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Detection and Quantification of Cardiotonic Drug Peruvoside Using HPTLC from *Thevetia neriifolia,* Juss Seed Extracts

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ABSTRACT : *Seeds of Thevetia neriifolia were reported as rich sources of cardiac glycosides, and peruvoside is one among the valuable cardiotonic drug used for the treatment of congestive heart failure. Samples were extracted with different solvent systems. Occurrence of peruvoside was analyzed by TLC technique; its presence was detected in chloroform and ethyl acetate fractions, both petroleum ether and methanol fractions gave negative results. Quantification of peruvoside in positive fractions was done using HPTLC fingerprinting. Densitogram showed scattered Rf values, and a comparison of Rf values of samples with standard peruvoside established the existence of different isomeric forms of the drug within same fraction. Extraction of peruvoside was partial with chloroform but complete with ethyl acetate. A total of 20% peruvoside can be extracted successively using chloroform (3-7%) and ethyl acetate (12-16%) from all three studied morphovariant plants. Considering the valuable therapeutic applications of cardiac glycosides, it is anticipated that isolation of this powerful drug economically from the natural resources will help to compensate the high cost alternatives for treating congestive heart failures.*

KEYWORDS: *Cardiac glycosides, HPTLC, Isomers, Peruvoside, Thevetia neriifolia.*

I. INTRODUCTION

Cardiac glycosides are important class of naturally occurring secondary metabolites which have both beneficial and toxic effects on the heart, and have played an outstanding role in the therapy of congestive heart failure. More than 200 naturally occurring cardiac glycosides are reported from plant sources, including *Digitalis purpurea* (foxglove), *Nerium oleander* (common oleander), *Thevetia neriifolia* (yellow oleander), *Strophanthus gratus* (ouabain) and *Apocynum cannabinum* (dogbane), which are distributed in various parts like seeds, leaves, bark and latex. The seeds of *Thevetia* contain many potent cardiac glycosides which are known to cause toxicological effects in human and livestock when ingested [1-2]. The dosage of cardiac glycosides is very critical, in low concentration it act as cardiotonic, and in slightly higher doze it becomes a cardiotoxin. About seventeen cardiac glycosides including thevetin, thevetoxin, peruvoside, ruvoside and neriifolin were isolated from all parts of *Thevetia* plant, especially from leaves, bark and seeds [3-6]. Excellent historical records concerning the therapeutic and toxic properties of this plant are available since long back when De vry isolated and named Thevetin from defatted seed mare in 1863. Chen & Chen [7] separated Thevetin in crystalline form; Rangaswami and Venkata [8] isolated and named peruvoside. Thevetin is a mixture of thevetin A and thevetin B. Both these triglycosides on enzymatic degradation yields monosides - peruvoside and neriifolin. Peruvoside is one among the useful cardiac glycoside in the management of congestive heart failure in man and experimental models [9-11]. This drug is used in the treatment of mild cardiac insufficiency and weak heart and shows relatively high degree of therapeutic index compared to digoxin. The advantage of Peruvoside over digitalis is that it is completely absorbed by the intestinal tract with wide safety margin and does not accumulate in the body with repeated doses [12]. In West Germany, peruvoside is marketed under name "Encordin"[13]. It is reported that *T. neriifolia* contains powerful cardiotonic drugs which are distributed throughout the plant, but mainly concentrated in the seeds. The roots, stems and kernels contain 1-5% of bitter principles, of which Peruvoside and neriifolin are the major components; their action is more or less equal to ouabain. This principle is more rapidly eliminated, though there is little difference between effective and toxic doses [14]. Recent investigators reported the anticancerous activity of peruvoside as it can potentially inhibit cell proliferation of both androgen sensitive and resistant prostate cancer cells without triggering severe cytotoxicity[15]. Interest in cardiac glycosides, used in patients with congestive heart failure, has increased because of their established anticancer [16] and antiviral activities [17]. Similar therapeutic necessities add more value for isolation of this potent drug from natural resources.

Cardiac glycosides consist of a glycone (sugar) and an aglycone (non sugar) moiety. The active principles of cardiotonic glycosides are of cardinolide type; these are steroid derivatives to which a 5-membered unsaturated lactone ring is attached at the 17 position. The absence of an unsaturated lactone ring renders the glycosides cardio-inactive. The aglycone part of peruvoside is cannogenol and glycone part is L-thevetose; and pharmacological activity resides in aglycone part. These steroidal cardiotonic glycosides specifically exert definite action on heart muscles. The plant *Thevetia neriifolia*, Linn (= *T. peruviana*, fam: Apocynaceae) is included in the list of toxic plants even though enormous medicinal properties were cited in various literatures. This evergreen garden shrub commonly known as "yellow oleander" possesses large showy flowers in three different shades- yellow, orange and white. Plants blossom and produce fruits throughout the year. Each fruit contain 1-4 seeds which are the store house of powerful cardiotonic drugs with many potentialities. Seeds are odourless, tastes bitter and produce numbness when chewed. Each seed contain two cotyledons with approximately 60-63 % oil useful for cooking and soap industry. In view of the vast literature accumulated over centuries related to the therapeutic significance of cardiotonic drugs, especially peruvoside, we focused our study to quantify it using HPTLC techniques, from plants growing in present climatic conditions. A comparison between seeds of morphovariant plants also carried out to know better availability of drug within these plants. Quantification of peruvoside from other parts of plant especially leaves, flowers and fruit rind are also underway.

II. MATERIALS AND METHODS

Mature fruits were collected from different fields of middle Kerala. The plants were authenticated by Dr. Sunil C N and the voucher specimens are maintained at St. Teresa's College herbarium for future reference. Seeds were collected by mechanical cracking of the inner fruit wall. Coarsely powdered 30g sample was subjected to petroleum ether extraction to remove oil. Remaining residue was extracted with chloroform, ethyl acetate and methanol in a soxhlet apparatus for 15-18 h, fractions were evaporated to dryness, weighed and kept in labeled specimen bottles for further analyses. Standard drug Peruvoside (CAS Number 1182-87-2) with 90% purity was purchased from Sigma –Aldrich. USA. The authentic standard appeared as light yellow powder, empirical formula is $C_{30}H_{44}O_9$ with molecular weight 548.66. Drug was available as 100mg pack (Product Number P7897) and stored at $4^{\circ}C$. Standard was prepared at a concentration of 1mg/ml and all samples were diluted to get a final concentration of 10mg/ml. Preliminary phytochemical screening were carried out using standard protocol [18] to identify different classes of primary and secondary metabolites.TLC plates were developed in different solvent systems of varying polarity. Concentrated sulphuric acid was used as spraying reagent for the detection of peruvoside [19]. The instrument used for quantification of peruvoside was CAMAG-HPTLC system (Germany) comprising Linomat V automatic sample applicator, Camag TLC scanner with win CATS planar chromatography manager software. The samples were spotted in the form of bands of width 6 mm in precoated silica gel aluminum plates 60 F 254 TLC (E MERCK KGaA) of 10 x 10cm size. The distance between two tracks was 10 mm. An application volume of 5µl was applied at a position of 10mm from the bottom on the plate and the length of chromatogram run was 80mm from the application position. The chromatogram was developed in a Camag twin-trough glass chamber of 20 x 10cm size, using a linear ascending technique. The chloroform methanol (8:2) mobile phase was saturated for 10 min in the developing chamber at room temperature. The developed chromatogram was scanned using CAMAG TLC Scanner-3 at a speed of 20mm/s at 220 wavelengths. The scanner converts bands into peaks, peak height and area which were related to the concentration of the substance on the spot. Developed plates were visualized and documented using digital documentation system in a visualizer under UV light at 254nm, 366 nm and white light. When individual component does not respond to UV, then derivatization was done with suitable visualizing agent, the Libermann Burchard reagent [20]. Images were captured prior and after derivatization. Standard drug peruvoside and six samples were loaded simultaneously in the same plate as nine tracks.

III. RESULTS AND DISCUSSION

Phytochemicals from natural sources are gaining much importance because of their vast chemical diversity, and hence led to the increasing demand of herbal medicines. To assure quality and purity of phytochemicals, the techniques usually employed are sequential preliminary screening, TLC and sophisticated analytical techniques like High Performance Thin Layer Chromatography- HPTLC. Analysis of all fractions of seed extract revealed the presence of carbohydrates, proteins, alkaloids, terpenoids, steroids and cardiac glycosides as primary and secondary metabolites. In the present study, cardiac glycosides were separated by TLC technique using chloroform: methanol, ethyl acetate: chloroform: water and Toluene: butanol solvent systems in varying proportions. Effective separation was achieved in chloroform: methanol (8:2) mobile phase. Of the four fractions assayed, chloroform and ethyl acetate fraction showed the presence of peruvoside as a lemon yellow band with spraying reagent, concentrated sulphuric acid having an Rf value of 0.65, whereas

petroleum ether and methanol fraction gave negative results. Hence, HPTLC analysis was carried out using these two positive fractions. Presence of peruvoside in the samples was detected by comparing the Rf values of samples with standard. A comparative analysis was made between chloroform and ethyl acetate fractions of the seeds from three morphoforms by parallel loading of samples.

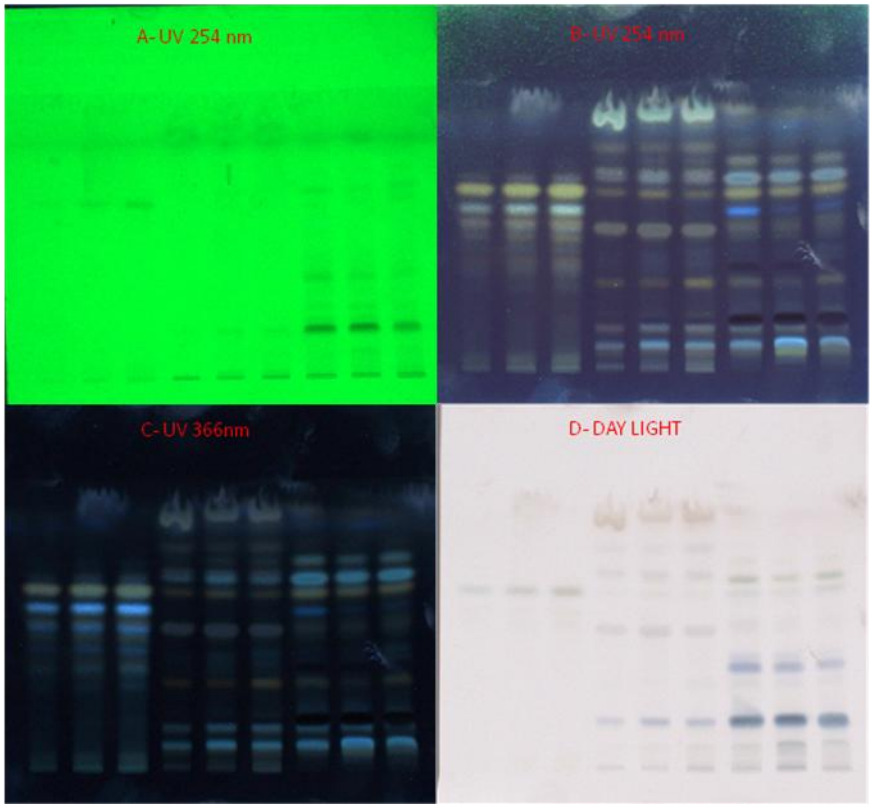

Fig.1: Comparative chromatogram of standard Peruvoside and other cardiac glycosides of *T. neriifolia* seed extracts

A. HPTLC profile of *Thevetia neriifolia* seed extracts of yellow, orange and white forms at 254nm wavelength before derivatization
(Tracks 1-3 standard drug peruvoside; 4-6 chloroform fractions; 7-9 ethyl acetate fractions).B-D. HPTLC profile after derivatization under 254nm, 366nm wavelength and under day light.

Fig 1 (A-B) represents the HPTLC fingerprinting of three morphovariants at 254 nm before and after derivatization with Libermann Burchard reagent. This reagent gave a prominent pale yellow colored band with standard peruvoside in first three tracks (Fig 1 B-D). Track 4-6 represents the images of chloroform extract and 7-9 tracks images of EA extract. From the images it is clear that extractability of peruvoside with EA was higher compared to chloroform. Estimation of peruvoside drug was done in positive fractions of seed extracts of *Thevetia* using HPTLC technique. Using scanning densitometry, HPTLC chromatogram can be converted into densitogram, in which all spots will be observed as peaks [21]. Densitogram compute the quantity of an analyte as percentage area that comes between start Rf and end Rf. Peruvoside started to elute at Rf 0.58, comes maximum at 0.61 and ends in 0.66 (Fig 2A). Standard also showed slight difference in Rf in all three tracks in same developing plate (Table 1). A minor deviation of 0.02 in the Rf can be considered as negligible variation. A reasonable acceptance criterion would be that Rf values of the same substance do not vary more than 0.02 from plate to plate [22]. Chloroform extract of yellow phenotype showed the presence of 10 peaks indicated the existence of 10 different types of cardiac glycosides (Fig 2B), where as orange and white forms contain 13 peaks each (Fig 2 C-D) within Rf range from 0.01 to 0.99. Lower number of bands was observed in the yellow form compared to the other two forms. Similarly, EA fractions also carried 12, 13 and 14 forms of cardiac glycosides including the analyte under study.

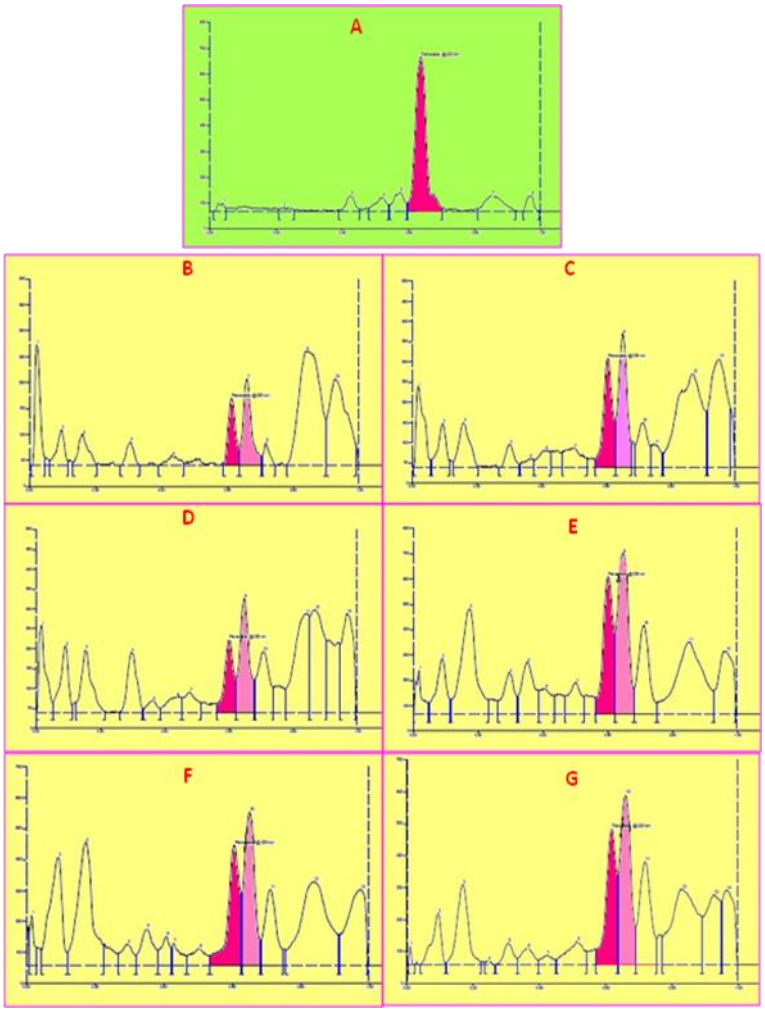

Fig 2. Comparative chromatogram of standard drug peruvoside with CH and EA fractions of seed extracts of *T. neriifolia.*

A. Chromatogram of standard peruvoside

B – D. Chloroform fractions of yellow, orange and white variants.

E – G. Ethyl acetate fractions of yellow, orange and white variants.

Quantity of an analyte can be calculated from various parameters and data obtained from densitogram. Thus, chloroform fraction of yellow form hold 7.27% area within Rf 0.59 and 0.64, and hence contain 1.3% of desired drug peruvoside (Table 1). Similarly, CH fraction of orange form contains a greater quantity of peruvoside (3.4%) when compared to white form (2.32%). Present study also revealed that a very good percentage (7.47, 5.74, and 5.83 %) of peruvoside can be extracted using EA from all three variants than CH. Paper chromatographic studies by Bisset revealed a maximum of 15 cardenolides in fresh seeds with a low proportion of mono glycosides. Our study also exposed that the composition of glycoside extract from the seeds of an orange and yellow flowered specimen was essentially similar. The literature also suggests that the cardenolide content and composition in the seeds of three color varieties were more or less similar [23].Consolidated table 1 (part B) represents the quantification of a similar compound within the same Rf value limit of standard when analysed. This similarity of compounds 8, 9 and 10 can be interpreted as various isomeric forms of peruvoside. This second compound started to separate at Rf 0.63 that comes within the standard range of 0.58-0.66. This drug can exist in four forms A, B, C and D depending upon the functional group it carries, according to Huanh [24]. Peruvoside A carries CHO, B with CH_3, C contains CH_2OH and D carries COOH as functional groups. Loi *et al* [25] reported that peruvoside B has a remarkable tranquilizing effect, and clinical trials on 357 cases for congestive heart failure showed 78.4% effective rate.

Table 1. Comparative densitogram showing Rf values and quantity of peruvoside (in %) in chloroform and ethyl acetate fractions of seed extract of yellow, orange and white variants.

Stand /sample	Peak No.	Total peaks	Start Rf	Max Rf	End Rf	Area	Area %	Assigned substance	Quantity in %
Part A									
PERU	-	-	0.6	0.64	0.7	16471.1	64.37	Peruvoside	-
PERU	-	-	0.59	0.63	0.66	19003.0	49.03	Peruvoside	-
PERU	-	-	0.58	0.62	0.66	23372.2	48.36	Peruvoside	-
YSCH	6	10	0.59	0.61	0.64	2387.5	7.27	Peruvoside-1	1.30
OSCH	8	13	0.57	0.61	0.63	6231.0	10.88	Peruvoside-1	3.40
WSCH	8	13	0.56	0.60	0.62	4244.9	7.18	Peruvoside-1	2.32
YSEA	8	12	0.56	0.60	0.62	13679.0	12.55	Peruvoside-1	7.47
OSEA	9	13	0.54	0.61	0.63	10504.3	11.22	Peruvoside-1	5.74
WSEA	9	14	0.57	0.62	0.64	10674.5	13.27	Peruvoside-1	5.83
Part B									
YSCH	7	10	0.64	0.66	0.71	3551.6	10.82	Peruvoside-2	1.94
OSCH	9	13	0.63	0.65	0.68	7060.9	12.33	Peruvoside-2	3.86
WSCH	9	13	0.63	0.65	0.68	6789.4	11.49	Peruvoside-2	3.71
YSEA	9	12	0.63	0.65	0.68	17333.7	15.9	Peruvoside-2	9.47
OSEA	10	13	0.63	0.65	0.68	12288.3	13.13	Peruvoside-2	6.71
WSEA	10	14	0.64	0.66	0.69	13933.6	17.32	Peruvoside-2	7.61

YSCH, OSCH, WSCH – Chloroform fraction of Yellow, Orange and White variants.
YSEA, OSEA, WSEA – Ethyl Acetate fraction of Yellow, Orange and White variants

Studies conducted by Abe *et al* reported different isomeric forms of cardenolide glycosides while isolating and identifying seventeen cardiac glycosides using NMR technique from *Thevetia* leaves. Siddiqui *et al* documented the presence of different functional groups in peruvoside such as hydroxyl group, methyl group and aldehyde group in *Thevetia* leaves while summarizing his studies using ^{1}H and ^{13}CNMR techniques. From their studies, it was proven that the cardiac glycosides present in this plant can occur in several isomeric forms. Such variations will presumably be due to seasonal, ecological or any of the many other factors which can affect the production of cardenolides in these plants [3]. Most glycosides occur in nature in different isomeric forms due to the tautomeric shift of functional groups. The second compound named as Peruvoside-2 in the table accounts a higher quantity than peruvoside-1. Our study revealed that a total 20% extractable peruvoside was present in the seeds in different isomeric forms. Said & Rahman [26] isolated 22.5% of peruvoside in pure form by enzymatic hydrolysis of the defatted seed kernel followed by TLC in CH: MT (9:1) solvent system.

IV. CONCLUSION

Congestive cardiac failure is one of the most common causes for death worldwide. Peruvoside, a powerful cardiac glycoside was quantified from various fractions of seed extracts of *Thevetia* phenotypes using HPTLC technique. Studies showed that the glycoside mixtures present in the seeds were essentially similar compounds that occur in various inter convertible forms. Availability of high amount (20%) of natural cardio tonic drug peruvoside in the seeds adds therapeutic significance of *T. neriifolia* in the treatment of congestive heart failure. In modern drug analysis, a natural compound having pharmacological value can be converted into many lead compounds. In India, potential therapeutic knowledge and industrial facilities should be utilized for the isolation of powerful cardiotonic drugs on commercial scale to reduce the import of expensive drugs like digoxin.

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