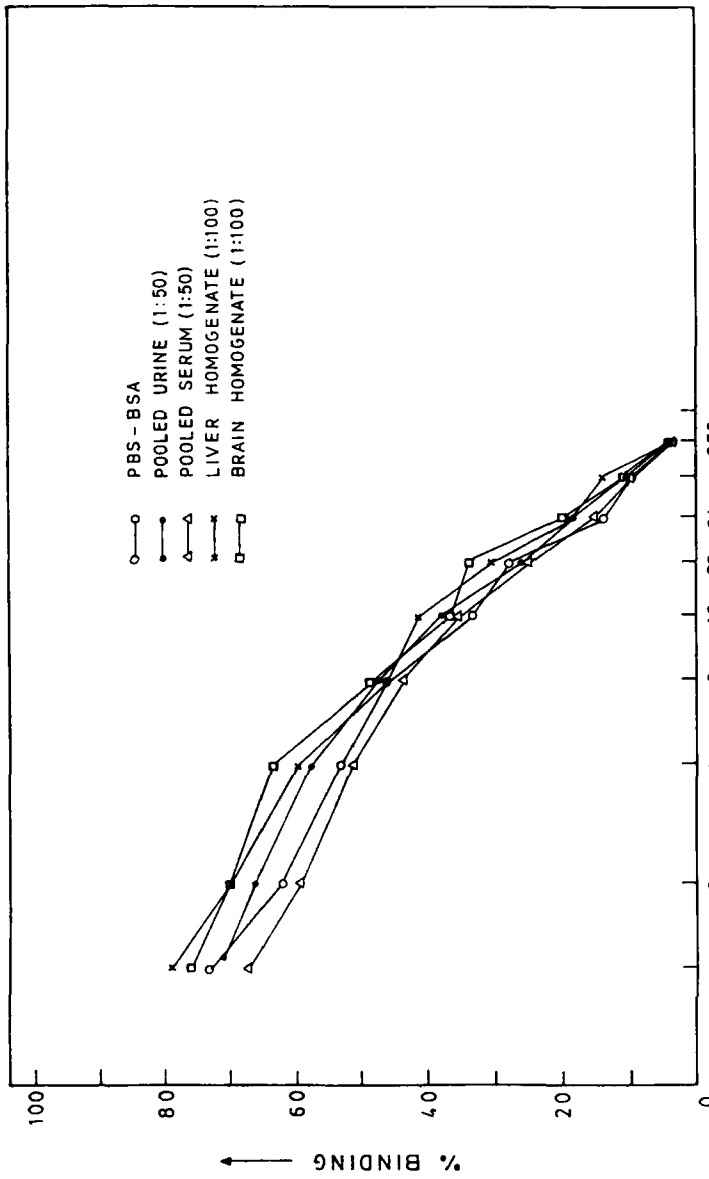

FIGURE 4 Effect of different matrices on competitive ELISA for thevetin.

TABLE 2

Cross - reactivity of anti-thevetin antibody during ELISA as tested with thevetin structural analogues

Compounds tested	% Cross reactivity*
Ruvoside+	75.0
Thevetigenin++	75.0
Nerifolin+	50.0
Peruvoside+	50.0
Digitoxin	35.0
Cerberin+	30.0
Digoxin	7.0
Gitoxin	1.6
Oleandrigenin	1.3
Oleandrin	1.0
Ouabain	1.0

* Thevetin was assigned a value of 100

\+ Glycosides from T.nerifolia plant

++ Genin part of the glycoside thevetin

in Figure 1 is a small molecular weight glycoside containing vicinal hydroxyl groups in its carbohydrate moiety. Periodate conjugation was used here to couple the glycoside thevetin with BSA. In the present study it was through the gentiobiose moiety of thevetin. This method of conjugation, using a reactant ratio of BSA : thevetin of 1:60, yielded a good

TABLE 3

Drugs, hormones and plant toxins tested and found negative for cross-reactivity with anti-thevetin antibody during ELISA

Drugs*	Drugs+	Hormones	Phytotoxins
Amylobarbitone	Bactrim DS	FSH	Brucine
Butobarbitone	Chymoral Forte	LH	Cleistanthin A
Chlordiazepoxide	Digene	Progesterone	Cleistanthin B
Chlorpromazine hydrochloride	Doxt	Prolactin	Ellagic acid
Chlorpromazine sulphoxide	Erythromycin	T3	Strophanthin
Cyclobarbitone calcium	Fepanil	T4	Strychnine
Dextropropoxyphene hydrochloride	Hetrazan	TSH	
Diazepam	Redoxon	Testosterone	
Dicoumarol	Supradyn		
Diphenhydramine hydrochloride	Wymox		
Heptabarbitone			
Imipramine			
Indomethacin			
Lorazepam			
Nitrazepam			
Nortriptyline			
Oxazepam			
Oxyphenbutazone			
Pentobarbitone sodium			
Phenacetin			
Phenobarbitone sodium			
Promethazine hydrochloride			
Quinal barbitone sodium			
Temazepam			
Thiopentane sodium			
Trimipramine maleate			

* Drugs encountered in toxicology

\+ Commonly used drugs

immunogen since the conjugate had 18 thevetin residues per molecule of BSA. Other coupling procedures like those involving mixed anhydride, carbodiimide, or glutaraldehyde would not have been feasible due to the lack of functional groups like - COOH or $-NH_2$ in the thevetin molecule.

For effecting the conjugation of thevetin to the enzyme penicillinase, again periodate was preferred, with a ratio of pencillinase : thevetin of 1:22, since our earlier experience with a different hapten proved to be successful (25). Self polymerisation of thevetin is not possible under these conditions since it is devoid of amino groups. Penicillinase was chosen as the marker enzyme since it is known to be stable at ambient temperature over the pH range of 5 to 9, it has a high turnover number, and it is not present in human beings (26). Steriods (27) and lignan lactones (21) have been subjected to carbodiimide mediated conjugation to penicillinase, but thevetin is the first cardiac glycoside to be labelled with penicillinase through the periodate method.

The coefficients of variation for the intra- and inter- assays were less than 10%. Matrix effect on the ELISA was found to be negligible, thereby demonstrating the applicability of the method to clinical and forensic cases.

The cross-reactivity with ruvoside, thevetigenin, nerifolin, peruvoside and cerberin all of which share the genin skeleton of thevetin, all marker molcules of

T.nerifolia, would in fact be an advantage during analyses for poisoning by yellow oleander (T.nerifolia). The cross-reactivity of digitoxin (a non - T.nerifolia glycoside with the genin part identical to but the carbohydrate residue distinctly different from thevetin B) requires cautious interpretation.

It is of significant interest however to note that other phytotoxins, hormones and drugs of possible occurrence in clinical and forensic specimens show no cross-reactivity. This non-interference by other compounds makes the present ELISA very attractive in affording discrimination of yellow oleander poisoning from others.

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