Isolation and quantification of six cardiac glycosides from the seeds of *Thevetia peruviana* provide a basis for toxological survey

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In order to support toxicological assessment of poisoning from the ingestion of plant material containing thevetia cardiac glycosides, a new isolation method has been developed for thevetia glycosides from the seeds of the Yellow Oleander [*Thevetia peruviana* (Pers.) K. Shum, Apocynaceae] with yellow and orange flowers, respectively, using accelerated solvent extraction. The relative proportions of four cardiac-active thevetia glycosides (thevetin A and B, acetylthevetin A and B) and two additional, cardiac-inactive thevetia glycosides (thevetin C and acetylthevetin C) in the extract were determined using an LC-ESI\(^+\)-MS/MS technique. Absolute quantification of thevetin B was achieved using a pure standard of this compound isolated by preparative high performance liquid chromatography.

Keywords: Accelerated solvent extraction, LC-ESI\(^+\)-MS/MS, *Thevetia peruviana* seeds, quantification, Yellow Oleander

*Thevetia peruviana* (Yellow Oleander) is a well-known evergreen ornamental shrub, but also grows widespread as a wild plant in the tropics and subtropics. The accidental or intentional intake of parts of the plant can lead to intoxication at a certain specific dose and the victim may die from cardiac arrest. This effect has been traced back to the fact that *Thevetia peruviana* contains cardiac glycosides, which are formed in each part of the plant although in different abundance and proportions\(^1\) with the seeds being highly enriched in these constituents compared to the rest of the plant. Eddleston *et al.*\(^2\) quoted that poisoning became a popular method of self-harm in northern Sri Lanka, where the plant is common, after the adverse effect of intake became public knowledge. German suicide forums, for instance, discuss the use of yellow oleander seeds as an agent for suicide because the seeds can easily be purchased via the internet.

Beyond this general knowledge there is no information, however, on the number of seeds that have to be ingested to reach the lethal dose. As Eddleston *et al.*\(^3\) noted, there is no obvious relationship between the amount of seeds ingested and the severeness of the poisoning effect. Bandara *et al.*\(^4\) explained the lack of correlation by, *e.g.*, the variability of glycoside concentration among seeds and the mode of ingestion (whole or crushed seeds). They also published a compilation of case reports and studies of Common and Yellow Oleander poisoning.

Chen and Chen\(^5\) provided a first estimate of the toxicity of “thevetin” without differentiating among the individual glycosides in the thevetin mixture. They noted that “thevetin” is approximately 1/8 to 1/7 as toxic as “digitalis”. Two years later, Tschesche\(^6\) reported the minimal systolic dose of “thevetin” to be 0.92 mg/kg for cat and 0.0045 mg/g for frog. Corresponding values for digitoxin were 0.33 mg/kg cat and 0.008 mg/g frog, respectively. Later, a lethal dose of 0.889 ± 0.0316 mg/kg for cat was noted by Helfenberger and Reichstein\(^7\) for “thevetin”; a similar value was given by Kramer\(^8\). Klupp\(^9\) specified minimal lethal dose values for thevetin B (1. *Figure 1*) to be 3.5 mg/kg for guinea pig and for thevetin A (2. *Figure 1*) to be 13.5 mg/kg for guinea pig, *i.e.* relatively high values compared to the minimal lethal dose of 0.2 mg/kg for guinea pig for convallatoxin\(^9\). In contrast to this, Pathare *et al.*\(^10\) reported thevetin A to be more active than thevetin B showing that there is an urgent need of a better knowledge of the glycoside composition of thevetia seeds and the activity of individual glycosides for the
purpose of reliable toxicological surveys. As a prerequisite, the reliable qualitative and quantitative analysis of the thevetia glycosides is important. This is particularly significant because cardiac glycosides are relevant for toxicological as well as medical purposes and because it is known that structurally related cardiac glycosides like digitoxin, biosynthesised by the foxglove, applied as therapeutic agents to medicate cardiac insufficiency, have therapeutic indices very close to the toxic dose.

We have performed an analytical baseline study for toxicological assessment of poisoning with thevetia cardiac glycosides to narrow this gap. As we found that the isolation method for thevetia glycosides published in the literature is not sufficiently effective, we developed an improved extraction and purification method for the target compounds in seeds of *Thevetia peruviana*. Furthermore, we determined relative proportions of individual thevetia glycosides in the seed extract, the purity of which was estimated as well.

Results and Discussion

Qualitative analysis of the isolated product

The seed extracts of yellow as well as orange blooming *Thevetia peruviana* contain mixtures of at least six different primary glycosides. Their structural characterization by Nuclear Magnetic Resonance (NMR) measurements and mass spectrometric analysis of the primary cardiac glycosides and their aglycones was reported previously. Thevetin B (1), thevetin A (2), acetylthevetin B (3), and acetylthevetin A (4, Figure 1) are primary glycosides with an unsaturated lactone ring. Two additional, structurally related glycosides, i.e. thevetin C (5) and acetylthevetin C (6), do not exhibit the unsaturated lactone ring but contain the same sugar moieties.

At that time, we were unable to unequivocally assign the NMR signals to the 20S and 20R epimers, respectively, because the amounts of thevetin C (5) and acetylthevetin C (6) isolated from the thevetia seeds were insufficient for comprehensive Nuclear Overhauser Enhancement Spectroscopy (NOESY) or Heteronuclear Multiple Bond Correlation (HMBC) experiments. However, Pauli *et al.* in the course of a study on the occurrence of progesterone and related animal steroids in two higher plants published NMR data of structurally related spiro steroids, including 5 and 6 (underlined) – their structures 10a and 10b).
purified from the seeds of (a) yellow and (b) orange blooming *Thevetia peruviana*. During the ionisation process adducts were formed due to the attachment of ammonium ions present in the mobile phase. Sodium adducts, formed due to the ubiquity of sodium ions, are marked in Figure 2 as well. Obviously, both thevetia subspecies contain the same range of compounds, although in different relative proportions as already reported by Bisset\textsuperscript{15} based on results of paper chromatography. Thus, cases of thevetia intoxication due to seed ingestion can be revealed by an analytical survey of the pattern of these six compounds.

### Quantitative analysis of glycosides isolated from thevetia seeds

For quantitative assessment of thevetia cardiac glycosides in plant seeds, or forensic specimens, their relative proportions and absolute contents in the mixture have to be determined. Furthermore, the purity of the isolated product has to be established.

If mass spectrometry is to be used for quantification, the relative ionisation efficiencies of target compounds as well as the influence of the aglycone and the sugar moieties on the ion yield need to be established. Structural diversity, especially polarity, has an influence on the ionisation response. Three of the thevetia glycosides are acetylated at the C2′ position of the thevetose. In addition, these six glycosides contain three different aglycones.

#### Digitalis glycoside standards

Differences in the aglycone or the sugar moiety are also known from digitalis glycosides (digitoxin, digoxin, digitoxigenin, digitoxigenin and α-acetyldigoxin). Digoxin (like digoxigenin) contains an additional hydroxyl group in the aglycone compared to digitoxin (or digitoxigenin). α-Acetyldigoxin features an acetylated sugar moiety in comparison to digoxin. We studied the mass spectral (ESI\textsuperscript{+}) behaviour of these glycosides to derive analogies for the analysis of thevetia glycosides under identical analytical conditions, as digitalis glycosides have a reasonably close structural relationship to the thevetia glycosides. Measurements with equimolar amounts of the compounds showed that the sugar moieties have a

#### Table I — Comparison of \(^1\text{C}\) and \(^1\text{H}\) NMR data relating to the spiro functionality of the epimers of 5 and 6 (CD\(_3\)OD, 500 MHz), respectively, data set of Kohls et al.\textsuperscript{13}, with the spirophanthigenin epimers 7a and 7b (CD\(_3\)OD, 400 MHz), data set of Pauli et al.\textsuperscript{14}.

<table>
<thead>
<tr>
<th>Carbon number</th>
<th>5 (20R+S) (\delta_C\delta_H)</th>
<th>6 (R+S) (\delta_C\delta_H)</th>
<th>7a (20R) (\delta_C\delta_H)</th>
<th>7b (20S) (\delta_C\delta_H)</th>
</tr>
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<tbody>
<tr>
<td>23</td>
<td>178.97 q 178.95 q</td>
<td>178.90 q</td>
<td>178.90 q</td>
<td>178.24 q</td>
</tr>
<tr>
<td>22</td>
<td>38.91 1.75 38.88 1.77</td>
<td>41.62 2.67 38.08 2.797</td>
<td>2.517</td>
<td></td>
</tr>
<tr>
<td>21</td>
<td>78.08 4.26/4.10 78.09 4.10</td>
<td>75.70 4.427 77.97 4.261</td>
<td>4.261</td>
<td></td>
</tr>
<tr>
<td>20</td>
<td>76.11 4.44 76.10 4.43</td>
<td>4.359 4.099</td>
<td></td>
<td></td>
</tr>
<tr>
<td>19</td>
<td>90.04 q 90.06 q</td>
<td>88.67 90.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>18</td>
<td>88.55 q 88.56 q</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>17</td>
<td>72.75 3.47 72.75 3.47</td>
<td>72.43 4.111 72.66 4.158</td>
<td>4.158</td>
<td></td>
</tr>
<tr>
<td>16</td>
<td>38.88 1.77 38.88 1.77</td>
<td>38.52 38.38</td>
<td></td>
<td></td>
</tr>
<tr>
<td>15</td>
<td>76.11 4.44 76.10 4.43</td>
<td>76.10 4.43 76.10 4.43</td>
<td>76.10 4.43 76.10 4.43</td>
<td></td>
</tr>
<tr>
<td>14</td>
<td>90.04 q 90.06 q</td>
<td>88.67 90.07</td>
<td></td>
<td></td>
</tr>
<tr>
<td>13</td>
<td>88.55 q 88.56 q</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>12</td>
<td>72.75 3.47 72.75 3.47</td>
<td>72.43 4.111 72.66 4.158</td>
<td>4.158</td>
<td></td>
</tr>
<tr>
<td>11</td>
<td>88.55 q 88.56 q</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
| q Quaternary carbon atom. | --- Not determined because of low intensity.
strong influence on the ionisation behaviour and, accordingly, on the type of quasi-molecular ions ([M+H]⁺ or [M+NH₄]⁺) and their yields, respectively. Digoxigenin and digitoxigenin predominantly yielded protonated quasi-molecular ions, whereas their glycosides form mainly ammonium adducts. Peak area-injected amount relationships for equimolar solutions of two aglycones and three glycosides are shown in Figure 3 based on the peak area data in Table II. Within the groups of aglycones and glycosides, respectively, the ionisation efficiencies and hence the ion yields are largely uniform. Still, α-acetyldigoxin with the less polar sugar moiety gave a slightly higher yield than the other two glycosides. Apparently, the extent of ionisation increases with decreasing polarity of the sugar moiety. In contrast, an additional hydroxyl group in the aglycone does not appear to have an influence on the ion yield.

Relative proportions of thevetia glycosides in the isolated mixture

Figure 4 shows the ammonium adduct quasi-molecular ion traces of the thevetia glycosides from High Performance Liquid Chromatography-Mass Spectrometry (LC-MS) analysis. The sodium adduct quasi-molecular ion chromatograms, for which only mass and response data are provided, are very similar, but their signal intensities (Figure 4, right) do not exceed 5% of the abundance of the ammonium adducts.

The relative proportions of the thevetia glycosides in the isolated mixture were estimated using the observation that they show identical ionisation behaviour as outlined before. Individual signal areas of their molecular ion adducts were integrated, and the relative ratios were calculated on the basis of the sum of ammonium and sodium adducts (cf. Figure 2). Because 2 co-elutes with glycoside 5, and 4 with glycoside 6, respectively, the ¹³C₂ isotope contributions of 2 ([M+NH₄]⁺ = m/z 890.4) and 4 ([M+NH₄]⁺ = m/z 932.4) to the molecular ion adducts of 5 ([M+NH₄]⁺ = m/z 892.4) and 6 ([M+NH₄]⁺ = m/z 934.4) were used to correct the peak areas of the two spiro compounds 5 and 6. The relative proportions of
The six thevetia glycosides in the seeds of the yellow oleander are listed in Table III.

The results are similar to those published by other authors previously under the following assumptions: The yellow flowering oleander is more common than the orange flowering subspecies, hence in the absence of specific information most of the descriptions in the literature are supposed to refer to the yellow flowering oleander. Singer and Studer\textsuperscript{16} were encouraged by Bloch \textit{et al.}\textsuperscript{17}, who had determined the molecular formula of C\textsubscript{42}H\textsubscript{64-66}O\textsubscript{19} for 2, to further separate the thevetin glycoside mixture. After clean-up of the crude mixture by counter current partition, they concluded that 2 and 1 occurred in a one-to-two abundance relation. Assuming that 2 and 5 were not separated under the chromatographic conditions used by Singer and Studer\textsuperscript{16}, a ratio of 2:1 for 1 to the sum of 2 and 5 fits to the results of this survey (cf. Table III, yellow flowering oleander). The suggested molecular formula of C\textsubscript{42}H\textsubscript{64-66}O\textsubscript{19}}

**Table II** — Peak areas of the quasi-molecular ion mass traces of equimolar amounts of digitalis glycosides

<table>
<thead>
<tr>
<th>Injected Component</th>
<th>Peak area (nmol)</th>
<th>1\textsuperscript{st} run</th>
<th>2\textsuperscript{nd} run</th>
<th>3\textsuperscript{rd} run</th>
<th>Mean value [RSD (%)]</th>
</tr>
</thead>
<tbody>
<tr>
<td>[Digoxigenin + H]\textsuperscript{+}</td>
<td>111.92</td>
<td>117.07</td>
<td>115.29</td>
<td>114.76 [2.3]</td>
<td></td>
</tr>
<tr>
<td>[Digoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>44.12</td>
<td>40.31</td>
<td>44.45</td>
<td>42.96 [5.4]</td>
<td></td>
</tr>
<tr>
<td>Digitoxigenin + H]\textsuperscript{+}</td>
<td>116.43</td>
<td>112.93</td>
<td>115.55</td>
<td>114.97 [1.6]</td>
<td></td>
</tr>
<tr>
<td>[Digitoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>39.99</td>
<td>43.17</td>
<td>41.45</td>
<td>41.54 [3.8]</td>
<td></td>
</tr>
<tr>
<td>[α-Acetyldigoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>49.62</td>
<td>52.84</td>
<td>50.41</td>
<td>50.96 [3.3]</td>
<td></td>
</tr>
<tr>
<td>Digoxigenin + H]\textsuperscript{+}</td>
<td>294.13</td>
<td>289.74</td>
<td>285.51</td>
<td>289.79 [1.5]</td>
<td></td>
</tr>
<tr>
<td>[Digoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>107.48</td>
<td>105.16</td>
<td>102.21</td>
<td>104.95 [2.5]</td>
<td></td>
</tr>
<tr>
<td>Digitoxigenin + H]\textsuperscript{+}</td>
<td>303.07</td>
<td>315.25</td>
<td>304.48</td>
<td>307.60 [2.2]</td>
<td></td>
</tr>
<tr>
<td>[Digitoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>116.65</td>
<td>109.99</td>
<td>108.18</td>
<td>111.61 [4.0]</td>
<td></td>
</tr>
<tr>
<td>Digitoxigenin + NH\textsubscript{4}\textsuperscript{+}</td>
<td>120.62</td>
<td>113.48</td>
<td>115.01</td>
<td>116.37 [3.2]</td>
<td></td>
</tr>
<tr>
<td>[Digitoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>141.26</td>
<td>140.66</td>
<td>146.37</td>
<td>142.76 [2.2]</td>
<td></td>
</tr>
<tr>
<td>Digitoxigenin + H]\textsuperscript{+}</td>
<td>419.79</td>
<td>414.80</td>
<td>427.97</td>
<td>420.85 [1.6]</td>
<td></td>
</tr>
<tr>
<td>[Digitoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>156.05</td>
<td>157.56</td>
<td>151.72</td>
<td>155.11 [2.0]</td>
<td></td>
</tr>
<tr>
<td>[α-Acetyldigoxin + NH\textsubscript{4}\textsuperscript{+}]</td>
<td>163.34</td>
<td>164.12</td>
<td>166.02</td>
<td>164.49 [0.8]</td>
<td></td>
</tr>
</tbody>
</table>

**Figure 4** — LC-MS quasi-molecular ion mass chromatograms of the ammonium adducts of thevetia glycosides from the seeds of the Yellow Oleander plus digitoxin with their relative intensities (counts) and relative retention times. For comparison, the exact masses and counts of the sodium adducts are given as well; the mass traces (not shown) mirror those of the ammonium adducts. For abbreviations see Figure 2.
quantification. In this manner, nearly 17% RSD for Voigtländer may be acceptable as this component has a very low proportion of only 3%. Estimation of purity of the isolated thevetia glycoside mixture

Table III — Relative proportions, as mean values, of thevetia cardiac glycosides in the seeds of *Thevetia peruviana* with (a) yellow and (b) orange flowers

<table>
<thead>
<tr>
<th>Component</th>
<th>Mean value (%)</th>
<th>RSD (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Thevetin B (1)</td>
<td>43 [3.7]</td>
<td>43 [7.4]</td>
</tr>
<tr>
<td>Thevetin A (2)</td>
<td>12 [7.7]</td>
<td>27 [8.1]</td>
</tr>
<tr>
<td>Acetylthevetin B (3)</td>
<td>25 [2.3]</td>
<td>8 [9.5]</td>
</tr>
<tr>
<td>Acetylthevetin A (4)</td>
<td>7 [2.1]</td>
<td>4 [10.0]</td>
</tr>
<tr>
<td>Thevetin C (5)</td>
<td>9 [10.0]</td>
<td>15 [9.4]</td>
</tr>
<tr>
<td>Acetylthevetin C (6)</td>
<td>5 [2.7]</td>
<td>3 [16.9]</td>
</tr>
</tbody>
</table>

*From six measurements. The sum differs from 100% due to rounding.*

Thevetin B (2) (C$_{14}$H$_{20}$O$_{19}$) and 5 (C$_{14}$H$_{20}$O$_{19}$) and supports the assumed coelution indirectly. Voigtländer et al. isolated 3 in a 20% yield from the thevetin mixture, which is reasonably similar to the proportion of 25% obtained in this survey (cf. Table III, yellow flowering oleander).

In the seeds of both oleander varieties, 1 is the dominant glycoside, whereas 4 and 6 have the lowest proportions in the mixtures. This may explain why Voigtländer et al. only assumed the presence of 4. It is conceivable that 4 co-eluted with one of the other components and in this manner was not detected in their chromatographic analysis.

For the proportion of 6, derived from the orange blooming kind, the Relative Standard Deviation (RSD) is relatively high. Peters et al. discussed acceptance criteria relating to validation procedures and stated a precision of an analytical method as acceptable within 15% RSD and 20% near the limit of quantification. In this manner, nearly 17% RSD for 6 may be acceptable as this component has a very low proportion of only 3%.

Estimation of purity of the isolated thevetia glycoside mixture

The pure thevetin B (1) reference material, which so far is not commercially available, served as external standard for estimating the purity of the isolated thevetia glycoside mixture using the LC-MS/MS method in Multiple Reaction Monitoring (MRM) mode described by Kohls et al. We determined a mean value of 89.7 µg thevetin B in 1 mg thevetia glycoside mixture/mL methanol. As thevetin B represents 43% of all thevetia glycosides in the mixture (Table III), the thevetia glycosides constitute 208.6 µg in 1 mg isolated raw mixture.

Thus, a purity of about 21% was estimated for the thevetia cardiac glycoside mixture extracted from the plant seeds and purified as described in the Experimental Section later on.

Significance for toxicological assessment

A thevetia glycoside mixture was obtained from *Thevetia peruviana* seeds by an improved isolation technique based on accelerated solvent extraction. LC-ESI-MS measurements, based on the analytical method developed for the main compound, thevetin B (1), revealed that both types of *Thevetia peruviana* (yellow and orange blooming) contain the same six thevetia glycosides in their seeds, differing, however, substantially in relative proportions.

The structural diversity of the thevetia glycosides is similar to that in the group of digitalis glycosides. Standards of these latter compounds were used for examining their ionisation behaviour during ESI mass spectrometry. The ionisation behaviour was shown to be identical within the group of digitalis glycosides, whereas the aglycones behaved differently due to the significant influence of the sugar units on the ionisation process. The ionisation behaviour within the group of thevetia glycosides was supposed to be analogous to that of the digitalis glycosides. Based on this assumption, the relative proportions of each thevetia glycoside in the isolated glycoside mixture was estimated. Using thevetin B (1) as external standard, we assessed the purity of the isolated thevetia glycoside mixture to be about 20%.

Only four of the six thevetia glycosides, namely thevetin B (1), thevetin A (2), acetylthevetin B (3), and acetylthevetin A (4) comprise the unsaturated lactone ring necessary for the cardio-active effect. As noted by Cruz et al., glycosides containing a spiro aglycone do not exhibit a significant cardio-active effect. Thus, the pattern of the six-component mixture may serve as a qualitative indicator of thevetia ingestion. However, for quantitative analysis in forensic studies, only the four cardio-active glycosides with the unsaturated lactone ring should be taken into account. So far, only the content of thevetin B (1) can be determined quantitatively. As the metabolism in the human organism after oral ingestion is supposed to be different within the group of thevetia glycosides, the content of the remaining thevetia glycosides cannot be calculated by using their relative proportions in relation to thevetin B (1) in plant seeds. A more rigorous method validation for quantification of every thevetia cardiac glycoside...
would require the availability of at least each of the four cardio-active, but preferably all six thevetia glycosides as pure compounds in sufficiently large amounts.

The attempt to deduce the potency of each thevetia glycoside on a biochemical level by comparison with other cardiac glycosides is unlikely to be successful, although general facts of compound behaviour have been reported. For instance, it is known that the presence of sugar moieties is not a prerequisite for the cardio-active effect\textsuperscript{22}. However, they have an influence on the physical and chemical properties of the glycosides and in this manner influence, \textit{e.g.}, absorption, protein binding, distribution in the organism, biodegradation, and elimination. With increasing polarity of the molecule, for example, protein binding becomes weaker and, thus, elimination occurs faster. This corresponds to the observation that in the case of intoxication with a highly polar glycoside the patient recovers faster than in the event of ingestion of a less polar glycoside\textsuperscript{23}. Protein binding properties of the highly polar thevetia glycosides are yet unknown\textsuperscript{24}.

Another important pharmacokinetic factor is the absolute solubility of cardiac glycosides in water and their dissolution rates\textsuperscript{23}. It is not possible to simply estimate the solubility from the constitution of a molecule. As an example, digoxin and gitoxin are structural isomers and contain only one additional hydroxyl group compared to digitoxin; solubility in water, however, decreases from digoxin over digitoxin to gitoxin. Solubility increases significantly after methylation at C4 of the terminal digitoxose in digoxin (= \(\beta\)-methyldigoxin)\textsuperscript{25}. Sticher\textsuperscript{23} also noted that the decay rate and elimination half-life depends on the metabolism of the different substances. For most part, polar glycosides are eliminated unaltered by humans through urination.

Experimental Section

Chemicals and standards

For extraction, preparative High Performance Liquid Chromatography (HPLC) and LC-MS analysis, mass spectrometry-grade solvents were used. Digitalis glycosides were obtained from Sigma-Aldrich (Schnelldorf, Germany).

Plant material

Two kinds of \textit{Thevetia peruviana} seeds were extracted. Those from \textit{Thevetia peruviana} with orange flowers were derived from Réunion island (Pacific Ocean, tropics), and the seeds from a yellow blooming plant were collected in Dehradun (North India, subtropics). All seeds were purchased from Sunshine Seeds (Ahlen, Germany; www.sunshine-seeds.de).

Accelerated Solvent Extraction (ASE)

Extraction of the target compounds was carried out with an Accelerated Solvent Extractor (ASE 200, Dionex Corporation, Sunnyvale, USA). After removing the nutshells, the seeds were ground and degreased with \(n\)-hexane (ultrasonic bath, 10 min). The clear yellow supernatant was decanted and the procedure repeated three times. After the fourth extraction, the supernatant was colourless. A further extraction step followed in the same way, but with dichloromethane as solvent. The supernatant was cloudy. The absence of cardiac glycosides in the organic extracts was proven with a negative \textit{Liebermann-Burchard colouring reaction} (no blue colour\textsuperscript{25}), originally developed for confirming the presence of steroids. It was shown that a primary double bond in the steroid skeleton is not mandatory for colour formation. Neither the lactone ring nor the sugar moiety affect the reaction as verified with digitoxin. The colour reaction was used as a process control during enrichment and purification of the thevetia glycosides.

The degreased seed material was air dried and mixed with annealed sea sand to achieve a better permeability. This mixture was treated by accelerated solvent extraction with methanol as solvent (10\textsuperscript{7} Pa, 120°C). The extraction cycle (5 min) was run three times. The extracts produced a blue colouration in the \textit{Liebermann-Burchard reaction}. The combined extracts were evaporated under vacuum to give a yellow-brown residue, which was subsequently redissolved in warm absolute ethanol. The solution was stored at RT overnight. Any precipitate was discarded. Diethyl ether was added to the yellow supernatant which led to the formation of a white precipitate. It was separated by centrifugation and stored in diethyl ether. The procedure was repeated several times until no precipitation occurred anymore. The diethyl ether was removed from the combined precipitates and the product dried under vacuum. The isolated white powder was stored under vacuum over Sicapent\textsuperscript{®} (\(P_2O_{10}\)), because in its raw state it appeared to decompose under ambient conditions. Raw glycoside yields ranged from 3% to 6% of the degreased and dried seed material.
Preparative HPLC

A pure standard of thevetin B (1, Figure 1) was obtained by separation of the raw thevetin mixture on a Pursuit 5µ PFP preparative column (250 mm x 10 mm ID; Varian, Darmstadt, Germany) connected to a security guard column (MetaGuard 4.6 mm Pursuit 5µ PFP, Varian, Darmstadt, Germany) in a LaChrom Elite® chromatographic module (VWR-Hitachi, Darmstadt, Germany), comprising an L-2000 piston pump equipped with a degasser. For space reasons the module had an external column thermostat (THERMOTECNIC products, Langenzersdorf, Austria) set to 35°C. The chromatographic system in addition was equipped with a Foxy Jr® fraction collector (Teledyne ISCO, Lincoln, USA). Both the fraction collector and the chromatographic system were controlled by the EZChrom Elite™ 3.1.7 software. Isocratic elution at a flow rate 3.0 mL/min was achieved with a mobile phase of water/methanol/acetonitrile, 3:1:1 (v/v/v). Compound 1 was collected in the time window of 11.0–11.4 min (whole run time 25 min/cycle). The solvent was removed from the eluate by rotary evaporation and the residue redissolved and stored in methanol.

Analytical methods

For chromatography, a phenyl-hexyl column (150 x 4.60 mm ID, Phenomenex® LUNA® 5µ, Aschaffenburg, Germany) combined with a corresponding security guard column (4.0 x 3.0 mm ID, Phenomenex®, Aschaffenburg, Germany) was used. Separation was carried out with a Waters 2695 Separation Module (Manchester, UK) equipped with quaternary pump, column oven (30°C), autosampler (15°C) and degasser. The mobile phase binary system was composed of (A) water/methanol, 95:5 (v/v), and (B) acetonitrile/methanol, 50:50 (v/v), and contained ammonium formate (10 mM) and formic acid (0.1%). Separation at a flow rate of 0.8 mL/min started with 50% B, was quickly ramped to 80% B, kept for 2 min, and then linearly ramped to 100% B in 5 min, followed by fast reduction to 50% B, then held again for 3 min. The flow was split to 0.4 mL/min after chromatography before direction into the mass spectrometer.

A Micromass® Q-ToFmicro (Waters, Manchester, UK) hybrid quadrupole time-of-flight mass spectrometer equipped with a Z-electrospray ion interface coupled to the separation module was used in the positive ion mode (ESI+) and controlled by the MassLynx 4.1™ software. Parameters of chromatogram and full-scan spectra acquisition were: Capillary voltage 2.7 kV, sample cone voltage 20 V, extraction cone voltage 0.5 V, desolvation temperature 220°C, source temperature 120°C, cone gas (N2) 15 L h⁻¹, desolvation gas (N2): 250 L h⁻¹, scan range: m/z 100 – m/z 1000.

Conclusion

In conclusion, it is quite obvious that there are still open questions regarding the behaviour of thevetia cardiac glycosides on a biochemical level. The improved isolation method presented here, providing a method for making reference standards available, is a first step on the way to determining the activity of each individual thevetia glycoside and, as a consequence, to evaluating the results of quantitative analysis in a toxicological survey.

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References


