QUANTIFICATION OF PERUVOSIDE (A CARDIOTONIC DRUG FOR CONGESTIVE HEART FAILURE) USING HPTLC FINGERPRINTING FROM WITHERED FLOWERS OF THEVETIA NERIIFOLIA, JUSS

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<th>ARTICLE INFO</th>
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<td><strong>Article history</strong></td>
<td>Thin-layer chromatography is widely used for metabolite analysis. Here it was used for identification and quantification of peruvoside, an effective cardiotonic drug from withered flowers of three morphoforms of <em>Thevetia neriifolia</em> (yellow, white and orange flowered) by densitometric analysis. Preliminary phytochemical screening conducted revealed the presence of metabolites like alkaloids, flavonoids, steroids, terpenoids, saponins and cardiac glycosides. Soxhlet extracts of flower samples and authentic standard of peruvoside were separated by normal-phase TLC as well as HPTLC with chloroform: methanol- 8:2 (v/v) as mobile phase. The separated compounds were detected with sulfuric acid and Liebermann Burchard reagent spray. These conditions enabled the detection of reference drug peruvoside at Rf range of 0.63-0.71±0.01 and 11-14 different types of cardiac glycosides from other components of the crude extracts at Rf range of 0.01 to 0.99. Two major analogues of peruvoside were detected in chloroform fraction (9.26-12.41%) and ethyl acetate fraction (0.18-1.28%). The three morphoforms of <em>Thevetia neriifolia</em> showed appreciable amount of peruvoside with their quantities varying from 13.69% in yellow flowers, 11.59% in white flowers to 10.1% in orange flowers. The results hence show that isolation of therapeutically significant cardiac glycosides can be effectively achieved from even the withered flowers using HPTLC method.</td>
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| **Keywords** | Cardiotonic, Chromatogram, HPTLC, Isomers, Peruvoside, Withered Flowers. |

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INTRODUCTION

Cardiac glycosides have been used for decades to treat congestive heart failure [1]. They are important class of secondary metabolites naturally distributed among a group of flowering plants, particularly abundant in some members of Apocynaceae (Apocynum cannabinum, Strophanthus gratus, Thevetia nerifolia and Nerium oleander) and Scrophulariaceae (Digitalis purpurea, D. lanata). In animal group a few poisonous toads (Bufo sp.) and some mammals produce these glycosides [2-3]. All cardiotonic steroids are characterized by their abundance in nature, diversity in structure, potential for chemical modification and wide use in cardiology for heart failure management [4]. Among the natural cardiotonic drugs used for treating weak hearts the most important ones are digitoxin, digoxin, digitalis and ouabain, in addition to nerifolin and peruvoside obtained from oleander sps.

Peruvoside, a valuable member of cardiac glycoside family, isolated by Rangaswami and Rao [5] from the kernels of Indian indigenous plant Thevetia nerifolia Juss, was proved to have positive inotropic effect and negative chronotropic effect on failing human heart [6]. In West Germany, peruvoside is marketed under the trade name “Encordin” [7]. Extensive pharmacological investigations have been conducted by many researchers on experimental models to show cardiotonic efficacy of the metabolite. Effects of peruvoside and nerifolin on isolated heart of dogs, cats and guinea pigs showed similarity in action to that of digitalis [8]. Major advantage of peruvoside over digitalis is that it has good absorbability from the gastrointestinal tract and a much wider margin of safety [9].

The taxon Thevetia nerifolia contain many potent cardiac glycosides, chiefly thevetin which on hydrolysis yield two triosides - Thevetin A and B. Further enzymatic hydrolysis of Thevetin A yields nerifolin, cerberin, peruvoside and ruvoside [10-11]. This metabolite is distributed throughout the plant including seeds, bark and flowers [12]. Plants of Thevetia nerifolia blossom throughout the year and have three morphoforms which produces flowers of different shades - yellow, orange and white. After pollination and fertilization, corolla droops and wither keeping embryo intact on plants. Withered corolla carries five epipetalous stamens, a massive stigmatic head and a broken style. In nature flowers are meant for reproduction to perpetuate species, but when they fall off, it became a floral waste. However, the withered flowers of this species were analyzed for the presence of the cardiac glycoside peruvoside, thereby not hampering with the natural phenomenon of seed setting and propagation.

To meet the ever increasing demand for cardiotonic drugs, isolation of powerful compounds from natural resources through cost-effective method is essential. At the same time therapeutic use of herbal cardiac glycosides also continues to be a source of toxicity today. Hence it is of utmost importance to elucidate methods to properly quantify the metabolite before they can be used for curative purposes. Here we describe a simple and relatively inexpensive method for detection and quantification of peruvoside and other cardiac glycosides in the flower extracts of three morphovariant plants of Thevetia nerifolia using TLC analyses and HPTLC profiling technology.

MATERIALS AND METHODS

Plant Material and sample preparation: Plant specimens (Thevetia nerifolia) were collected from different localities of Trichur district, Kerala. They were authenticated by Dr. Sunil CN (Associate Professor in Botany, SNM College, Malanikkara) and the voucher specimens (STHAPC 2458a-c) are deposited in the Herbarium of Department of Botany, St. Teresa’s College, Ernakulam for future reference. Withered flowers were collected every evening from their natural habitat and washed carefully to remove adhering soil particles, drained and dried in shade for 10-12 days. Samples were powdered thoroughly in a homogenizer and kept in airtight containers. Precisely weighed powdered samples (40g) were subjected to successive hot extraction using 350ml each of petroleum ether (60-80°C, PE), chloroform (CH), ethyl acetate (EA) and methanol (MT) in a soxhlet apparatus for 15-18 hours till the solvent became colorless. Solvents were allowed to evaporate and different fractions were dried and stored at 4°C in airtight labeled vials for further studies.

Standards and Materials: Reference drug peruvoside (CAS Number 1182-87-2) was purchased from Sigma– Aldrich, USA (purity of 90%, isolated from its natural source Thevetia nerifolia). Drug stored at 4°C was diluted to working concentration of 1mg/ml. All samples were dissolved in corresponding solvents to get a final concentration of 10mg/ml.

Preliminary phytochemical screening: Studies were conducted as per the standard protocol of Harborne [13]. Detection of cardiac glycosides in various fractions of extract was done by Thin Layer Chromatographic (TLC) technique. Chloroform methanol (8:2) mixture was employed as mobile phase to attain well separated bands of cardiac glycoside series. Samples were spotted on silica gel coated TLC plates using capillary tubes and were subjected to elution. Visualization was done by spraying the developed TLC plates with concentrated sulfuric acid [14].

HPTLC analysis: Studies were carried out by the method of Patel et al [15], using CAMAG Linomat instrument. Utilization of minimum quantity of sample for analysis is the advantage of this technique. Samples were loaded in pre-coated silica gel 60 F254 (E Merck KGAa) plates of 10 x 10cm size. An application volume of 5 µl was loaded as bands of 6.0mm width using a 100µl syringe of a distance from 10mm from the bottom of the plate. All six samples (CH and EA fractions of three color variants) and standard were chromatographed on same plate at a distance of 10mm between the tracks. The plate was placed in a twin trough developing glass tank (20 x 10 cm) after saturation with solvent phase chloroform: methanol (8:2) for 20 min. The developed plate was taken out when chromatogram run reached up to of 80mm and dried at 80°C for 3 min. Using CAMAG TLC scanner-171019, all nine tracks were scanned at a scanning speed of 20mm/s using D2 &W lamp at 220 nm lambda max from the application position of 10.0mm to solvent front position of 85.0mm. The scanner converts bands into peaks, peak height and peak area which were related to the concentration of the substance on the spot. The developed plate was derivatized by applying Liebermann Burchard spraying reagent. It was then kept in an oven at 120°C for 20 min. Dried plate was visualized in CAMAG visualizer and images were captured, prior and after derivatization, using a high resolution 12 bit digital camera type Dxa252 at all illumination modes (under UV light at 254nm and 366 nm and white light). Compounds in Track 4-9, similar to the reference material (track 1-3) in Rf value, sequence and color of bands
was compared and analyzed. Quantity of the analyte of interest in the samples was calculated by considering the sample taken initially and dilution factors.

RESULTS

Preliminary phytochemical analyses showed the presence of common classes of secondary metabolites in majority of samples analysed. Positive results were obtained for alkaloids (Marqui’s test, Dragendorff’s test), flavonoids (NaOH test, H₂SO₄ test), terpenoids (Salkowski test), cardiac glycosides (Keller Killiani test) and steroids (Liebermann Burchard test) for PE, CH and EA fractions. Methanol fractions gave negative results for the above classes of compounds, but responded positively for phenolic compounds, tannins (FeCl₃ test), saponins (Froth test) and coumarins (Na₂SO₄ test).

All extracts (PE, CH. EA & MT) of three morphoforms were analyzed for cardiac glycosides using TLC method. Peruvoside was observed as lemon yellow band with Rf value 0.63-0.65, besides other major visible spots with green, blue green, violet and reddish brown shades. Petroleum ether and methanol fraction did not show the presence of peruvoside in detectable amount in TLC plate, whereas, clear visible major and minor spots were observed in CH and EA fractions respectively. Hence, HPTLC was carried out with positive fractions (CH & EA) of all three variants for quantification of cardiotonic drug peruvoside.

Fig 1. Comparative chromatogram of standard drug Peruvoside and other cardiac glycosides profile of *T. neriifolia* flower extracts

A. HPTLC profile of *Thevetia neriifolia* flower 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 day light, 254nm, 366 nm wavelengths.

The overall intensity of bands in the fingerprints varies considerably due to quantitative differences in cardiac glycosides (Fig 1). The chromatograms presented here demonstrate that cardiac glycosides can be quantified easily using chloroform-methanol (8:2) mobile phase. The reference drug purchased from Sigma, isolated from *Thevetia neriifolia* possesses 90% purity. So, the densitogram of peruvoside contain 4-5 minor peaks, in addition to a major reference peak (Fig 2a). Reference compound began to elute at Rf 0.64, with a peak of 0.68 and ended at Rf 0.72 in the first track, but minute variability was observed in subsequent two tracks.

HPTLC studies revealed the presence of different forms of cardiac glycosides in both fractions of flower extracts. Densitogram of chloroform fractions revealed that flowers of yellow, orange and white variants showed the presence of 12, 11 and 12 peaks respectively, clearly demonstrating the existence of 11 to 12 different types of cardiac glycosides in this fraction. All these samples showed a significant peak corresponding to standard drug which started elution at Rf 0.59 and completed at Rf 0.68 with a maximum Rf of 0.65(Table 1). The quantity of drug calculated at this Rf value was 6.33, 4.67 and 5.74% respectively for all three variants. Similarly, a second compound having Rf value between 0.68 and 0.74 was also observed in all the three samples, with a belated elution time than the standard. This was considered as an isomer of peruvoside and named as peruvoside 2. Both compounds showed tailing at one side when compared to the standard and these compounds were present in CH fraction in almost equal amounts (Fig 2 b, d, f). The highest quantity appeared in yellow flowers (12.41%), followed by white (11.41%). Orange flowers recorded the least quantity (9.26%).
Table 1. Comparative densitogram data showing Rf values and quantity of peruvoside (in %) in chloroform fraction of yellow, orange and white flowers.

<table>
<thead>
<tr>
<th>Sample</th>
<th>Peak No.</th>
<th>Total peaks</th>
<th>Start Rf</th>
<th>Max Rf</th>
<th>End Rf</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
<th>Quantity in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ref:Peru</td>
<td>-</td>
<td>-</td>
<td>0.64</td>
<td>0.68</td>
<td>0.72</td>
<td>16809</td>
<td>49.31</td>
<td>Peruvoside</td>
<td>-</td>
</tr>
<tr>
<td>Peru</td>
<td>-</td>
<td>-</td>
<td>0.63</td>
<td>0.68</td>
<td>0.71</td>
<td>23209.5</td>
<td>41.78</td>
<td>Peruvoside</td>
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<tr>
<td>Peru</td>
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<td>-</td>
<td>0.63</td>
<td>0.67</td>
<td>0.70</td>
<td>26066.7</td>
<td>41.79</td>
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<td>-</td>
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<tr>
<td>YFCH</td>
<td>7</td>
<td>12</td>
<td>0.59</td>
<td>0.65</td>
<td>0.68</td>
<td>11819.7</td>
<td>13.55</td>
<td>Peruvoside</td>
<td>6.33</td>
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<tr>
<td>OFCH</td>
<td>6</td>
<td>11</td>
<td>0.59</td>
<td>0.65</td>
<td>0.68</td>
<td>8727.5</td>
<td>12.44</td>
<td>Peruvoside</td>
<td>4.67</td>
</tr>
<tr>
<td>WFCH</td>
<td>7</td>
<td>12</td>
<td>0.60</td>
<td>0.66</td>
<td>0.68</td>
<td>10721.4</td>
<td>12.46</td>
<td>Peruvoside</td>
<td>5.74</td>
</tr>
<tr>
<td>YFCH</td>
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<td>12</td>
<td>0.68</td>
<td>0.71</td>
<td>0.74</td>
<td>11349.7</td>
<td>13.01</td>
<td>Peruvoside-2</td>
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<tr>
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<td>7</td>
<td>11</td>
<td>0.68</td>
<td>0.70</td>
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<td>8573.1</td>
<td>12.22</td>
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<td>4.59</td>
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<tr>
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<td>10585.9</td>
<td>12.30</td>
<td>Peruvoside-2</td>
<td>5.67</td>
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YFCH, OFCH, WFCH- Chloroform fraction of Yellow, Orange and White Flowers.

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

a) Chromatogram of standard compound peruvoside.  b – g). Chromatogram of Chloroform and Ethyl acetate fractions of yellow (b-c), Orange, (d – e) and white (f – g) variants.

The quantity of peruvoside extracted from EA fraction is presented in Table 2. A maximum of 14 peaks were observed in yellow flowers, followed by 13 peaks in orange and 12 in white samples (Fig 2c,e,g). The results revealed that the residue contained minor quantities of peruvoside even after chloroform extraction. The drug present in EA fraction started to elute at Rf 0.63 ± 0.02 and ended at 0.68. This compound was identical in Rf value with the standard drug peruvoside. Minute quantities of analogue peruvoside 2 also appeared in this fraction, except for the white variant. In EA samples, peruvoside quantity decreased in the following order: yellow (1.28 %) > orange (0.84%) > white (0.18%).
Table 2. Comparative densitogram data showing Rf values and quantity of peruvoside (in %) in Ethyl Acetate fraction of flower extract of yellow, orange and white variants.

<table>
<thead>
<tr>
<th>Sample</th>
<th>No.</th>
<th>Total peaks</th>
<th>Start Rf</th>
<th>Max Rf</th>
<th>End Rf</th>
<th>Area</th>
<th>Area %</th>
<th>Assigned substance</th>
<th>Quantity in %</th>
</tr>
</thead>
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<tr>
<td>YFEA</td>
<td>10</td>
<td>14</td>
<td>0.61</td>
<td>0.65</td>
<td>0.68</td>
<td>909.8</td>
<td>1.73</td>
<td>Peruvoside</td>
<td>0.49</td>
</tr>
<tr>
<td>OFEA</td>
<td>10</td>
<td>13</td>
<td>0.64</td>
<td>0.66</td>
<td>0.68</td>
<td>301.3</td>
<td>0.62</td>
<td>Peruvoside</td>
<td>0.16</td>
</tr>
<tr>
<td>WFEA</td>
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<td>0.63</td>
<td>0.65</td>
<td>0.68</td>
<td>328.2</td>
<td>0.66</td>
<td>Peruvoside</td>
<td>0.18</td>
</tr>
<tr>
<td>YFEA</td>
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<td>14</td>
<td>0.69</td>
<td>0.72</td>
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<td>2.81</td>
<td>Peruvoside-2</td>
<td>0.79</td>
</tr>
<tr>
<td>OFEA</td>
<td>11</td>
<td>13</td>
<td>0.69</td>
<td>0.72</td>
<td>0.75</td>
<td>1275.8</td>
<td>2.63</td>
<td>Peruvoside-2</td>
<td>0.68</td>
</tr>
</tbody>
</table>

DISCUSSION

A wide variety of chromatographic methods have been used for the separation of cardiac glycosides, including thin layer (TLC) [12] and high performance liquid chromatography (HPLC) [16]. More sophisticated technique of HPTLC finger printing was used in this study for detection and quantification of cardiotonic steroids among oleander flower variants, a technique usually employed for isolation and estimation of individual compounds from plant extracts [17].

Cardiotonic steroids usually act as emetics and diuretics for centuries. The well known use of digitalis glycosides to slow down the rate and strengthen the contractility of the failing heart is widely accepted. The results of the present study revealed that a high percentage of cardiac glycosides exist in all flower morphoforms which can be extracted using different organic solvents. Extraction of peruvoside was not complete with chloroform; and the remaining compound present in the residue was squeezed out by the middle polar solvent ethyl acetate.

Plants contain different enzymes that can change the chemical nature of glycosides by the action of various factors like light, temperature, nature of solvents, etc. during extraction. A detailed analyses of chromatogram revealed that compounds of similar Rf values were present in all six samples. Minor variations in Rf values of the same compound in the same plate can be interpreted 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 [18]. So, from the results it is clear that the separation of glycoside mixtures from three flowers were more or less the same. Small 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 [12], which results in the formation of newer isomeric compounds with transferable functional groups. A maximum of 13.69% of peruvoside was extracted using both solvents from yellow variant, and CH showed higher efficacy in extraction compared to EA. The variations in number of peaks within the same solvent fraction may due to inter convertibility of molecules of a compound into various isomeric forms. Previous studies using seed extract gave similar results where two identical compounds within the Rf value of standard drug peruvoside was found in almost equal amount [19]. Investigations conducted by earlier researchers using $^1$H and $^{13}$CNMR techniques revealed the presence of hydroxyl group, methyl group and aldehyde group in peruvoside isolated from fresh uncrushed Thevetia leaf samples [20]. Depending upon the side group it carries, the compound was named as peruvoside A, B, C & D [21]. Evidence for the presence of an aldehyde group in peruvoside was observed by Rangaswami and Rao. Studies to detect the functional groups of these two analogues through FTIR analysis are in progress.

In many plants, cardiac glycosides and a number of related inter convertible compounds are present. By chemical structure, heart glycosides are esters of aglycones and residues of mono-, di-, tri- or tetra sugars are bonded between each other by glycoside linkages. As already known, the active principles of cardiotonic glycosides are aglycone steroid skeleton 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 [21]. In general, all Thevetia glycosides are closely related to each other chemically, and both aglycones and glycosides undergo isomeric changes in the presence of bases. Monoglycosides usually do not occur as such in the seeds and are formed as a result of enzymic hydrolysis of triosides [22].

Congestive heart failure is a condition in which the cardiac output is inadequate to body demands and there is poor cardiac contractility and relaxation, resulting in symptoms of low cardiac output and congestion [3]. Digitoxin and other cardiotonic steroids are widely prescribed for the treatment of heart failure and associated edema [23], but their narrow therapeutic index needs strict supervision for administration. The therapeutic effect derives from their ability to block the Na’/K$^+$ pump in cardiac cells and subsequently alter local Ca$^{2+}$ concentrations [24]. Adverse reactions to digoxin are usually dose dependent and occur at dosages higher than those needed to achieve a therapeutic effect [25]. In addition to the efficacy in congestive heart failure treatment and as anti-arrhythmic agents, the emerging role of peruvoside as effective drug in the prevention /treatment of proliferative diseases such as cancer, more specifically in the selective control of human tumor [26] need to be emphasized. Interestingly, Pongrakhananon, [27] pointed out that the concentration required to treat cancer was lower than of that used to treat cardiac disorders. Broad anticancer effect of cardiac glycosides may result from enhanced degradation of key cancer gene products [28]. Likewise, peruvoside can effectively block cell proliferation without triggering general cytotoxic response on androgen resistant prostate cancer cells [29]. Recent researchers reported the potential role of cardiac glycosides in accelerating wound healing property [30], lowering intra ocular pressure to slow down the onset and progression of glaucomatous blindness [31], effects on cellular transcriptome and other RNA binding proteins [24] and as anticancer agents [27, 29]. All these studies reveal the significance of cardiac glycosides including peruvoside in the ongoing era.
CONCLUSION
Safe, inexpensive and effective indigenous remedies are gaining popularity among the people of both urban and rural areas. Our investigations revealed that a series of 11-14 cardiac glycosides exist in flowers of Thevetia neriifolia irrespective of color variants. Chloroform and ethyl acetate were the better solvents for extraction than petroleum ether and methanol. Higher yield (10.1 - 13.69%) of cardiotonic drug peruvoside from the withered flowers shows that this untapped resource can be economically and effectively exploited for the isolation of cardiac glycosides which has immense implications in therapeutic field.

Conflict of interest
We declare that we have no conflict of interest in any matter.

REFERENCES