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Anthraquinones from *Isoplexis isabelliana* cell suspension cultures

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Abstract

A new compound, 3,6 (or 7)-dihydroxy-4-methoxy-2-methylanthraquinone for which we propose the name 6 (or 7)-hydroxydigitolutein, and eight known anthraquinones were isolated from the EtOH 70% extract of *Isoplexis isabelliana* cell suspension cultures. Five of them: 1-hydroxydigitolutein, phomarin (= digito-emodin), digiferruginol, ω-hydroxypachybasin and digiferrol have been isolated from the genus *Isoplexis* for the first time. The other three: digitolutein, madeirin and 3-methoxy-2-methylanthraquinone have been reported from the leaves of some *Isoplexis* species. © 1999 Elsevier Science Ltd. All rights reserved.

Keywords: *Isoplexis isabelliana*; Scrophulariaceae; Cresta de gallo; Cell suspension cultures; Anthraquinones; 3,6 (or 7)-dihydroxy-4-methoxy-2-methylanthraquinone

1. Introduction

Isoplexis isabelliana (Webb and Berthel.) Masf., ''cresta de gallo'', is one of the four species comprising the genus *Isoplexis* (Lindl.) Loudon (Scrophulariaceae). Like *I. canariensis* (L.) G. Don f. and *I. chalcantha* Svent. and O'Shan. it is endemic to the Canary Islands. The fourth, *I. sceptrum* (L. f.) Loudon, is from the island of Madeira (Pérez de Paz & Roca, 1982). From their leaves, anthraquinones and/or cardenolides closely related to those found in the Mediterranean *Digitalis* L. (Scrophulariaceae) have been isolated (Pavanaram, Hofer, Linde & Meyer, 1963; Freitag, Spengel, Linde & Meyer, 1967). *I. isabelliana* has been used in folk medicine for its diuretic, toxic and hypoglycemic properties (Darias, Abdala, Martín & Ramos, 1996). Previous investigations on the leaves of this taxon have resulted in the isolation of anthraquinones and C_{21}-steroids, and in the detection of some sapogenins (Pavanaram et al., 1963; Freitag et al., 1967). Our report, which constitutes the first study on constituents from cell suspension cultures of *I. isabelliana* presents the isolation and structure elucidation of a new anthraquinone and eight known ones, five of them reported for the first time for the *Isoplexis* genus.

2. Results and discussion

A 70% EtOH extract of freeze dried cells from *I. isabelliana* cell suspension cultures produced 0.2 g anthraquinone per 100 g DW. This extract was subjected to CPC and afforded 66 fractions. The fractions were combined according to TLC results, evaporated, redissolved in MeOH and chromatographed by HPLC. Peaks collected from HPLC yielded nine anthraquinones (Table 1), one of them, isolated and identified as 3,6 (or 7)-dihydroxy-4-methoxy-2-methylanthraquinone (**1**), is new. The known isolated anthraquinones were 1,3-dihydroxy-4-methoxy-2-methylanthraquinone (**3**), 4,7-dihydroxy-2-methylanthraquinone (**4**), 1-hydroxy-2-hydroxymethylanthraquinone (**5**), 4-hydroxy-2-hydroxymethylanthraquinone (**6**) and 1,4-dihydroxy-2-hydroxymethylanthraquinone (**7**), isolated from the genus *Isoplexis* for the first time, and 3-hydroxy-4-

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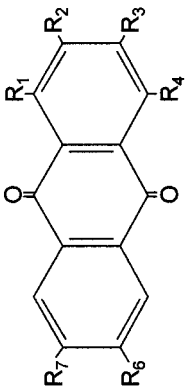
Table 1
Anthraquinones isolated from *Isoplexis isabelliana* cell suspension cultures



Compound	R_1	R_2	R_3	R_4	R_6	R_7	Compound name	Identified by
1	H	CH_3	OH	OCH_3	OH(or H)	H(or OH)	6 (or 7)-Hydroxydigitolutein	UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
2	H	CH_3	OH	OCH_3	H	H	Digitolutein	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
3	OH	CH_3	OH	OCH_3	H	H	1-Hydroxydigitolutein, 3-methylpurpurin 1-methyl ether	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
4	H	CH_3	H	OH	H	OH	Phomarin, digito-emodin	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
5	OH	CH_2OH	H	H	H	H	Digiferruginol	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS, authentic sample
6	H	CH_2OH	H	OH	H	H	ω-Hydroxypachybasin	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
7	OH	CH_2OH	H	OH	H	H	Digiferrol, 2-hydroxymethylquinizarin	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
8	OH	CH_3	H	OCH_3	H	H	Madeirin	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS
9	H	CH_3	OCH_3	H	H	H	3-Methoxy-2-methylanthraquinone	TLC, UV–VIS, ^{1}H-NMR, LC/ESI-MS/MS

methoxy-2-methylanthraquinone (**2**), 1-hydroxy-4-methoxy-2-methylanthraquinone (**8**) and 3-methoxy-2-methylanthraquinone (**9**), previously reported from the leaves of some *Isoplexis* species. The eight anthraquinones were identified from their ^{1}H-NMR spectra and by TLC and UV λ_{max}^{MeOH} comparison with data reported in the literature. As expected, all of them quenched fluorescence in UV_{254}. LC/ESI-MS and LC/ESI-MS/MS data of the isolated compounds have not been reported before.

Compound **1** showed an absorption maximum at 288 nm in the ultraviolet region suggesting that might be a digitolutein-like compound. The absence of an absorption band at ca 410 μm indicates that no hydroxyl group is an α-position (Bick & Rhee, 1966). The ^{1}H-NMR data showed, like in digitolutein (Imre, 1972; Zhang, Fox & Hadfield, 1996), a three-proton singlet at δ 2.32 (2-CH_3), a three-proton singlet at δ 4.00 (4-OCH_3) and one-proton singlet at δ 7.80 (H-1). The remaining aromatic signals are typical for a 6 or 7 substituted C-ring, having one doublet at δ 7.73 ($J =$ 2.6 Hz), a doublet of doublets at δ 7.20 ($J = 2.6$ and 8.6 Hz) and a doublet at δ 8.26 ($J = 8.6$ Hz). The spectral evidence thus points to a 3,6 (or 7)-hydroxy-4-methoxy-2-methylanthraquinone but it can not distinguish between them. IR spectral data could help to assign the hydroxyl group to the 6 or 7 position but unfortunately the amount of compound **1** available was too small to perform such an analysis. The structure was further confirmed by mass spectrometry. LC/ESI-MS gave an ion at m/z 285 $[M + H]^+$ which fits the proposed structure. LC/ESI-MS/MS analysis of this compound showed the molecular ion as base peak and fragment ions at m/z 267 $[M + H - 18]^+$ which is attributed to loss of H_2O, 255 $[M + H - 30]^+$ loss of OCH_3, and 239 $[M + H - 18 - 28]^+$ loss of H_2O and CO from the molecular ion.

The fact that most of the anthraquinones isolated from *I. isabelliana* occur in the genus *Digitalis* is one more proof of their close chemical relationship.

3. Experimental

3.1. General

CPC: modular Sanki Centrifugal Partition Chromatograph (type LLN). Power supply (Model SPL), loop sample injector plus flow director (Model FCU-V) equipped with a 3.4 ml loop and a triple head constant flow pump (Model LBP-V). Centrifuge (Model NMF) in which of the 12 cartridges only 6 (total internal volume 125 ml) were used. Panasonic Pen-recorder (Model VP-67222A) connected to a UVIS 200 detector. Fraction collector LKB 2211 Superrac. The pressure was limited to 60 bar. Flow

rate 2 ml min^{-1}. Fractions size 8 ml. HPLC: Waters 710B WISP and Waters 990 photodiode array detector. A guard column packed with octadecyl silica (5 μm particle size) and a 5 μm Hypersil C_{18} separation column (Shandon), 250 × 4.6 mm (i.d.) were used. The eluent consisted of MeOH–H_2O–HOAc (69: 30: 1) (pH 3.5) and a flow rate of 0.5 ml min^{-1} was used. Detection was performed from 220–799 nm. UV–VIS: MeOH. ^{1}H-NMR: 300 MHz in $CDCl_3$ as solvent, with TMS as internal standard. LC/ESI-MS and LC/ESI-MS/MS: HPLC performed as described above, ESI-MS and ESI-MS/MS measurements acquired using a Finnigan MAT TSQ-700 equipped with a custom made Electro Spray Interface (ESI). The repeller and ring-electrode voltage was optimized for anthraquinones. TLC: silica gel 60 F_{254} with concentration zone. Solvent systems: Toluene–MeOH (9: 1) (solvent I), EtOAc–MeOH–H_2O (81 : 11 : 8) (solvent II) and $CHCl_3$–MeOH (9 : 1) (solvent III). Spot detection: daylight, UV light at 254 and 366 nm, exposure to NH_3 vapor and spray with a solution of NaOH in MeOH (5% w/v).

3.2. Plant material

Cell suspension cultures from *I. isabelliana* were initiated in Murashige and Skoog medium (Murashige & Skoog, 1962) supplemented with 5 μM 2,4-D and 10 μM kinetin (Arrebola & Verpoorte, 1996). After 14 days from the last subculture, cells were harvested, frozen in liquid N_2, freeze dried and stored at −20°C until used. Seeds used for initiating cell suspension cultures were supplied by Dr Alicia Roca (Jardín Botánico Canario "Viera y Clavijo" del Excmo. Cabildo Insular de Gran Canaria). A voucher specimen of a plant obtained from these seeds has been deposited by Wim Snoeijer in the Onderzoekinstituut Rijksherbarium/Hortus Botanicus (L.), herbarium Pharmacognosy, Leiden University.

3.3. Extraction, analysis of the anthraquinone content and isolation

2.11 g DW cells were soaked in 70% EtOH for 96 h at room temperature. The EtOH 70% extract was concentrated under reduced pressure to give a residue (0.96 g) which was redissolved in 20 ml EtOH 70%. The absorption at 434 nm was determined and the anthraquinone content estimated (Zenk, El-Shagi & Schulte, 1975; Koblitz, 1988). For the separation of anthraquinones five isocratic CPC two-phase systems, chosen according to different polarity of the two phases and their overall polarity, were tested: *n*-hexane–EtOAc–MeOH–H_2O (9 : 1 : 5 : 5), $CHCl_3$–MeOH–H_2O–HOAc (5 : 6 : 4 : 0.5), $CHCl_3$–MeOH–H_2O (5 : 5 : 3), $CHCl_3$–MeOH–H_2O–*n*-ProH (9 : 12 : 8 : 1) and EtOAc–*n*-ProH–H_2O (7 : 3 : 9). The partition coefficient of each anthraquinone in these two-phase systems was compared by means of TLC. The best separation was obtained in the *n*-hexane–EtOAc–MeOH–H_2O (9 : 1 : 5 : 5) system, thus this system was chosen for further separation of the extract. Since separation of compounds was better in the upper phase, ascending mode was used for the first fifty fractions, afterwards the mode of elution was changed to descending mode to collect the rest of the fractions. Sixty-six CPC fractions were collected and subjected to TLC. Those fractions showing the same spots on TLC were combined and evaporated under reduced pressure The residues were redissolved in MeOH and further chromatographed by HPLC. Peaks detected by HPLC were collected and resulted in the isolation of nine compounds.

3.4. 3,6 (or 7)-Dihydroxy-4-methoxy-2-methylanthraquinone (6 (or 7)-hydroxydigitolutein) (**1**)

LC/ESI-MS *m/z* 285 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 285 $[M + H]^+$ (100), 267 (4), 255 (7), 239 (13). ^{1}H-NMR (300 MHz, $CDCl_3$): δ 2.32 (3H, *s*, 2-CH_3), δ 4.00 (3H, *s*, 4-OCH_3), δ 7.20 (1H, *dd*, $J =$ 2.6 and 8.6 Hz, H-6 or 7), δ 7.73 (1H, *d*, $J = 2.6$ Hz, H-8), δ 7.80 (1H, *s*, $J = 3$ Hz, H-1), δ 8.26 (1H, *d*, $J =$ 8.6 Hz, H-5).

3.5. 3-Hydroxy-4-methoxy-2-methylanthraquinone (digitolutein) (**2**)

TLC: *Rf* 0.39 (solvent I), *Rf* 0.63 (solvent II), *Rf* 0.57 (solvent III), NH_3 vapor red. LC/ESI-MS *m/z* 269 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 269 $[M + H]^+$ (100), 254 (97.5), 237 (9.5), 223 (24), 209 (2), 195 (4.5).

3.6. 1,3-Dihydroxy-4-methoxy-2-methylanthraquinone (1-hydroxydigitolutein, 3-methylpurpurin 1-methyl ether) (**3**)

TLC: *Rf* 0.43 (solvent I), *Rf* 0.64 (solvent II), *Rf* 0.62 (solvent III), NH_3 vapor red. LC/ESI-MS *m/z* 285 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 285 $[M + H]^+$ (6), 270 (100), 242 (12), 224 (7).

3.7. 4,7-Dihydroxy-2-methylanthraquinone (phomarin, digito-emodin) (**4**)

TLC: *Rf* 0.36 (solvent I), *Rf* 0.64 (solvent II), *Rf* 0.51 (solvent III), daylight yellow, UV_{366} orange, NH_3 vapor and NaOH red. LC/ESI-MS *m/z* 255 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 255 $[M + H]^+$ (100), 237 (4), 227 (9), 209 (4).

3.8. 1-Hydroxy-2-hydroxymethylanthraquinone (digiferruginol) (**5**)

TLC: *Rf* 0.62 (solvent II), *Rf* 0.50 (solvent III). LC/ESI-MS *m/z* 255 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 255 $[M + H]^+$ (7), 237 (100), 226 (1), 209 (6).

3.9. 4-Hydroxy-2-hydroxymethylanthraquinone (ω-hydroxypachybasin) (**6**)

TLC: *Rf* 0.29 (solvent I), *Rf* 0.62 (solvent II), *Rf* 0.49 (solvent III), daylight yellow, UV_{366} orange, NH_3 vapor and NaOH red. UV–VIS λ_{max}^{MeOH} nm: 223, 246sh, 254, 281sh, 326sh, 332sh, 387sh, 402. LC/ESI-MS *m/z* 255 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 255 $[M + H]^+$ (100), 254 (5), 237 (12), 225 (6), 209 (6).

3.10. 1,4-Dihydroxy-2-hydroxymethylanthraquinone (digiferrol, 2-hydroxymethylquinizarin) (**7**)

TLC: *Rf* 0.36 (solvent I), *Rf* 0.63 (solvent II), *Rf* 0.49 (solvent III), UV_{366} yellow-orange, NaOH red. LC/ESI-MS *m/z* 271 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 271 $[M + H]^+$ (43), 253 (32), 252 (33), 242 (100), 241 (72), 225 (97), 214 (9).

3.11. 1-Hydroxy-4-methoxy-2-methylanthraquinone (madeirin) (**8**)

TLC: *Rf* 0.48 (solvent I), *Rf* 0.60 (solvent II), *Rf* 0.62 (solvent III), daylight orange, UV_{366} yellow-orange, NH_3 vapor and NaOH red. LC/ESI-MS *m/z* 269 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 269 $[M + H]^+$ (24), 254 (100), 241 (1), 239 (4).

3.12. 3-Methoxy-2-methylanthraquinone (**9**)

TLC: *Rf* 0.45 (solvent I), *Rf* 0.63 (solvent II), *Rf* 0.61 (solvent III), daylight yellow, UV_{366} yellow-orange, NH_3 vapour and NaOH yellow. LC/ESI-MS *m/z* 253 $[M + H]^+$. LC/ESI-MS/MS *m/z* (rel. int.) 253 $[M + H]^+$ (100), 238 (40), 223 (6.5), 210 (3.5).

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