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Original Article

COMPARATIVE ANALYSIS OF CARDIAC GLYCOSIDES AND QUANTIFICATION OF PERUVOSIDE FROM *THEVETIA NERIIFOLIA*, JUSS FRUIT RIND EXTRACTS THROUGH HPTLC FINGERPRINTING

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ABSTRACT

Objective: Present investigation was carried out to determine the cardiac glycoside profile and to quantify the powerful cardiotonic drug peruvoside in the fruit wall of *Thevetia neriifolia*, Juss a medicinal plant used in indigenous system of medicine since decades.

Methods: Shade dried powdered samples were subjected to soxhlet extraction using different solvents, and various forms of cardiac glycosides were analysed in all fractions using TLC plates. Positive samples for peruvoside were subjected to HPTLC fingerprinting on silica gel 60 F_{254} plates using chloroform: methanol (8:2v/v) mixture as mobile phase.

Results: Fingerprint profile showed the presence of 6-14 cardiac glycosides in all three morphovariat plants. Densitometric analysis at 220 nm showed a peak at Rf 0.67±0.01 to the reference drug peruvoside, and in all samples analysed an identical compound corresponding to peruvoside was eluted within a range of 0.63 to 0.75 with minor variations. It was noticed that peruvoside was the third largest cardiac glycoside extracted from the fruit rind using chloroform.

Conclusion: In all studied morphoforms, about 3.25% peruvoside was detected in the rind which was the neglected and unused portion of fruit. Using fingerprinting technique, all cardiac glycosides can be effectively separated and utilized for pharmaceutical purposes.

Keywords: Cardiac glycosides, Fruit rind, HPTLC fingerprinting, Morphoform, Peruvoside.

INTRODUCTION

Cardiac glycosides are potent group of naturally occurring drugs and their distribution in plants is restricted to a few families and genera. Among angiosperms, these secondary metabolites are mostly concentrated within the members of Apocynaceae, Scrophulariaceae, Asclepiadaceae and Liliaceae. Plants use these compounds for defense purposes against herbivores [1]. The extensive use of digitalis related glycosides for treating heart failure are well accepted from centuries back, but narrow therapeutic index between effective and toxic dozes makes their use more vigilant [2]. The term cardiac glycoside was derived from the Greek word '*kardia*', meaning heart.

A great majority of drugs are either naturally derived or synthesized from plant related compounds. *Thevetia neriifolia*, Juss. (*T. peruviana*), a popular medicinal plant belongs to family Apocynaceae, contains many powerful cardiotonic drugs. In addition to the well documented folk uses of the plant in indigenous system of medicine [3-5], extensive pharmacological assays have been conducted by many researchers using peruvoside and related glycosides on failing human heart and experimental models, which showed better cardiotonic activity [6-7]. Naturally occurring digitalis-like glycosides continues to be isolated from different plant and animal species; though digoxin and its acetyl and methyl derivatives are the cardiac glycosides most currently used in therapeutics [2]. Recent researches revealed promising role of cardiac glycosides in the treatment of anticancer therapy [8-10]. Their novel role in healing several other diseases have also been already established [11-13]. Most of the *Thevetia* glycosides has been isolated and identified from different parts of the plant including fresh uncrushed leaves, seeds, flowers and stem bark [14-18]. The plant commonly called 'yellow oleander' is cultivated as an ornamental for its attractive, light scented flowers in yellow, orange and white forms. These color variants are considered as three morphoforms. Mature seeds are protected by fleshy mesocarp and hard stony endocarp. The green colored outer pulpy fruit wall (rind) rots away easily from fallen fruits. Seeds are reported to be a store house of various cardiac glycosides, however, phytochemical studies related to fruit rind remains scanty. Hence, the present investigation was carried out to categorize various phytochemicals, mainly cardiac glycosides in the fruit wall of all three morpho-variants and to quantify a potent cardiotonic drug peruvoside in light of the previous documentations regarding the therapeutic significance of peruvoside and other cardiac glycosides.

MATERIALS AND METHODS

Mature fruits of *Thevetia neriifolia* were collected from various fields of Trichur district, Kerala. The authenticated voucher specimens (2458a-c) were kept in the Herbarium cabinet of Botany Department, St. Teresa's College, Ernakulam. Ripened fruits were collected, washed carefully and kept in shade for 2-3 days for the ease of separation of fleshy mesocarp from stony endocarp. Pulpy fruit rinds were dried in shade for 3-4 weeks and powdered. Precisely weighed powdered samples (40g) were subjected to successive soxhlet extraction using 350 ml each of Petroleum Ether (PE, 60-80°C), Chloroform (CH), Ethyl Acetate (EA) and Methanol (MT) for 15-18 h in a soxhlet extractor. The fractions were dried, weighed and kept in labeled vials at 4°C for further analyses. Solvents and routine chemicals were procured from Merck Chemicals, Mumbai.

Preliminary phytochemical screening was conducted systematically as per the standard protocol [19]. All four fractions (PE, CH, EA, MT) from three color variants were analysed for the presence of primary and secondary metabolites like carbohydrates, proteins, starch, alkaloids, terpenoids, phenolics, steroids, saponins, cardiac glycosides and anthraquinons.

Biological reference material, Peruvoside isolated from *Thevetia neriifolia* was procured from Sigma- Aldrich, USA as 100 mg pack (CAS Number 1182-87-2) of 90% purity. All twelve samples (four extracts each from three plants) and reference drug were loaded side by side on TLC plates and from repeated experimental trails

separation of various cardiac glycosides was achieved on chloroform methanol (8:2) mobile phase. Presence of peruvoside and other members of cardiac glycoside family were detected using spraying reagent sulfuric acid [20]. Quantification of peruvoside and other cardiotonic steroids was done by HPTLC fingerprinting using Camag HPTLC system (Germany). Samples of CH and EA fractions from the three variants were prepared by dissolving 10mg of extract in 1 ml of corresponding solvent, while 1mg/ml dilution was made for reference compound. Sample solutions were centrifuged at 3000 rpm for 3 min and spotted (5 µl) as bands of 6 mm width in precoated silica gel aluminum plates 60 F_{254} (E Merck KGaA). Camag twin-trough developing chamber was saturated with 10 ml chloroform: methanol (8:2) mixture mobile phase for 10 min at room temperature. The developed chromatogram was dried at 80ºC for 3 min and scanned using CAMAG TLC Scanner-3 at a speed of 20 mm/s at 220 nm.

Plates were visualized and documented using digital documentation system in a visualizer under UV light at 254 nm, 366 nm and white light. Post chromatographic derivatization was carried out by spraying visualizing agent- Liebermann Burchard reagent onto the plate. The plate was dried again at 120ºC for 20 minutes and images were captured. From the recorded data, quantity of peruvoside was calculated in percentages and comparative table of other cardiac glycoside series was prepared. Six unknown samples and reference compound were chromatographed side by side on the same plate; and fingerprints were compared with respect to number, sequence, position and color of separated zones [21].

RESULTS AND DISCUSSION

Qualitative phytochemical screening for the presence of diverse groups of primary and secondary metabolites was carried out in all four fractions (PE, CH, EA & MT) of three studied morphovariant plants. The results revealed the presence of carbohydrates, alkaloids, flavonoids, steroids, saponins, terpenoids, and cardiac glycosides in various fractions. All these classes of phytochemicals have a wide range of bioactivities, so the plant is proved to be a valuable reservoir of several compounds with immense therapeutic value [22-24]. TLC analysis was employed to detect the presence of various cardiac glycosides, since the study was focused on these metabolites especially peruvoside. In silica gel plates the Rf (retardation factor) value of peruvoside was found as 0.63-0.65 when chloroform: methanol (8:2) mixture was used as mobile phase. For all samples analysed, positive results were obtained for CH and EA fractions, while PE and MT fractions showed negative result for peruvoside. Appearance of purplish brown and blue green bands was considered as positive evidence for the presence of various cardiac glycosides; but peruvoside was observed as deep yellow colored band with spraying reagent concentrated H_2SO_4 [25] in chloroform and ethyl acetate fractions. These positive samples were further analysed *via* HPTLC fingerprinting for the simultaneous separation and estimation of various cardiac glycosides and quantification of peruvoside using biological reference material. Extensive scientific research on this unattended fruit wall exposed the occurrence of enormous biological potential in the form of cardiac glycosides and other phytochemicals.

Densitogram of HPTLC revealed that chloroform extract of yellow and white variants (YRCH & WRCH) showed the presence of 9 types of cardiac glycosides as 9 peaks with different Rf values ranging from 0.03 to 1.00. Lesser diversity was observed in ORCH with 6 compounds (Table 1). Comparison of compound 1 in all three CH samples with respect to the Rf value and peak area, it is understood that this compound is identical in all three color variants. Higher percentage in ORCH may be due to the fact that it does not have undergone hydrolysis and formed compound 2 as observed in YR and WR. Compound 3 was observed in WR only, because it may be another hydrolysis product of compound 1. Almost similar Rf values were detected for compound 4 in all samples, but minor quantity of compound 5 was present in YR only. Subsequently eluted compound (6) in YR and WR illustrated quite similarity to reference drug and the elution time in WR prolonged further at the tail end when compared to reference compound. Peruvoside purchased from Sigma showed an elution phase from 0.63 to 0.74 (Fig 2A). Samples from all three variants possessed similar compound with elution period 0.63 to 0.75 with a ±0.03 variation. Minor variations can be neglected as all compounds in the same developing plate show this deviation. Similar Rf values showed by the remaining three compounds (7, 8 & 9) revealed the relationship of *Thevetia* glycosides in nature structurally and functionally. Of course, all these cardiotonic steroids have similar function. All these derivatives are formed from the parent compound Thevetin [26]. Fractionation and hydrolysis of Thevetin leads to the formation of a variety of compounds with interconvertable and rearrangable functional groups and other molecules. In chloroform extract, peruvoside was quantified as the third major compound in all studied variants, and all investigated samples showed nearly the same fingerprint (Fig 2 B-D). Usually, different developmental stages of plants or different plant organs may vary considerably in their qualitative and quantitative phytochemical composition. This difference in constituent profile of the plant material may be determined by several environmental factors (temperature, light, rain, soil pH) harvesting time, drying methods, extraction methods, duration, enzymatic reaction during storage etc. [21].

Table 1: Comparative evaluation of Rf values of Cardiac Glycosides present in CH fraction of three color variants of *Thevetia neriifolia* fruit rind.

	YRCH (9)				ORCH(6)				WRCH(9)			
Peak no.	**Start Rf**	**Max Rf**	**End Rf**	**Area %**	**Start Rf**	**Max Rf**	**End Rf**	**Area %**	**Start Rf**	**Max Rf**	**End Rf**	**Area %**
1	0.03	0.05	0.10	8.96	0.03	0.05	0.08	10.48	0.01	0.05	0.08	7.05
2	0.23	0.26	0. 32	3.09	-	-	-	-	0.23	0.25	0.26	0.89
3	-	-	-	-	-	-	-	-	0.33	0.36	0.39	1.91
4	0.40	0.45	0.50	10.06	0.41	0.44	0.49	4.15	0.40	0.44	0.49	10.26
5	0.51	0.52	0.54	0.92	-	-	-	-	-	-	-	-
6	0.58	0.62	0.64	3.50	-	-	-	-	0.59	0.65	0.67	5.48
7	0.65	0.71	0.74	14.80	0.63	0.71	0.73	16.95	0.68	0.70	0.75	7.94
8	0.74	0.77	0.80	8.51	0.74	0.76	0.80	9.66	0.75	0.76	0.78	0.79
9	0.81	0.86	0.91	36.25	0.80	0.86	0.88	25.12	0.79	0.85	0.87	32.09
10	0.91	0.93	1.00	13.92	0.88	0.93	1.00	33.64	0.87	0.90	0.98	32.71

YRCH, ORCH, WRCH – CH fraction of Rind from Yellow, Orange and White variants (Compounds having similar Rf values are considered as same and tabulated in single row)

Fingerprint analysis of EA fraction of fruit rind (Fig E-G) is tabulated in Table 2. Compound 1, extracted by EA (Table 2) with elution range of 0.1 to 0.7 showed the highest area of 35-38 %, and can be considered as identical to compound 1 present in CH fraction when Rf value was considered. This compound was more soluble in ethyl acetate than chloroform. Similarly, Compound 2 and 12 appeared as another chief glycoside in all three samples. Minute quantities of some analytes (3, 6, 10, 14) were detected in all samples, where as a few (4, 5, 7, 8, 9, 11, 13) were present in particular samples only. It was noticed that moderate amount of peruvoside was observed in

all three samples (compound 10) and quantity varied in the following order - yellow> orange > white. In white variant, a total of three compounds (Rf 0.59 to 0.77) were separated within the Rf range of reference compound (Rf 0.63 to 0.74). Each morphovariant plant contains more than a dozen similar but different compounds in one way or the other. The complex molecules have a tendency of biotransformation into different derivatives due to the action of endogenous enzymes [27]. Definitely, this genus contains many known and unknown cardiac glycosides as complex mixtures that occur together in the same plant.

Table 2: Comparative evaluation of Cardiac Glycosides present in EA fraction of three color variants of *T. neriifolia* fruit rind

	Yrea (9)				Orea (12)				Wrea (11)			
Peak No.	**Start Rf**	**Max Rf**	**End Rf**	**Area %**	**Start Rf**	**Max Rf**	**End Rf**	**Area %**	**Start Rf**	**Max Rf**	**End Rf**	**Area %**
1	0.03	0.05	0.07	37.72	0.01	0.05	0.07	36.64	0.02	0.04	0.07	35.16
2	0.08	0.10	0.14	25.70	0.07	0.10	0.13	24.99	0.07	0.08	0.11	10.28
3	0.15	0.18	0.20	2.08	0.13	0.16	0.18	4.12	0.11	0.12	0.14	1.16
4	0.20	0.22	0.25	1.28	-	-	-	-	0.20	0.25	0.30	5.24
5	-	-	-	-	0.36	0.38	0.41	0.98	-	-	-	-
6	0.42	0.45	0.50	2.42	0.41	0.45	0.47	2.92	0.42	0.45	0.46	1.50
7	-	-	-	-	-	-	-	-	0.54	0.56	0.58	4.84
8	-	-	-	-	0.58	0.61	0.63	3.81	0.59	0.62	0.66	6.59
9	-	-	-	-	0.68	0.68	0.69	1.12	0.66	0.69	0.72	5.98
10	0.69	0.73	0.76	4.31	0.70	0.71	0.75	3.79	0.72	0.74	0.77	3.15
11	0.76	0.77	0.79	0.90	0.75	0.76	0.79	1.15	-	-	-	-
12	0.83	0.88	0.95	23.75	0.83	0.88	0.92	16.93	0.83	0.88	0.95	25.18
13	-	-	-	-	0.93	0.94	0.96	2.51	-	-	-	-
14	0.96	0.98	1.00	1.83	0.97	0.98	1.00	1.03	0.98	0.99	0.99	0.91

YREA, OREA, WREA – EA fraction of Rind from Yellow, Orange and White variants

Results presented in Table 3 revealed that all morphovariant plants contained almost same amount of peruvoside. Higher amount can be extracted using chloroform than ethyl acetate.

Two similar compounds having Rf values within the range of standard values are considered as same and may be formed by the rearrangement of functional groups that could occur due to various reasons. Detailed studies are needed for each component to prove the structural similarities or variations, if any. Similar analogues were observed in our earlier studies with seed and flower extracts [28-29].

The description of the chromatogram prior and subsequent derivatization with Liebermann Burchard reagent was presented in Fig 1. The chromatogram of the reference compound (track 1-3) exhibits a prominent band in derivatized plate which can be visualized at both lower (254 nm) and higher (366 nm) wavelengths. Likewise, eluted compounds with identical nature can be recognized by looking its position and band color in samples. Bands were narrower, but visible in CH (track 4-6) and EA (track 7-9) samples in the chromatogram against the sharp and prominent ones of standard compound.

Table 3: Quantification of Peruvoside in CH and EA fractions of fruit wall of three color variant plants

Sample	Start Rf	Max Rf	End Rf	Area %	Area	% of peru	Total
Peru	0.63	0.67	0.74	57.8	15544.4	-	-
YRCH	0.65	0.71	0.74	14.8	4696.1	2.72	3.26*
ORCH	0.63	0.71	0.73	16.95	4558.5	2.64	
WRCH	0.59	0.65	0.67	5.48	1319.0	0.76	
„	0.68	0.70	0.75	7.94	1908.6	1.11	3.24**
YREA	0.69	0.73	0.76	4.31	925.1	0.54	
OREA	0.68	0.68	0.69	1.12	236.9	0.14	
„	0.70	0.71	0.75	3.79	801.8	0.46	3.07***
WREA	0.59	0.62	0.66	6.59	1091.9	0.63	
„	0.66	0.69	0.72	5.98	990.6	0.57	

Total Peruvoside present in *Yellow, ** Orange, ***White variants

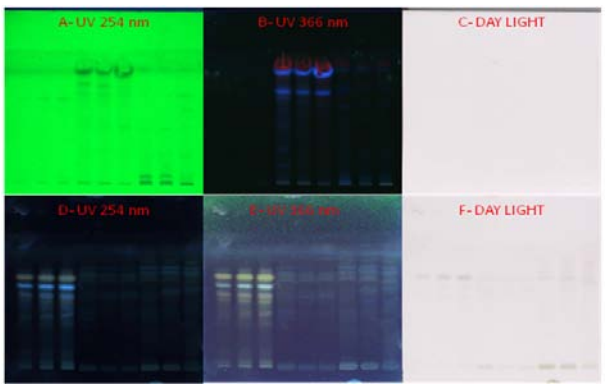

Fig. 1: Chromatographic plate of standard drug Peruvoside and other cardiac glycosides of *T. neriifolia* fruit wall extracts before and after derivatization.

A-C HPTLC fingerprints of *T. neriifolia* fruit wall extracts of yellow, orange and white forms before derivatization under 254 nm, 366 nm wavelength and day light (Tracks 1-3 Standard drug peruvoside; 4-6 chloroform fractions; 7-9 ethyl acetate fractions). D-F HPTLC profile after derivatization with Liebermann Burchard Reagent, photo documentation under 254 nm, 366 nm wavelength and day light.

Cardiac glycosides are a group of natural products with specific action on myocardial contraction. In large dozes, they are toxic and bring about cardiac arrest but in lower doses, they are important drugs in the treatment of congestive heart failure and associated atrial fibrillation [30]. Use of cardiac glycosides of biological origin in treating failing heart was widely accepted from nineteenth century. Peruvoside possess a very good cardiotonic activity and *Thevetia* glycosides (Peruvoside, Theveneriin, Neriifolin) can be converted to digitoxigenin and digitoxose by enzymatic reaction and by selective 1, 2-beta hydroxylation to yield digoxin [31]. Huge quantities of cardiac glycosides available in decaying fruit wall can thus be converted into commercial products for valuable cardiotonics.

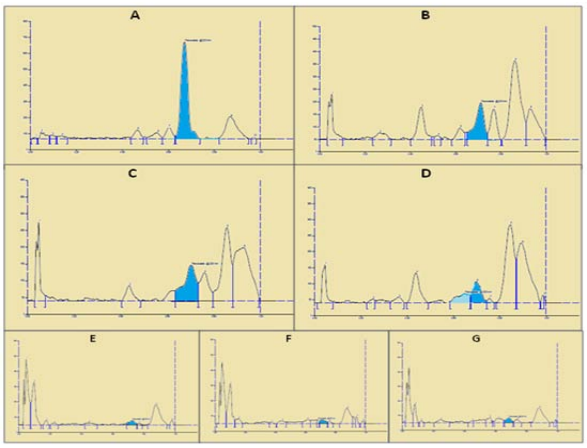

Fig. 2: Comparative chromatogram display of standard drug peruvoside, CH and EA fractions of fruit wall extracts of *T. neriifolia* at 220 nm wavelength showing the separation of various cardiac glycosides.

A. Chromatogram of standard compound peruvoside, B-D. Chloroform fraction of Yellow, Orange and White Variants. E-G. Ethyl Acetate fraction of three variants.

CONCLUSION

Present study shows that fourteen different types of cardiac glycosides were present in the fruit wall of *Thevetia neriifolia*. These glycosides can be extracted using easily accessible solvents. As expected, all three morphovariants contained an identical array of some cardenolides. Peruvoside was detected as the third major compound in the cardiac glycoside family and was present in all three morphovariants in almost equal amount. Commercial exploitation of these powerful secondary metabolites from the ignored rind definitely provides cheap and easily reachable medicines for common people. In fact, the lower availability of natural and therapeutically valuable metabolites makes them very costly. In this context, the occurrence of a higher quantity of cardiac glycosides in the neglected fruit rind makes the plant more significant for the extraction and isolation of all available compounds for the fruitful conversion into commercial products of cardiotonic series.

CONFLICT OF INTEREST

We have no conflict of interest.

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