In vivo *and in vitro* studies on *Thevetia* Species Growing in Egypt
I: Isolation, Identification, and Quantification of cardiac glycosides in *in vivo* and *in vitro* cultures of immature seeds.

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ABSTRACT: *In vivo* and *in vitro* extracted cardiac glycosides of immature seeds (IS) cultures of *Thevetia neriifolia* Jussieu. and *T. thevetioides* Kunth. were chemically identified. Calli were grown on modified Murashige & Skoog (MS) medium supplemented with 1mg/l 2,4-dichlorophenoxyacetic acid (2,4-D) +3mg/l kinetin (Kin). The content of cardiac glycosides in IS cultures of *T. neriifolia* and *T. thevetioides* were monitored by HPLC. Two major compounds were detected and isolated from IS extracts i.e. digitoxigenin and thevetin B. The different structures of the *in vivo* and *in vitro* isolated compounds were verified by means of MS and NMR spectral analysis, as well as those compounds were identified and determined using HPLC technique. [Journal of American Science 2010;6(11):390-395]. (ISSN: 1545-1003).

Key words: Cardiac glycosides, callus, *Thevetia spp.*, HPLC, MS medium, immature seed cultures

INTRODUCTION

Thevetia neriifolia Juss. and *T. thevetioides* Kunth. belonging to Apocynaceae family, which are commonly known as the dogbane or oleander family (Omino and Kokwaro, 1993). Apocynaceae plants are distributed in tropical America and the West Indies and widely grown in various parts of the world for ornamental purpose (Rizk and Al-Nowaihi, 1989; Sabira *et al.* 1993). It has been used in folk medicine as a purgative, emetics, and a remedy for intermittent fever (Githens, 1948). Moreover, It is having cardiotonic activity (Aleshkina and Berezhinskaya, 1962), anticancer (Cardellina *et al.* 1993), neuroprotection against ischemic stroke, as well as insecticidal properties (James *et al.* 2006). The toxicity of *Thevetia* species attributed to their cardenolide content in their tissues (Goncalves *et al.* 2003; Gaillard *et al.* 2004; Gaillard *et al.* 2004). Cardiac glycosides Cerberin (monoacetylneriifolin), ruvoside and perusitin were isolated from the seeds of *Thevetia peruviana* (Siddiqu *et al.* 1992). Abe *et al*., (1994) isolated sixteen cardenolide glycosides and one pregnane glycoside from the frozen fresh leaves of *Thevtia neriifolia*. Siddiqui *et al* (1992) isolated neriifoside from the fresh uncrushed leaves of *Thevetia neriifolia*, in addition to four triterpenes; oleanolic acid, ursolic acid, α-amyrin acetate and β-amyrin acetate. Also, cerberoside, 2-*O*-acetylcerberoside, neriifolin, thevetin A&B, peruvoside, digitoxigenin were isolated (Balsam and Kufner 1971; Seitz and Ripphahn 1975; Said, 1985; Decosterd *et al.* 1994). The triterpenes, sterol, Fatty acids and flavonoid constituents of *Thevetia peruviana* fruit pericarp and flowers were also studied (Qazi *et al*. 1973; Rao *et al.* 1975; Schum-Obasi *et al.* 1990).

In vitro culture technology has been proven to be effective in some cases for the production of secondary metabolites such as taxol (Oksman-Caldentey and Inzé 2004). Moreover, Dantas *et al.* (1994) studied six strain of *T. neriifolia* cell suspension cultures for cardenolides production. Even after two years of subculture, cardenolides proved to be present in all these strains. The cardenolides content varied from one strain to another according to the nature of the original explants. Furthermore, Lopes *et al.* (2001) reported that some compounds found in the intact plant could accumulate in cultured cells. Cardenolides of *T. neriifolia*, were accumulated in cultured cells during one year of cultivation.

In the present work, cardiac glycoside in seed cultures of *T. neriifolia* and *T. thevetioides* were identified and compared qualitatively and quantitatively with those isolated previously from *in vivo* seeds of the same plants.

MATERIALS AND METHODS

General procedures

^{1}H NMR (500 MHz, CD_3OD) and ^{13}C NMR (125 MHz, CD_3OD) were recorded on a JEOL LA500 MHz, Germany spectrometer with TMS as an internal standard. The FAB-MS spectrum was taken on a JEOL JMS_AX500 mass spectrometer MS-FAB09A positive. HPLC was carried out on Agilent a series 1100 interface with stationary phase (RP18), injection volume (10 µl), oven temperature (25°C), diode array detector (254 nm), flow rate (1ml / min) and mobile phase: MeOH/H_2O (1:1) under gradient conditions**.** Column chromatography was carried out on silica gel 60 (Merck; 230 - 400 mesh). TLC: pre-coated silica gel 60F_{254} plates (Merck); CC: silica gel type 60 (Merck). MS**:** Murashige and Skoog medium (Duchefa Biochemie The Netherlands).

Plant materials

Immature seeds (IS) of *T. neriifolia* Juss*. and T. thevetioides* Kunth were collected from Al-Orman garden, Giza, Egypt in March 2006. They were identified by Prof. Dr. K. H. El-Batanouny, Botany Department, Faculty of Science, Cairo University. Voucher specimens are deposited at the Herbarium of NRC, Dokki, Cairo, Egypt.

Authentic compounds

The reference of cardiac glycosides (peruvoside and neriifolin) were purchased from Sigma Chemicals Co., St. louis Mo. USA. Thevetin B and digitoxigenin were separated and identified throughout this work.

Initition of IS calli cultures

Calli cultures of *T. neriifolia and T. thevetioides* derived IS were performed as described by Taha *et al*. (2010).

Extraction and isolation of cardiac glycosides

In vitro derived calli of *T. neriifolia and T. thevetioides and in vivo* IS were lyophilized and powdered. Then they were percolated in methanol (3x3L) at room temperature for 24 hrs and filtered. The percolation was repeated three times and the methanolic extracts for each plant were combined and evaporated *in vacuo* at 45 ºC. The dry crude extract was defatted with petroleum ether. The residue was dissolved in MeOH/H_2O (1:1) and extracted with chloroform. The chloroform extracts were combined together, dehydrated and the solvent distilled of *in vacuo* at 45 ºC to give total cardenolides (Fried and Sherma, 1994 and Abdel-Azim *et al.,* 1996).

Identification of the isolated compounds

All isolated compounds were identified by FAB-MS, ^{1}H-NMR and ^{13}C NMR spectroscopy. Furthermore, the determination of cardiac glycosides was carried out using co-chromatography TLC and HPLC techniques with samples of reference compounds.

HPLC analysis

One gram each of air-dried powdered of *in vivo* and *in vitro* derived calli of IS of *T. neriifolia and T. thevetioides* was accurately weighed. Each sample was extracted with 50 ml 70% MeOH till exhaustion. The obtained methanolic extract was evaporated till dryness. The residue was re-dissolved in 1 mL methanol and filtered. The filtrate was used for quantitative determination of the isolated compounds using HPLC (10 µL was injected). Which, then they were performed on RP18 column using water (A) and methanol (B) as solvents and detected at the wave length of 220 nm. The following gradient was employed: 10% B for 25 minutes, 100% B within 30 minutes, then isocratic elution at 20% B for 20 minutes. Standard curves of authentic compounds and calculations of unknown amounts of cardiac glycosides in calli and regenerated culture samples were done using routine protocols as described by Scott (1996).

Statistical analysis of data

All experiments were statistically analyzed using the F-test according to Steel and Torrie (1960). ANOVA was determined and the LSD was calculated at P=0.05. The data presented are the means of five replicates ± standard error (SE).

RESULTS AND DISCUSSION

Isolation and structure elucidation

The crude cardenolides extract (5 g) were applied onto the top of a silica gel column 50x5 cm. Elution was carried out using $CHCl_3$ followed by gradual increasing of the proportions of MeOH till 100% MeOH. The course of the chromatographic fractions (100 ml each) was monitored by silica gel for TLC and developed using solvent system $CHCl_3$: MeOH (8:2). The chromatoplats were visualized using Kedde's reagent (Wagner and Bladt 1995), which give violet colour with cardiac glycosides. Similar fractions were combined and concentrated to dryness under reduced pressure. Thevetin B (compound I) was isolated in a pure form *T. neriifolia* and having R_f values 0.12. Also digitoxigenin (compound II) was isolated from *T. thevetioides* and having R_f values 0.81.

Compound (I)

Compound I was identified as thevetin B (**Fig. 1**) by comparing its spectroscopic measurements with that published with Rodrigo *et al.* (2005). Its FAB-MS (positive mode) spectrum showed a molecular ion peak at *m/z* 858 (calcd. 858.96), which is corresponding to the molecular formula $C_{42}H_{66}O_{18}$. ^{1}H- NMR spectrum (CD_3OD), showed two methyl protons at δ 0.8 and 0.9 corresponding to C_{18} and C_{19}, respectively. Signals due to the cardenolides ring were identified at δ 5.00 ($H_{21}\alpha$), 4.8 ($H_{21}\beta$) and 5.8 (H_{22}). In addition to the signal detected at δ 2.7, which is corresponding to H_{17}. The three anomeric protons of the three sugar units were identifies at δ 4.3, 4.5 and 5.11, respectively. The oxygenated methines were also identified at δ 3.54 for H_3 and δ 3.54 for C-3'-OMe.

Compound (II)

Compound II was identified as digitoxigenin (**Fig. 1**) by comparing its spectroscopic measurements with that published with Rodrigo *et al.* (2005). Its FAB-MS (positive mode) showed a fragment ion peak at *m/z* 374.25 (calcd 374.51), which is corresponding to the molecular formula $C_{23}H_{34}O_3$. ^{1}H- NMR spectrum (CD_3OD), showed two methyl signals at δ 1.05 and 0.99 corresponding to H_{18} and H_{19}, respectively. Signals due to the cardenolides ring were identified at δ 6.15 (H_{22}), 5.06 ($H_{21}\alpha$) and 4.67 ($H_{21}\beta$), in addition to H_{17} at δ 2.84**.** ^{13}C-NMR spectrum (CD_3OD), showed an ester carbonyl at δ 172.1 (C_{23}) and the vinylic carbons were identified at δ 117.7 (C_{22}) and 172.0 (C_{20}). Additional oxygenated carbons were observed for C_{14} (δ 86.0) and C_{21} (δ 75.8). Also, the two methyl carbons were identified at δ 16.4 (C_{18}) and δ 24.1 (C_{19})**.**

The results obtained are in agreement with those reported by Mahran *et al.* (1971), who isolated thevetin B from seeds of *T. neriifolia*, and Perez *et al.* (1993), who isolated digitoxigenin from seeds of *T. thevetioides*.

Inititation of IS calli cultures

The highest value of IS calli of *T. neriifolia and T. thevetioides* (Fig. 2 A and B) was recorded with MS+ 1mg/l 2,4-D + 3mg/l Kin as described by Taha *et al.* (2010).

Qualitative and quantitative determination of cardenolides

1. Qualitative determination

TLC chromatoplates as a preliminary screening to check the presence of the examined cardenolids compounds in calli and regenerated shootlet extracts. The chloroform extracts were compared with authentic samples using system solvent chloform/methanol (8:2) and visualized with Kedde's reagent. The spots corresponding to R_f values 0.12, 0.60, 0.67 and 0.81 were referred to thevetin B, neriifolin, peruvoside and, digitoxigenin, respectively. The four compounds were detected in all the examined calli and regenerated shootlet extracts.

2. Quantitative determination (HPLC technique)

As shown in Table 1 thevetin B, digitoxigenin, peruvoside and neriifolin *in vivo* and *in vitro* seeds cultures of *T. neriifolia* and *T. thevetioides* were illustrated in (Table 1). The amount of thevetin B, digitoxigenin, peruvoside and neriifolin of *in vivo* seeds of *T. neriifolia* plant were 0.19, 0.11, 0.39 and 0.25 mg/g DW, respectively**.** On the other hand, the amount of these compounds of *in vivo* seeds of *T. thevetioides* plant were 0.99, 0.15, 0.24 and 0.39 mg/g DW, respectively. The highest concentration of thevetin B (85 mg/g DW) and digitoxigenin (0.028 mg/g DW) was recorded in *T. neriifolia* IS calli cultures and that of peruvoside (0.017 mg/g DW) and neriifolin (0.032 mg/g DW) was found in *T. thevetioides* .

Concerning, the accumulation of cardenolides in *T. neriifolia* calli cultures and in close of our obtained results Dantas *et al*. 1994 and Lopes *et al.* 2001 reported that some compounds of cardenolides were accumulated in cultured cells of *T. neriifolia*. Morevor, some studies by Fett-Netto *et al.* (1992) reported that 2,4-D and Kin combination were more effective in accumulation of secondary metabolites *viz*. taxol and related taxanes in cell suspension of *Taxus* species. It is interesting to note that the technique used in this experiment, together with other reported techniques e.g. callus and cell suspension cultures (Stuhlemmer *et al*. 1993) can be offered alternative sources for large scale production of cardiac glycosides.

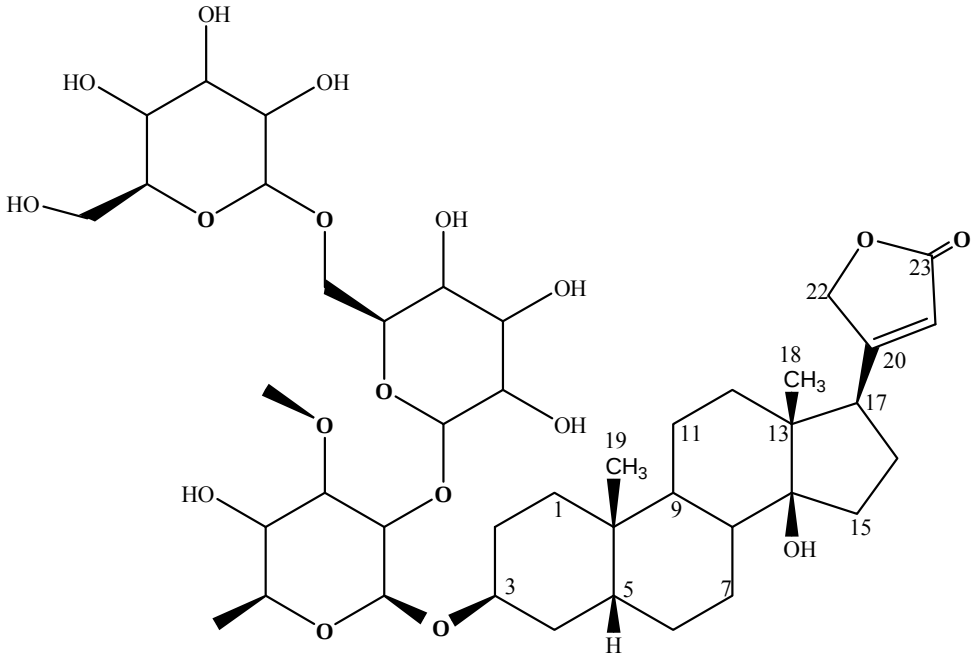

Fig. 1 Thevetin B = (Compound I)

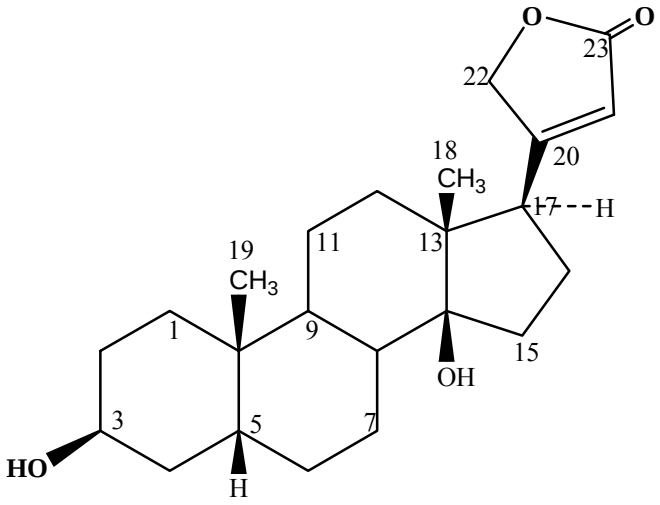

Fig. 2 Digitoxigenin = (Compound II)

Table 1: Concentrations of the cardiac glycosides (mg/g DW)

Extract	Thevetin B	Digitoxigenin	Peruvoside	Neriifolin
In vivo seeds*	0.19	0.12	0.39	0.25
In vivo seeds**	0.99	0.15	0.24	0.39
IS calli cultures*	0.085	0.028	0.013	0.019
IS calli cultures **	0.047	0.027	0.017	0.032

where:
Thevetin B (R_t = 9.48)**,** digitoxigenin (R_t = 11.01)**,** peruvoside (R_t = 11.32) and neriifolin (R_t = 12.72)**.**
(*) *T. neriifolia;* (**) *T. thevetioides*

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