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Original Article

HPTLC PROFILING ON CARDIAC GLYCOSIDES OF *THEVETIA PERUVIANA* LEAF EXTRACTS OF THREE MORPHOVARIANT PLANTS GROWN IN KERALA, INDIA

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ABSTRACT

Objective: *Thevetia peruviana*, an invasive plant with several cardioactive glycosides are grown in different localities of Kerala, India with three colour blooms. Present study was carried out to detect various cardiac glycosides in air dried samples; and soxhlet extracts of shade dried, powdered leaves of the three morphovariants, using spectrophotometry and chromatography.

Methods: Determination for total cardiac glycosides in dried samples were carried out at 495 nm, using Baljet's reagent. After preliminary screening on silica coated micro TLC glass plates using chloroform-methanol mobile phase (8:2), separation of various cardiac glycosides and quantification of 'Peruvoside' (a valuable cardiotonic drug), was achieved using HPTLC, on silica gel $60F_{254}$ plates at 220 nm, for CH (chloroform) and EA (ethyl acetate) fractions.

Results: About 2.5-3.37 % of total cardiac glycosides were detected in powdered leaves. Among the successive extracts, both CH (9-11) and EA fractions (8-12) occupied different forms of cardiac glycosides, and a good amount of peruvoside was detected either in single or in two isomeric forms. The quantity of the drug varied in the order white (8.52 %)>orange (7.16)>yellow (4.97 %) among the three studied plant samples.

Conclusion: Peruvoside, a notable drug for congestive heart failure has been reported to have anticancerous properties apart from its cardiotonic efficacy. Hence, the leaves can be effectively utilized for the successful isolation of this potent drug and other glycosides, without endangering the existence of this taxon.

Keywords: Cardiac glycosides, Densitogram, HPTLC, Peruvoside.

INTRODUCTION

Majority of medicinal plants contain many remarkable compounds with exploitable biological properties, sometimes beyond the practical reach of synthetic chemicals. Cardiac Glycosides, of course, were used as potent heart stimulants from ancient time. Plants with these phytochemicals are spread over not less than 34 genera in about 11 unrelated families, including Apocynaceae, Asclepiadaceae, Liliaceae and Ranunculaceae [1]. They are used to treat severe chronic cardiac insufficiency, certain forms of cardiac arrhythmia and cardiac shock, and are considered as a single group due to its similar pharmacological characteristics [2]. *Thevetia peruviana* K. Schum (= *T. neriifolia*, Juss) occupied a significant place among the many genera of Apocynaceae rich in cardiac glycosides. The genus, commonly known as 'yellow oleander' belongs to the dogbane family, distributed in the tropical and subtropical regions of the world, is now naturalized in Indian subcontinent and elsewhere. It grows up to a height of 10-15 feet and bears simple, alternate, dark green lanceolate foliage, clustered together on slender branchlets. The showy blooms usually in yellow, orange or white colours are developed on axillary cymes. All plant parts, especially the seeds, were reported to possess a huge quantity of therapeutically valuable cardiac principles.

Chemical examination of *Thevetia* seed glycosides dates back to 1863 when DeVry first reported the presence of cardiac glycosides and isolated 'Thevetin' from its seeds. Since then, there has been considerable published literature regarding the therapeutic and toxicological aspects of this plant. As many as 15 components were isolated from fresh seeds that include Thevetin A, Thevetin B, peruvoside, thevebiocide, thevenerin, neriifolin and cerberoside [3]. Similarly, about 16-17 compounds (neriifoside, peruvoside, oleanolic acid, ursolic acid, lupeol acetate, α-amyrin acetate, β-amyrin acetate, etc.) were recovered from the fresh and frozen leaves of this plant [4, 5]. Two new cardenolides identified were termed as Thevetin C and acetyl thevetin C, among the six compounds isolated from its leaves [6]. All these studies revealed that the plant is a natural source of many cardiac glycosides, which could find a useful role in congestive heart failure.

Heart diseases are increasing day by day due to many known and unknown reasons. Availability of life saving drugs is beyond the reach of common people. In order to compensate the increasing demand of cardiotonic drugs, isolation of powerful compounds from natural resources through cost-effective method is essential. Moreover, plants are the chief source of cardiac glycosides and their synthesis is difficult due to their high structural peculiarity. Since there are overwhelming reports regarding the existence of a series of glycosides in various plant parts, it is relevant to make a comparative quantitative study regarding the occurrence of various cardiac glycosides, principally 'Peruvoside' in the leaves of three colour variants grown in Kerala, the Southern State of India.

MATERIALS AND METHODS

Plants are commonly cultivated as ornamentals in Trichur Dist, Kerala. Fresh twigs were collected from three morphovariant plants; and the authenticated herbarium specimens (STHAPC 2458a-c) are maintained in the Department of Botany, St. Teresa's College, Ernakulam. Materials were washed thoroughly in running tap water to remove adhering dust particles and other contaminants. To avoid the loss of phytoconstituents *via* latex of wounded ends, twigs were allowed to dry in shade for 2-3 weeks, at room temperature. Withered leaves were collected from dried twigs, finely powdered and kept in airtight containers till further assays were done.

Quantitative estimation of total cardiac glycosides

One gram each of powdered samples was soaked overnight in 10 ml of 70 % alcohol and filtered. Based on lactone ring, total cardiac glycosides in the raw samples were estimated using Baljet's reagent, described by El-Olemy *et al.*, [7]. One ml of extract was purified using 12.5 % lead acetate and 4.77 % Na_2HPO_4 solution before the addition of freshly prepared Baljet's reagent (95 ml of 1 % aqueous picric acid+5 ml 10 % aqueous NaOH). The intensity of the color produced is proportional to the concentration of the glycoside. The absorbance was measured using spectrophotometer at 495 nm against blank (distilled water) and the concentration of the glycoside

was calculated. Appearance of deep orange color, a character of digitalis cardiac glycosides, indicated the presence of lactone ring in the agycone part of cardenolides, after the reaction.

Soxhlet extraction

Accurately weighed leaf samples (30 g) were extracted successively with 300 ml each of PE (60-80 °C, petroleum ether), CH (chloroform), EA (ethyl acetate) and MT (methanol) in a Soxhlet extractor for 12-18 h [8]. The extracts were concentrated to dryness, weighed and kept in labelled bottles at 4 ◦C for further studies.

Sample preparation

Preliminary phytochemical screening and Thin Layer Chromatography (TLC) were carried out using appropriate dilutions of samples in corresponding solvents. The suitable solvent system was optimized to achieve the best resolution for cardiac glycosides (chloroform-methanol 8:2) in favor of chromatographic analysis. For High Performance Thin Layer Chromatographic (HPTLC) studies, chlorophyll pigments were precipitated by treating the samples with 10 % lead acetate and a final concentration of 10 mg/ml was made ready for fingerprinting. Detecting reagents sulfuric acid and Libermann Burchard were used to perceive various cardiac glycosides, especially for 'Peruvoside' in TLC and HPTLC plates respectively. Reference compound 'Peruvoside' (Sigma-Aldrich, CAS Number 1182-87-2) was diluted to a concentration of 1.0 mg/ml.

HPTLC instrumentation

Analysis was performed using commercially available pre-coated silica gel $60F_{254}$ plates (E. Merck KGaA) on the Camag-HPTLC system (Germany). To estimate the quantity of peruvoside and other cardiac glycosides, an aliquot of 5 µl sample (three samples, each of CH and EA fractions) and standard drug were loaded in a plate of 10 x 10 cm size as 9 tracks of 6 mm band width. The plate was developed up to a height of 85 mm in a twin trough developing chamber saturated with 10 ml of chloroform methanol mixture (8:2) mobile phase. After drying, the developed plate was visualized under UV light at 254 and 366 nm wavelengths and day light. Densitometric scanning was performed in the absorbance mode at 220 nm at a scanning speed of 20 mm/s through a 4.00 x 0.30 mm dimension slit. The plate was derivatised with Libermann Burchard reagent and images were captured again at all illumination modes. Bands for 'Peruvoside' in the samples was ascertained by comparing the Rf values and spectra of samples with those of the standard compound [9].

RESULTS

Cardiac glycosides usually do not get precipitated with lead acetate. Using Baljet reagent, a total of 3.37, 2.52 and 3.2 % glycosides were estimated in the dried leaves of yellow, orange and white variants, respectively.

Preliminary screening experiments showed that, of the four fractions analyzed, 'Peruvoside' was detected in CH and EA fractions, whereas it was absent in PE and MT fractions. Nonpolar solvent PE usually acts as a defatting agent, and they do not dissolve cardiac glycosides. Consequently, these metabolites were quantified in positive fractions. Bands for 'Peruvoside' in HPTLC plates were detected as compact yellow ones under shorter (254 nm) and longer (366 nm) wavelengths without derivatization. Before routine analyses the method need to be validated in the pharmaceutical environment. Initially, the repeatability of 'Peruvoside' application and measurement of peak area was carried out using three replicates on the same plate with 5, 10 and 15 µg/spot, to generate calibration curve.

The linear regression analysis data for the calibration plots showed a good linear relationship with r^2 = 0.9921 in the concentration range 5-15 µl with respect to peak area. HPTLC profiling of standard drug and eluted compounds in various leaf samples is illustrated in fig.1.

Total cardiac glycosides eluted during chromatography from CH and EA fractions of yellow variant leaves (YLCH & YLEA) were tabulated in table 1. Both fractions showed 11 and 8 peaks, and each peak represents different forms of cardiac glycosides. Of which, only 6 compounds were identical with respect to the Rf value. The remaining ones were observed in any one of the solvent fractions. The elution phase of the biological reference compound was found in the range of 0.62 to 0.71 with±0.02 deviation. In the EA fraction, similar elution was achieved by a compound (peak no. 9) with a moderate area of 14.22 %; but in less polar solvent (CH), the same compound splits apart as two analogous or isomeric compounds (peaks 9,10), sharing the Rf range of standard compound. Similarly, in orange color variant, both fractions occupied 9 cardiac glycosides each. Out of this, 7 metabolites have comparable Rf values, including the analyte under study, and only two differed each other (table 2). Likewise, the occurrence of 'Peruvoside' appeared quantitatively more in EA fraction (17.25 % area) as a single compound, than CH which split into two isomers and appeared as two peaks (12.11 area %).

As expected, chromatogram of the white variant possessed a series of 10-12 cardiac glycosides (table 3). In contrast to the above two observations, here, the compound of interest in the CH sample did not convert into two isomeric forms and showed a major area of 13.6 %, but in EA fraction, two identical forms with a minor deviation in Rf value was quantified. Quantity of peruvoside was calculated taking various parameters like dilution factors, area of analytes, etc.

Table 1: Comparative densitogram showing Rf values and area of compounds (in %) in CH and EA fractions of leaf extract of yellow variant and standard 'Peruvoside'(fig. 1: B, G, J)

Peak	YLCH (11)					YLEA (8)				
No.	Start Rf	Max Rf	End Rf	Area	Area %	Start Rf	Max Rf	End Rf	Area	Area %
1	0.02	0.05	0.06	11018.7	30.30	0.00	0.04	0.05	9653.3	20.53
2	-	-	-	-	-	0.08	0.10	0.11	186.5	0.40
3	0.13	0.15	0.17	231.0	0.64	-	-	-	-	-
4	0.28	0.29	0.32	853.0	2.35	-	-	-	-	-
5	0.33	0.34	0.35	312. 3	0.86	-	-	-	-	-
6	-	-	-	-	-	0.38	0.45	0.46	1962.1	4.17
7	0.45	0.47	0.48	517.1	1.42	-	-	-	-	-
8	0.51	0.55	0.57	2287.9	6.29	0.49	0.57	0.57	3409.2	7.25
9	0.62	0.64	0.66	2717.3	7.47	0.60	0.64	0.69	6686.9	14.22
10	0.66	0.68	0.69	1307.1	3.59	-	-	-	-	-
11	0.69	0.70	0.75	2726.0	7.50	0.70	0.74	0.75	3567.8	7.59
12	0.79	0.85	0.92	7867.9	21.63	0.78	0.84	0.91	12519.8	26.62
13	0.92	0.98	1.00	6528.3	17.95	0.92	0.98	1.00	9042.9	19.23
Std	0.63	0.67	0.70	19400.7	48.37	-	-	-	-	-

YLCH, YLEA–chloroform (CH) and ethyl acetate (EA) fractions of leaves (L) of yellow(Y) variant

Table 2: Comparative densitogram showing Rf values and area of compounds (in %) in CH and EA fractions of leaf extract of orange variant (fig. 1: H. K)

Peak No.	OLCH (9)					OLEA (9)				
	Start Rf	Max Rf	End Rf	Area	Area %	Start Rf	Max Rf	End Rf	Area	Area %
1	0.01	0.04	0.07	9526.3	23.79	01	0.04	0.06	10174.4	16.55
2	0.18	0.23	0.25	387.6	0.97	-	-	-	-	-
3	-	-	-	-	-	0.35	0.37	0.40	1119.3	1.82
4	-	-	-	-	-	0.40	0.45	0.47	2220.7	3.61
5	0.47	0.56	0.57	3227.4	8.06	0.47	0.51	0.53	3430.1	5.58
6	0.57	0.60	0.62	2107.0	5.26	0.54	0.59	0.60	3550.6	5.78
7	0.62	0.64	0.66	3200.9	7.99	0.60	0.64	0.70	10603.9	17.25
8	0.66	0.67	0.70	1648.6	4.12	-	-	-	-	-
9	0.70	0.72	0.76	2552.2	6.37	0.70	0.74	0.79	9185.2	14.94
10	0.78	0.85	0.93	10327.3	25.79	0.79	0.84	0.92	13608.2	22.14
11	0.93	0.98	1.00	7067.0	17.65	0.93	0.98	1.00	7581.1	12.33

OLCH, OLEA–chloroform (CH) and ethyl acetate (EA) fractions of leaves (L) of orange (O) variant

Table 3: Comparative densitogram showing Rf values and area of compounds (in %) in CH and EA fractions of leaf extract of white variant (fig. 1: I, L)

Peak No.	WLCH (10)					WLEA (12)				
	StartRf	MaxRf	EndRf	Area	Area %	Start Rf	Max Rf	End Rf	Area	Area %
1	0.01	0.04	0.05	7736.0	18.58	0.01	0.05	0.06	12852.6	18.46
2	0.07	0.09	0.11	244.6	0.59	-	-	-	-	-
3	-	-	-	-	-	0.11	0.13	0.14	89.7	0.13
4	0.17	0.19	0.21	198.4	0.48	-	-	-	-	-
5	-	-	-	-	-	0.23	0.28	0.30	1076.9	1.55
6	-	-	-	-	-	0.30	0.33	0.36	1569.3	2.25
7	0.41	0.44	0.45	679.9	1.63	-	-	-	-	-
8	0.47	0.54	0.56	2824.4	6.78	0.45	0.49	0.50	1901.1	2.73
9	-	-	-	-	-	0.50	0.56	0.57	4604.9	6.61
10	0.57	0.59	0.61	1860.5	4.47	**0.57**	**0.62**	**0.65**	**9167.6**	**13.16**
11	0.62	0.64	0.71	5660.7	13.60	0.66	0.67	0.69	3525.4	5.06
12	0.71	0.72	0.75	1744.0	4.19	0.70	0.75	0.76	7116.5	10.22
13	0.77	0.85	0.92	12150.1	29.19	0.76	0.77	0.79	2644.5	3.80
14	-	-	-	-	-	0.79	0.83	0.92	16134.6	23.17
15	0.92	0.96	1.00	8529.6	20.49	0.92	0.96	1.00	8953.2	12.86

WLCH, WLEA–chloroform (CH) and ethyl acetate (EA) fractions of leaves (L) of white (W) variant

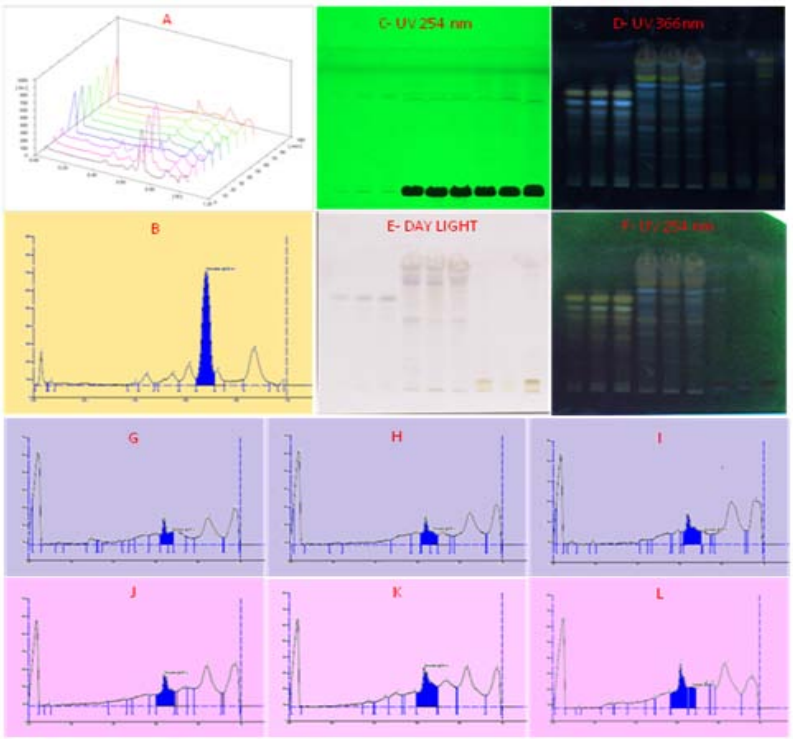

Fig. 1: HPTLC fingerprint analysis on Cardiac Glycosides of *Thevetia peruviana* leaf extracts of three morphovariant plants using standard drug 'Peruvoside'

A) 3D display of Chromatogram of leaf fractions (tracks 1-3 standard drug peruvoside; 4-6 chloroform fractions; 7-9 ethyl acetate fractions) B) Chromatogram of 'Peruvoside' at 220 nm at track 1; C-D) before derivatization at UV 254 nm and 366 nm; E-F) after derivatization at daylight and 254 nm; G-I) comparative densitogram display of CH fractions at tracks 4-6; and J-L) EA fractions at tracks 7-9 in the order yellow, orange and white forms, scanned at 220 nm.

Table 4: Quantity of 'Peruvoside' distribution in different parts of *T. periviana* morphovariants (leaves, flowers [10], rind [11] and seeds [12])

Sample	Quantity/ Sample(%)	Total quantity (%)	sample	Quantity/ Sample(%)	Total quantity (%)	sample	Quantity/ Sample(%)	Total quantity (%)
YLCH	1.87	4.97	OLCH	2.23	7.15	WLCH	2.63	8.52
YLEA	3.10		OLEA	4.92		WLEA	5.89	
YFCH	12.41	13.69	OFCH	9.26	10.1	WFCH	11.41	11.59
YFEA	1.28		OFEA	0.84		WFEA	0.18	
YRCH	2.72	3.26	ORCH	2.64	3.24	WRCH	1.87	3.07
YREA	0.54		OREA	0.60		WREA	1.2	
YSCH	3.24	20.18	OSCH	7.26	19.71	WSCH	6.03	19.47
YSEA	16.94		OSEA	12.45		WSEA	13.44	

Abbreviations for: mophovariants (Y-Yellow, O-Orange, W-White); Plant parts (L-Leaf, F-Flower, R-Rind, S-Seed) and Solvents (CH-Chloroform, EA-Ethyl Acetate).

DISCUSSION

There is no obvious pattern for the occurrence of various metabolites in different plants and plant parts. Around 2.5-3.4 % of total cardiac glycosides were quantified in air dried, powdered leaf samples, using Baljet's reagent. The varied distribution of cardiac glycosides was reported as 4.27 and 4.70 % of the raw seeds of *Thevetia* [13, 14]. Likewise, Sun and Libizor [15] noticed 3.6-4.0 % thevetin as the major glycoside in the seeds.

The results obtained during the ongoing investigations on the quantification of 'Peruvoside' in diverse plant parts of three morphoforms varied considerably, and are summarized in table 4. It is noteworthy to point out the efficacy of organic solvents-CH and EA in extracting maximum 'Peruvoside' without leaving any traces for further extraction by MT. An average quantity of extractable 'Peruvoside' was present in CH and EA fractions of leaf extracts (4.97-8.52 %), either in intact form or in two isomeric forms. Earlier studies revealed that around one fifth portion of CH and EA fractions of seeds [12] contained this valuable drug (19.47-20.18 %). Most surprisingly, a good amount (10.1-13.69 %) could be tapped even from withered flowers [10], and a fair amount (3.07-3.26 %) from unused and neglected fruit rind walls [11], clarified that they were distributed throughout the plant, and their intensity varied from organ to organ.

Cardiac glycosides are usually soluble in water and alcohol and insoluble in fat organic solvents with the exception of CH and EA. Compounds with higher number of sugar are soluble in water and lower number in CH [16]. Accordingly, many of the compounds extracted using CH and EA can be considered as stable compounds with lesser number of sugar molecules. Usually, the glycosides are susceptible to hydrolysis by enzymes during extraction process. The acetyl group and the glucose are split off, but fortunately the partly hydrolyzed glycosides such as digoxin and digitoxin are as biologically active as the parent compound [16].

Minute variations were observed regarding the number of cardiac glycosides present in the same fraction of morphovariant plants (YLCH-11, OLCH-9, WLCH-10). This variation could be more correlated with various enzyme actions and other metabolic changes that could happen from harvesting time onwards. The difference in the constituent profile of the plant material may be affected by several environmental factors (temperature, light, rain, soil pH, etc.) harvesting time, drying methods, extraction methods and its duration, enzymatic reactions during storage, etc. [17]. According to natural rules, cardiac glycosides vary in availability both qualitatively and quantitatively. Due to the complex chemical nature of this group, more cardiac glycosides are continuously added to the database, in addition to the existing molecules, and the possibility of biological conversion depends on a number of criteria. Among the possible views related to the variations observed in the number of glycosides, it will be relevant to discuss the nature of cardiac glycosides during hydrolysis.

Natural cardiac glycosides found in plants are the precursor molecules, which upon alkaline and enzymatic hydrolysis yield various secondary glycosides by giving up one or more sugar molecules, which on further hydrolysis produces stable tertiary compounds. Because of greater stability of secondary glycosides, and lesser absorption of primary glycosides, a higher content of the latter is not ideal for direct therapeutic usage [18]. Upon acid hydrolysis, the sugars are cleaved leaving the aglycone or genin, that makes it inactive [6]. In plants, the hydrolyzing enzymes usually co-exist with cardiac glycosides, but in different cells or compartments. Glycosides are stored in vacuoles, protected from hydrolytic enzymes of cytoplasm and usually specific enzymes hydrolyzes specific cardiac glycosides. Primary glycosides from its storage region pass across the tonoplast, with the aid of a specific carrier protein [19], and might be reached all over the plant body. In a number of species, the site of biosynthesis is restricted to one organ, whereas the products are accumulated all over the plant or in different organs and cells [20]. In *Thevetia peruviana*, the site of biosynthesis of cardiac glycosides could be considered as the photosynthetic vegetative part and might be transported to various reproductive parts where they accumulate, that accounts for the irregular distribution of 'Peruvoside' and other cardiac glycosides in various plant parts.

'Thevetin', the primary glycoside of *T. peruviana*, gives off two trioses: Thevetin A and B (secondary glycosides). Further hydrolysis leads to the production of tertiary glycosides: cerberin, neriifolin, peruvoside, ruvoside and peruvosidic acid [3]. Most of the extracted glycosides are the partial enzyme hydrolytic products of natural ones [2]. A wide range of semi synthetic and synthetic analogues have been prepared by introducing suitable modifications on isolated natural compounds, to determine the essential structural requirements for displaying cardiotonic activity, and to obtain derivatives with less toxicity [21]. To improve the pharmacological properties of natural cardiac glycosides, chemical and biochemical transformations are necessary. Furthermore, enzymatic biotransformation of peruvoside and neriifolin from yellow oleander gives digoxin [22]. The therapeutic potential of cardiac glycosides, including anticancer and antitumor effects was widely discussed by many investigators [23, 24].

CONCLUSION

Thevetia peruviana, 'the yellow oleander', one among the Indian indigenous plants, contain a mixture of several cardiac glycosides. Since different plant parts find use in various folk medicines and other clinical applications, and the plant material is the only industrial source for cardiac glycoside availability, it is important to establish the quality and quantity of various components in constituent plant parts.

About 15-18 types of cardiac glycosides exist in leaves and other parts which were distributed unevenly throughout the plant, some being common in all plant parts. Cardiac glycosides can undergo

hydrolysis at any time, from the time of harvest. If injury occurs to plant parts at the time of collection, the enzymes compartmentalized in cells mix with glycosides residing in the vacuole, leading to unpredictable breakdown of compounds. This results in the collapse of parent glycosides at various levels, which ultimately leads to the formation of different types of molecules or isomers, and hence, the process and method of extraction are very sensitive. Minor qualitative and quantitative variations occur in naturally distributed flora, unquestionably.

CONFLICT OF INTERESTS

No conflict in any matters

REFERENCES

1. Knight AP, Walter PG. Plants affecting the cardiovascular system. In: A Guide to Plant Poisoning of Animals in North America, Ithaca, New York, USA, International Veterinary Information Service; 2002.
2. Vardanyan R, Hruby V. Synthesis of Essential Drugs. 1st Edition. Elsevier, Amsterdam; 2006.
3. Bisset NG. Cardiac glycosides Part IV, Apocynaceae: A preliminary Paper Chromatographic Study of the Glycosides from *Thevetia peruviana* (Pers.) K. Schum. Ann Bogor 1961;4(2):145-52.
4. Abe F, Yamauchi T, Wan ASC. Cardiac glycosides from the Leaves of *Thevetia neriifolia.* Phytochem 1992;31(9):3189-93.
5. Siddiqui S, Siddiqui BS, Adil Q, Begum S. Cardenolides and Triterpenoids of the Leaves of *Thevetia neriifolia*. Phytochem 1992;31(10):3541-6.
6. Kohls S, Scholz-Bottcher BM, Teske J, Zark P, Rullkotter J. Cardiac glycosides from Yellow Oleander (*Thevetia peruviana*) seeds. Phytochem 2012;75:114-27.
7. El-Olemy MM, Al-Muhtadi FJ, Afifi AFA. Experimental Phytochemistry: A Laboratory Manual. Saudi Arabia, King Saud University Press; 1994.
8. Harborne JB. Phytochemical Methods: A Guide to Modern Techniques of Plant Analysis. 3rd Edn. New Delhi: Springer (India) private Ltd; 1973.
9. Patel RB, Patel MR, Batel BG. Chapter 3. Experimental aspects and implementation of HPTLC. In: Srivastava MM editor. High-Performance Thin-Layer Chromatography (HPTLC). New York, Springer, Heidelberg Dordrecht, London; 2011.
10. Nesy EA, Mathew L. Quantification of peruvoside (A Cardiotonic drug for Congestive heart failure) Using HPTLC fingerprinting from Withered Flowers of *Thevetia neriifolia*, Juss. Indo Am J Pharm Res 2014;4(06):2766-72.
11. Nesy EA, Mathew L, Comparative analysis of cardiac glycosides and quantification of peruvoside from *Thevetia neriifolia*, Juss fruit rind extracts through HPTLC Fingerprinting. Int J Pharm Pharm Sci 2014;6(8):412-5.
12. Nesy EA, Mathew L. Detection and quantification of cardiotonic drug peruvoside using HPTLC from *Thevetia neriifolia*, Juss Seed Extracts. Int J Pharm Sci Inv 2014;3(4):11-6.
13. Oluwaniyi OO, Ibiyemi SA. Extractability of *Thevetia peruviana* glycosides with Alcohol mixture. Afr J Biotechnol 2007;6(18):2166-70.
14. Ayinde BO, Ogunwole OA, Majekodunmi BC, Oikeh I. Proximate and cardiac glycoside composition of thevetia (*Thevetia neriifolia*. Juss) seed as affected by soaking in water, Brine and ethanol. J Agric Sci 2013;5(11):201-7.
15. Sun NC, Lizibor NI. The glycosides of *Thevetia peruviana*. Izuch. I. Ispol'3 Lekartstv Rastil Resursor USSR, Leningrad: medical South bay; 1964. p. 253-5.
16. Hollman A. Plants and cardiac glycosides. Br Heart J 1985;54:258-61.
17. Reich E, Schibli A. High-performance thin-layer chromatography for the analysis of medicinal plants. New York: Thieme Medical Publishers; 2006.
18. Shah B, Seth AK. Textbook of Pharmacognosy and Phytochemistry. 1st edition. New Delhi, Elseiver India Pvt. Ltd; 2010.
19. Kreis W, Reinhard E. Selective uptake and vacuolar storage of primary cardiac glycosides by suspension-cultured *Digitalis lanata* cells. J Plant Physiol 1987;128:311-26.
20. Wink M. Physiology of secondary product formation in plants; 1990. p. 23-41.
21. Melero CP, Medarde M, Feliciano S. A short review on cardiotonic steroids and their aminoguanidine analogues. Molecules 2000;5:51-81.
22. Kokate CK, Purohit AP, Gokhale SB. Pharmacognosy, 42nd Edn. Pune: Nirali Prakashan; 2008.
23. Montano JMC, Moron EB, Orta ML, Navas DM, Dominguez IG, Lazaro ML. Evaluating the cancer therapeutic potential of cardiac glycosides. BioMed Res Int 2014. Available from: URL: http://dx.doi.org/10.1155/2014/794930; 2014.
24. Newman RA, Yang P, Pawlus AD, Block KI. Cardiac glycosides as novel therapeutic agents. Molecular Inventions 2008;8(1):36-49.