

Cardiac glycosides from shoot cultures of *Cryptostegia grandiflora*

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ABSTRACT

Cardiac glycosides in shoot cultures of *Cryptostegia grandiflora* were identified when grown in modified MS medium. The change in shoot segments and cardiac glycosides content was followed between day zero and day 12 at 2-day intervals. The content of cardiac glycosides in leaves and shoot cultures of *Cryptostegia grandiflora* was monitored by HPLC. Two major compounds were detected and isolated from shoot cultures extract, named oleandrogenin 3-O- β – glucopyranosyl-(1 $\rightarrow$ 4)- β – cymaropyranosyl-(1 $\rightarrow$ 4)- β -digitoxopyranoside (cryptostigmin I) and oleandrogenin 3-O- β – glucopyranosyl-(1 $\rightarrow$ 4)- α -rhamnopyranoside (cryptostigmin II). The structures of the isolated compounds were verified by means of MS and NMR spectral analysis, as well as by comparison with authentic samples. The leaves and shoot cultures were analyzed for their cardiac glycosides content. The shoot cultures inoculated into MS-based culture media supplemented with 0.1 mg L⁻¹ BA, 30 g L⁻¹ sucrose, 0.1 g L⁻¹ myo-inositol and 0.1 g L⁻¹ ascorbic acid were found to contain a quantity of cardiac glycosides that was about four fold the cardiac glycosides content of leaves extract.

Key words: Cardiac glycosides, *Cryptostegia grandiflora*, HPLC, MS medium, shoot cultures

INTRODUCTION

Cryptostegia grandiflora R.Br. (Asclepiadaceae) is an ornamental plant commonly cultivated in Egypt and warm countries.^[1-3] The plant has widespread use in manufacturing of rubber and as a source of hydrocarbon fuels from its latex.^[4] A number of cardiac glycosides have been isolated and identified from the leaves of *C. grandiflora*.^[5,6] Moreover, the macro- and micro-morphological characters of the leaves and stems of *C. grandiflora* have also been investigated.^[7]

Plant cell and tissue cultures have been recognized as promising alternatives to whole plants in the production of secondary metabolites.

In the present work, cardiac glycosides in shoot cultures of *C. grandiflora* were identified and compared qualitatively and quantitatively with those isolated previously from leaves of the same plant.

MATERIALS AND METHODS

General procedures

¹H-NMR (400 MHz, Bruker, Germany) spectra were performed in C₅D₅N using TMS as internal standard. FAB-MS spectra were taken on a JEOL JMS-SX 102 spectrometer by direct inlet method at an ionizing voltage of 70 eV. Optical rotations ([α]_D) were measured with a Perkin Elmer polarimeter 341. The TLC was performed on silica gel 60 F₂₅₄-coated aluminum sheets (Merck, Darmstadt, Germany).

Plant material

Seeds of *C. grandiflora* R.Br. (Asclepiadaceae) were collected from the trees cultivated in the Experimental Station of Faculty of Pharmacy, Assiut University, Assiut, Egypt. The plant was identified by Prof. N. El-Keltawi, Department of Horticulture, Faculty of Agriculture, Assiut University, Assiut, Egypt.

Authentic compounds

The reference cardiac glycosides — cryptostigmin I and II previously isolated from *C. grandiflora*^[6] — were kindly provided by Prof. Kamel MS, Department of Pharmacognosy, Faculty of Pharmacy, El-Minia University, El-Minia, Egypt; and Prof. Assaf MH, Department of

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Initiation of shoot cultures

C. grandiflora seeds were surface-sterilized with 3.5% NaOCl for 10 minutes, followed by three washes with sterile distilled H₂O. For germination, the seeds were placed on a plant-growth regulator-free half-strength MS medium^[8] supplemented with 30 g L⁻¹ sucrose, 0.1 g L⁻¹ *myo*-inositol and 3.5 g L⁻¹ phytagel (Sigma, USA) as a gelling agent. The seeds were incubated at 25°C ± 2°C under a 16-hour photoperiod at 20 μmol m⁻² s⁻¹ irradiance. After germination, the upper parts of the seedlings were excised and transferred onto an MS medium supplemented with 0.1 mg L⁻¹ benzyladenine (BA), 30 g L⁻¹ sucrose, 0.1 g L⁻¹ *myo*-inositol and 3.5 g L⁻¹ phytagel for shoot multiplication. The cultures were grown at 25°C ± 2°C under a 16-hour photoperiod at 20 μmol m⁻² s⁻¹ irradiance.

For initiation of liquid shoot cultures, the shoots were cut into segments containing two leaves. The nodal segments were transferred into 50-mL Erlenmeyer flasks containing 20 mL of MS-based culture media supplemented with 0.1 mg L⁻¹ BA, 30 g L⁻¹ sucrose, 0.1 g L⁻¹ *myo*-inositol and 0.1 g L⁻¹ ascorbic acid. The flasks were incubated on a rotary shaker at 130 rpm under a 16-hour photoperiod at 60 μmol m⁻² s⁻¹ irradiance at 25°C ± 2°C. Subcultures were carried out every 6-8 days by cutting the shoots into segments containing two leaves and placing the segments into a fresh medium.

Extraction and isolation of cryptostigmin I and II

Leaves of *C. grandiflora* were collected from the shoot segments cultivated on modified MS medium as mentioned before, air-dried and powdered. The air-dried powdered leaves (200 g) were extracted with 1.4 L 70% EtOH. The dried ethanolic extract (43 g) was suspended in H₂O and defatted successively with *n*-hexane and CH₂Cl₂.^[6] The aqueous fraction was freeze-dried and re-dissolved in MeOH. The methanolic solution was subjected to MPLC on RP-18 column eluted with MeOH-H₂O (3:7) at a flow rate of 3 mL/min, where two compounds were isolated: cryptostigmin I and II (21 and 47 mg, respectively).

Identification of the isolated compounds

All isolated compounds were identified by HR FAB-MS and ¹H-NMR spectroscopy; also, their optical rotations ([α]_D) were determined. Furthermore, co-chromatography (TLC and HPLC) with samples of reference compounds was carried out.

Growth curve and cardiac glycosides content

Ten flasks containing 20 mL of MS-based culture medium supplemented with 0.1 mg L⁻¹ BA, 30 g L⁻¹

sucrose, 0.1 g L⁻¹ *myo*-inositol and 0.1 g L⁻¹ ascorbic acid were taken and the contents were inoculated with shoot segments containing two leaves. The flasks were incubated on a rotary shaker at 130 rpm under a 16-hour photoperiod at 60 μmol m⁻² s⁻¹ irradiance at 25°C ± 2°C. Cardiac glycosides were quantified spectrophotometrically using HPLC. Cryptostigmin II in a concentration of 1 μg/100 μL was used as a reference compound. Cardiac glycosides contents were determined from day zero to day 12 at 2-day intervals.

HPLC analysis

One gram each of air-dried powdered leaves of both aerial parts and shoot cultures (8 days old) was accurately weighed and separately transferred to a 250-mL flask. Each sample was extracted with 7 mL 70% EtOH till exhaustion, and the ethanolic 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 (30 μL was injected). This was performed on a nucleosile 100-5 C-18 column using water (A) and methanol (B) as solvents. The following gradient was employed: 10% B for 4 minutes, 15%-50% B within 30 minutes, then isocratic elution at 60% B for 6 minutes. The flow rate was 1 mL/min and the detection wavelength set to 220 nm. Cryptostigmin II in a concentration of 1 μg/100 μL was used as a reference compound. The quantity of each compound was estimated on the basis of its area with respect to the area of cryptostigmin II (0.3 μg) as external standard.

RESULTS AND DISCUSSION

From the shoot cultures of *Cryptostegia grandiflora*, two major compounds were isolated. They were identified as oleandrigenin 3-O-β-glucopyranosyl-(1→4)-β-cymaropyranosyl-(1→4)-β-digitoxopyranoside (cryptostigmin I) and oleandrigenin 3-O-β-glucopyranosyl-(1→4)-α-rhamnopyranoside (cryptostigmin II) [Figure 1] by comparison of their optical rotations, chromatographic and spectral data with those previously reported.^[6]

Cryptostigmin I

White powder, [α]_D²⁵ -15.4° (MeOH; *c* 5.70). HR FAB-MS (negative) *m/z*: 867.4374 (M-H)⁻ C₄₄H₆₈O₁₇ (req. 867.4378). ¹H-NMR (C₅D₅N): δ_H 6.29 (1 H, *s*, H-22), 5.64 (1 H, *m*, H-16), 5.42 (1H, *d*, *J* = 18.3 Hz, H-21), 5.38 (1H, *bd*, *J* = 9.3 Hz, H-1 Dig), 5.24 (1 H, *d*, *J* = 18.3 Hz, H-21), 5.04 (1 H, *d*, *J* = 7.8 Hz, H-1 Glc), 4.72 (1 H, *dd*, *J* = 4.7 and 9.5 Hz, H-1 Cym), 3.5 (3 H, *s*, OMe), 3.36 (1 H, *d*, *J* = 8.8 Hz, H-17), 2.79 (1 H, *dd*,

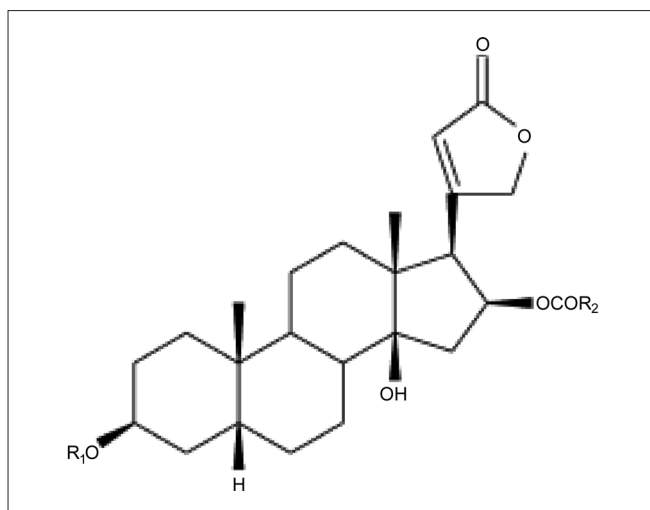


Figure 1: Chemical structures of cardiac glycosides

Compounds	R ₁	R ₂
Crptostigmin I	— Dig ⁴ Cym ⁴ — Glc	— CH ₃
Crptostigmin I	— Rha ⁴ — Glc	— CH ₃

$J = 8.6$ and 14.6 Hz, H-15), 2.01 (1 H, d , $J = 13.2$ Hz, H-15), 1.8 (3H, s , COMe), 1.58 (3 H, d , $J = 5.4$ Hz, Me-6 Cym), 1.43 (3 H, d , $J = 6.1$ Hz, Me-6 Dig), 1.03 (3 H, s , Me-18) and 0.86 (3 H, s , Me-19).

Cryptostigmin II

White powder, $[\alpha]_D^{25} -39.4^\circ$ (MeOH; c 3.73). HR FAB-MS (negative) m/z : 739.3573 $[M-H]^-$ C₃₇H₅₆O₁₅ (req. 739.3541). $^1\text{H NMR}$ (C₃D₅N): δ_H 6.31 (1 H, bs , H-22), 5.68 (1 H, m , H-16), 5.40 (1 H, dd , $J = 1.8$ and 18.3 Hz, H-21), 5.33 (1 H, bs , H-1 Rha), 5.21 (1 H, dd , $J = 1.5$, 18.3 Hz, H-21), 5.19 (1 H, d , $J = 7.7$ Hz, H-1 Glc), 4.18 (1 H, m , H-3), 3.38 (1 H, d , $J = 8.6$ Hz, H-17), 2.78 (1 H, dd , $J = 9.5$ and 15.7 Hz, H-15), 2.01 (1 H, dd , $J = 2.1$ and 15.3 Hz, H-15), 1.82 (3 H, s , COMe), 1.66 (3 H, d , $J = 6.4$ Hz, Me-6 Rha), 1.03 (3 H, s , Me-18), and 0.83 (3 H, s , Me-19) [Figure 1].

The cardiac glycosides content was correlated with the growth of shoot cultures. Figure 2 shows a characteristic increase in cryptostigmin II content between day 6 and day 8.

*The data are mean values of the two independent experiments.

The prepared sample for quantitative determination was subjected to HPLC (30 μL), and the quantity of each compound was estimated on the basis of its area with respect to the area of cryptostigmin II (0.3 μg) as external

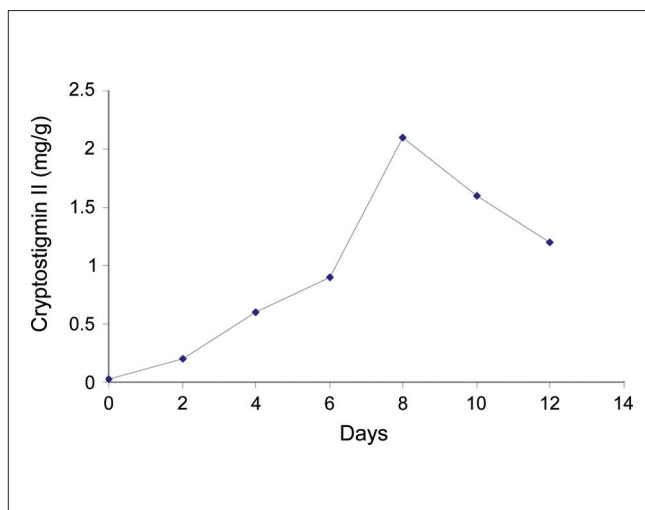


Figure 2: Changes in cryptostigmin II content of *Cryptostegia grandiflora* shoot cultures

Table 1: Concentrations of the isolated compounds (mg/g)

Extract	Cryptostigmin I	Cryptostigmin II
Aerial parts	0.033	0.067
Shoot cultures	0.11	0.23

standard. The results of quantitative determination of the isolated compounds in the aerial parts and shoot cultures are recorded in Table 1.

*The data are mean values of the two independent experiments.

As shown by the obtained results, the cardiac glycosides content in shoot cultures of *Cryptostegia grandiflora* was found to be four fold that in leaves extract.

It is interesting to note that the technique used in this experiment, together with other reported techniques e.g. callus and cell suspension cultures^[9-13] can be offered alternative sources for large scale production of cardiac glycosides.

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