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Original Article

Cardiac glycosides from *Cascabela thevetioides* by HPLC-MS analysis

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ABSTRACT

The present work investigates the chemical composition of seeds of *Cascabela thevetioides* (Kunth) Lippold, an ornamental shrub of México. Six thevetia cardiac glycosides or thevetosides (thevetin A, B, and C, acetylthevetin A, B and C) were identified from the methanol extract of seeds of *C. thevetioides* by High-Performance Liquid Chromatography–Mass Spectrometry and by comparison of mass spectral fragmentation patterns. Enzymatic hydrolysis of a sample of thevetosides from methanol extract of seeds and subsequent High-Performance Liquid Chromatography–Mass Spectrometry analysis yielded the monoglycosides neriifolin, acetylneriifolin and acetylperuvoside, previously reported for this plant. For the fisrt time thevetin A, B and C, and acetylthevetin A, B and C are reported as components of seeds of *C. thevetioides*.

Introduction

Cascabela thevetioides (Kunth) Lippold, the accepted name for *Thevetia thevetioides*, Apocynaceae, is an ornamental shrub of 3–6 m high which grows widespread as a wild plant and it is distributed in tropical regions of the center and south of México, central America and South America. In the Mexican Traditional Medicine, it is named as "Yollotl" (heart in Nahualtl language) and "Codo de fraile" (Friar's elbow) (Argueta et al., 1994; Rojas, 2009).

In México, seeds of *C. thevetioides* has been used against tooth pain, deafness, skin conditions, and its ingested to lose weight or deworming agent. However, the ingestion of seeds or other parts of the plant produces a clinical picture very similar to the digoxin poisoning: diarrhea, sickness, vomit, dizziness, abdominal pain and arrhythmia (Bandara et al., 2010; Essiett and Udolfa, 2014). Toxicity of *Cascabela* species to higher animals and humans is well documented and is attributed to cardiotonic glycosides whose steroidal aglycone is digitoxigenin (McLaughlin et al., 1980).

Phytochemical studies have demonstrated that whole plants of *Cascabela* genus, but specifically its seeds, are rich sources of cardiac glycosides structurally similar to the digitoxins. From *C. thevetioides* have reported the isolation of neriifolin, acetylneriifolin (McLaughlin et al., 1980) and peruvoside 2′-acetate (Voigtländer et al., 1968; Cruz et al., 1979), while from *Cascabela thevethia* (*Thevetia peruviana)*, the most studied plant of the *Cascabela* genus, have isolated iridoids, terpenoids, alkaloids, flavonoids, saponins, tannins and cardiac glycosides (Abe et al., 1996; Essiett and Udolfa, 2014).

Pharmacological studies have shown that neriifolin and acetylneriifolin have insecticidal and cytotoxic activity (McLaughlin et al., 1980), and ethanol extract have antitumor activity on Crown Gall Tumor Disc Bioassay (Galsky et al., 1980).

However, the most of cardiac glycosides of *C. thevetioides* have not yet been determinate. Therefore, the aim of this study is to determinate the cardiac glycosides of the methanol extract of *C. thevetioides* by High Performance Liquid Chromatography coupled with Mass Spectrometry (HPLC-MS).

Materials and methods

Plant material

Fruits of *Cascabela thevetioides* (Kunth) Lippold, Apocynaceae, were collected in Colima, México in August 2012. The seeds were manually separated from pericarp and dried in the shade. A sample of plant material was deposited at herbarium of University of Colima, Colima, México (voucher number AMB012012).

Chemicals

Acetonitrile and formic acid HPLC grade were purchased from Merck México (México). Hexane, methanol and chloroform anhydrous grade were purchased from Sigma–Aldrich México (México).

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Samples preparation

Thevetosides mixture

Dried seeds (1552 g) were separated from endocarp, milled in a mortar and extracted at room temperature with hexane (3 × 3 l, 72 h each) and with methanol (3 × 3 l, 72 h each); evaporation of the solvents in vacuum gave 490 g of hexane (31.57% yield) and 76.2 g of methanol (4.91% yield) extracts.

Methanol extract (62.4 g) were chromatographed using a silica gel 60S column (400 mm × 40 mm ID) and eluted with crescent polarity mixtures of chloroform/methanol. The Keddle reagent was used to monitor cardiac glycosides on TLC (Liu et al., 2013). The fractions collected with chloroform/methanol (80:20) yielded 14.4 g and chloroform/methanol (70:30) yielded 41.2 g of thevetosides mixture.

Hydrolyzed thevetosides mixture

A sample of chloroform/methanol (70:30) thevetosides mixture (500 mg) was enzymatically hydrolyzed, with fresh and milled seeds of *C. thevetioides* (10 g) as catalysator, in water (100 ml) and a top layer of hexane (3 ml) in an erlenmeyer flask for 4 days at 37 °C. Afterwards, the mixture of reaction was extract with ethanol (80 ml at 50% v/v) and vacuum filtering. Ethanol phase was extracted by chloroform (3 × 30 ml each). This chloroform phase was concentrated under reduced pressure obtaining 351.6 mg of a pale-yellow solid.

HPLC-MS analysis

Thevetosides mixture

The chromatographic separation of thevetosides mixture was achieved on a C18 column (100 mm × 2.1 mm ID, Waters Acquity UPLC BEH, 1.7 μm) equipped with a security guard column. The method was developed with a Waters Acquity UPLC system, equipped with a column oven (25 °C), auto sampler and degasser. For elution, a binary mobile phase consisting of (A) water containing formic acid (0.1%) and (B) acetonitrile containing formic acid (0.1%) was used. For thevetosides mixture, starting with 13% B, kept for 4 min and the linearly ramped to 40% B in 5 min; afterwards it was quickly ramped to 100% B and kept at this level for 1 min. At the end of the column the flow was split, and 0.3 ml/min were directed into the mass spectrometer. The samples were dissolved in A mobile phase. The total flow of chromatographic separation was 3 ml.

Hydrolyzed thevetosides mixture

For a pale-yellow solid from hydrolyzed thevetosides mixture, elution starting with 10% B, kept for 5 min and the linearly ramped to 50% B in 5 min and kept at this level for 2 min; afterwards it was quickly ramped to 90% B in 2 min to return to initial conditions. At the end of the column the flow was split, and 0.3 ml/min were directed into the mass spectrometer.

Mass spectrometry

MS was performed in a hybrid quadrupole time-of-flight mass spectrometer Waters Synapt G2-S spectrometer with the following settings: desolvation gas: 8 l/min; capillary voltage: 3.0 kV; ion source temperature: 100 °C; desolvation temperature: 300 °C; entrance potential: 1.0 V; and cone gas flow: 2 l/min. Nitrogen was used as the nebulizer, desolvation, and cone gas. Mass calibration (*m/z* 50–1500) was performed using a solution of sodium formate (100 ppm) The measurements were performed in positive ion mode (ESI^+) over a range of *m/z* 50–1500 and a resolution of 20,000.

Results and discussion

From the seeds of *C. thevetioides* were identified nine cardiac glycosides, six from methanol extract and three from a sample of hydrolyzed methanol extract catalyzed by the own seeds. All thevetosides are components already known and isolated from other *Cascabela* species.

Thevetosides mixture

The first compound shows in its ESI^+ mass spectra ion fragments at *m/z* 872, *m/z* 873 and *m/z* 890 that were assigned respectively to molecular ion $[M]^+$, protonated molecule $[M+1]^+$ and ammonium adduct $[M+NH_4]^+$. Additionally, shows ion fragments related to different sugar moiety cleavages, *m/z* 485 for a trisaccharide moiety elimination, *m/z* 323 for gentiobiose moiety (Kohls et al., 2012), *m/z* 485 for aglycone of glycoside and *m/z* 549 for thevetoside monoglycoside. Also, ions fragments in *m/z* 111 and *m/z* 85 are characteristics lactone ion fragments (DagerAlbalawi, 2016). Finally, the CO group elimination from *m/z* 353 to *m/z* 325 and the comparison with data reported by (Kohls et al., 2015, 2012) allowed identity it as thevetin A (**1**).

The second compound exhibit several analogies with **1**, particularly by showing the same sugar and lactone fragment ions at *m/z* 485 and *m/z* 323, and *m/z* 111 and *m/z* 85, respectively. This is evidence that both compounds are thevetosides with the same trisaccharide moiety. Ion fragments at *m/z* 858, *m/z* 876 and *m/z* 897 were assigned to molecular ion $[M]^+$, ammonium adduct $[M+NH_4]^+$ and potassium adduct $[M+K]^+$. The mass weight between this substance and **1** was 14 u, this mass difference was assigned to functional group change from aldehyde to methyl in its aglycone. These data were compared with reports by (Amaringo, 2011), allow identify to this compound as thevetin B (**2**).

The third compound from the fraction also showed the same sugar and lactone fragments ions of **1** and **2**, so that this compound is closely similar to **1** and **2** and differs in one or two functional group only. Ions fragments at *m/z* 892 and *m/z* 897 were assigned to ammonium adduct $[M+NH_4]^+$ and sodium adduct $[M+Na]^+$ respectively, the signal of molecular ion $[M]^+$ (expected at *m/z* 874) was very small and was not detected in the mass spectrum. Ion fragment at *m/z* 391 was assigned to aglycone after a loss of 485 u due to trisaccharide moiety. The mass pattern of the aglycone showed a loss of 30 u from *m/z* 355 to *m/z* 325 corresponding to CH_2O group elimination, this evidence and data reported by (Kohls et al., 2012) allow us to identify this compound as thevetin C (**3**).

From the fractions collected from chloroform/methanol (80:20) were identity three thevetosides with structure closely similar to thevetosides previously described.

The first one, showed ion fragments at *m/z* 915, *m/z* 937 and *m/z* 932 that were assigned respectively to molecular ion $[M]^+$, sodium adduct $[M+Na]^+$ and ammonium adduct $[M+NH_4]^+$. The ion fragments of **1** and this compound differ by 42 u, this mass difference was related to an acetyl group. The ion fragments at *m/z* 591 (monoglycoside) and *m/z* 389 (aglycone) confirm that the acetyl group is attached to thevetose moiety. Furthermore, this compound and **1** showed the same aglycone and lactone mass pattern. This evidence allows us to identify this compound as acetylthevetin A (**4**) (Kohls et al., 2015, 2012).

By the same way, the two last compounds were closely similar to **2** and **3** in structural constitution, mass pattern in aglycone and lactone moieties and differ in 42 u in sodium adduct $[M+Na]^+$ and ammonium adduct $[M+NH_4]^+$ (Table 1). Comparing these data with those reported by Kols and coworkers (Kohls et al., 2015, 2012), these compounds were identified as acetylthevetin B (**5**) and acetylthevetin C (**6**).

Table 1
Ionized molecule, ion fragments and adducts of thevetosides identified from *Cascabela thevetioides*.

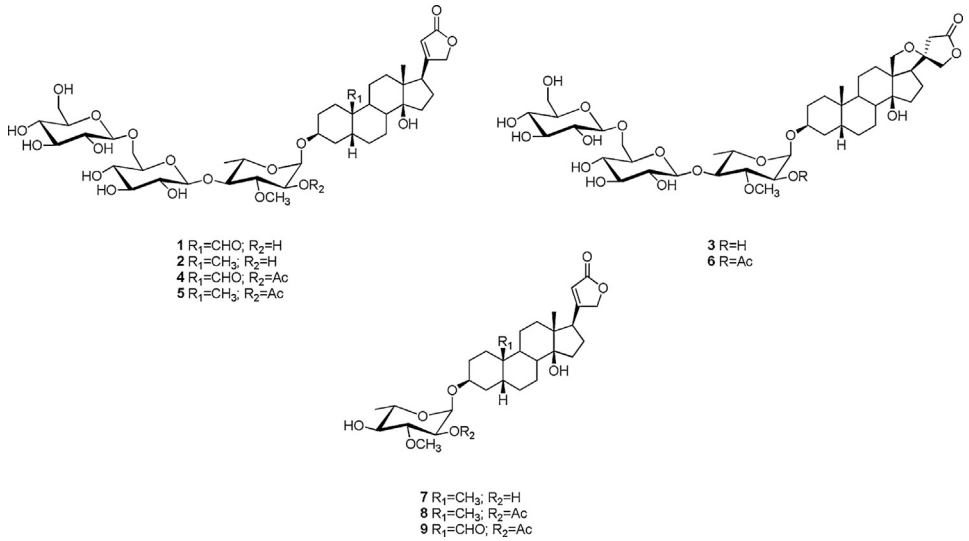
Ionized molecule, ion fragment or adduct	*m/z*								
	Thevetosides								
	1	2	3	4	5	6	7	8	9
$[2M+2]^{+}$							1170	1154	1182
$[M+K]^{+}$		897							
$[M+Na]^{+}$			897	937	923	939	557	599	613
$[M+NH_4]^{+}$	890	876	892	932	918	934			
$[M+1]^{+}$	873		875	915			535	577	591
$[M+1-(H_2O)]^{+}$							517		
$[M]\cdot+$	872	858	874	914	900				
$[M+1-(C_{20}H_{20}O_{10})]^{+}$	549	535		591	577	692			
$[C_{19}H_{32}O_{14}+1]^{+}$	485	485				527			
$[M+1-(C_{19}H_{32}O_{14})]^{+}$	389	375	391	389	375	391			
$[M+1-(C_6H_{12}O_4)]^{+}$							375		
$[M+1-(C_6H_{12}O_4)-(H_2O)]^{+}$							357		
$[M+1-(C_6H_{12}O_4)-(H_2O)-(H_2O)]^{+}$							339	339	
$[M+1-(C_9H_{14}O_5)-(H_2O)]^{+}$									371
$[M+1-(C_9H_{14}O_5)-(H_2O)-(H_2O)]^{+}$									353
$[M+1-(C_{19}H_{32}O_{14})-(H_2O)]^{+}$	371	357	373	371	357	575			
$[M-(C_{19}H_{32}O_{14})-(H_2O)-(H_2O)]^{+}$									
$[M+1-(C_{19}H_{32}O_{14})-(H_2O)-(H_2O)]^{+}$	353	339	355	353	339	355			
$[M+1-(C_{19}H_{32}O_{14})-(H_2O)-(H_2O)-(CO)]^{+}$	325								
$[M+1-(C_{19}H_{32}O_{14})-(H_2O)-(H_2O)-(CH_2O)]^{+}$			325						
$[C_{13}H_{23}O_9+1]^{+}$	323	323	323	365	365	365			
$[C_{15}H_{23}]^{+}$		203							
$[C_9H_{14}O_5+1]^{+}$				203	203	203		203	203
$[C_6H_7O_2]^{+}$	111	111	111	111				111	111
$[C_4H_5O_2]^{+}$	85	85	85	85	85	85		85	85

Hydrolyzed thevetosides mixture

After enzymatic hydrolysis of a thevetosides mixture sample three compounds were identified, that are described below. The first compound showed fragment ions at *m/z* 535, *m/z* 557 and *m/z* 1070 that were assigned to protonated molecule $[M+1]^{+}$, sodium adduct $[M+Na]^{+}$ and $[2M+2]^{+}$ adduct, respectively. Ion fragment for trisaccharide or acetyl moiety were not detected, and ion fragments at *m/z* 375, *m/z*357 and *m/z* 339 suggest a closely similar structure of its aglycone with **2**. The difference of 160 u between $[M+1]^{+}$ and *m/z* 375 (aglycone + 1) showed the presence of thevetose moiety. These data allow us to identify this compound as neriifolin (**7**), substance previously isolated from this plant by (McLaughlin et al., 1980).

The second compound of this mixture showed a mass pattern similar to **7** with a difference of 42 u in protonated molecule $[M+1]^{+}$ and sodium adduct $[M+Na]^{+}$, and a difference of 84 u in $[2M+2]^{+}$ adduct. Additionally, the presence of an ion fragment at *m/z* 203 showed the presence of an acetyl thevetose moiety; therefore, this substance was identified as acetylneriifolin (**8**), substance already known as component of this plant (McLaughlin et al., 1980).

The last compound showed three ion fragments at *m/z* 591, *m/z* 613 and *m/z* 1182 that were assigned to protonated molecule $[M+1]^{+}$, sodium adduct $[M+Na]^{+}$ and $[2M+2]^{+}$ adduct respectively. As in**7** and **8**, in this substance was not detected ion fragments for trisaccharide, however, an ion fragment at *m/z* 203 confirms that this substance it is an acetyl-monoglycoside thevetoside. Ion fragments at *m/z* 371 and *m/z* 353 suggest that share the same aglycone with **1**. This substance was isolated previously by Voigtländer and coworkers (Voigtländer et al., 1968) and named as acetylperuvoside or peruvoside 2′-acetate (**9**).



Conclusions

In conclusion, from methanol extract of *C. thevetioides* were identified six thevetosides termed thevetin A (**1**), thevetin B (**2**), thevetin C (**3**), acetylthevetin A (**4**), acetylthevetin B (**5**) and acetylthevetin C (**6**) by comparison of mass spectral fragmentation patterns of the glycosides and aglycones. Additionally, three monoglycosides thevetosides were identified after enzymatic hydrolysis termed neriifolin (**7**), acetylneriifolin (**8**) and acetylperuvoside (**9**). All substances are already known but **1**-**6** had not been reported as components of *C. thevetioides*.

Author's contributions

SB and EP-R contributed in running the laboratory work, extractions and acquisition of the data. JCT-C and JLB-L contributed in analysis and interpretation of data. JLB-L and AN drafted the paper.

Conflicts of interest

The authors declare no conflicts of interest.

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