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Phenolic glycosides from *Exostema mexicanum* leaves

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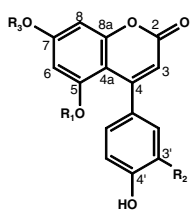
Phytochemical investigation of the leaves of *Exostema mexicanum* led to the isolation of two novel acylated flavonol glycosides **6**, **7** and three glycosides **1**–**4** structurally belonging to the group of 4-phenylcoumarins. One of them, 5-*O*-β-D-glucopyranosyl-4′-hydroxy-7-methoxy-4-phenylcoumarin (**2**), turned out to be new. Furthermore, the 4-phenylcoumarin aglycone 3′-hydroxy-4′,5,7-trimethoxy-4-phenylcoumarin (**5**) was obtained. The *in vitro* cytotoxicity of **3**–**5** against the cell line ECV-304 was evaluated; the aglycone **5** was highly cytotoxic, whereas the glycosidic compounds **3** and **4** were inactive.

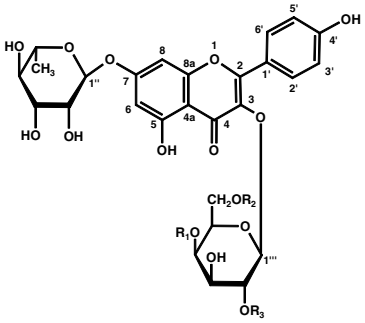
1. Introduction

The neotropical species *Exostema mexicanum* A. Gray, Rubiaceae, is found in the West Indies, Mexico and El Salvador. *E. mexicanum*, known in Mexico as "quina" and "melena de león", is locally used for the treatment of malaria and dengue fever. The stem bark of this shrub or tree, designated as copalchi bark, is commonly used in traditional medicine as a substitute for quinine. In Mexican folk medicine there are five different plants described as "copalchi". Members of the family Rubiaceae, as well as the Euphorbiaceae, belong to such "copalchi-bark" supplying plants (Noster 1992). Interestingly, these rubiaceous species do not produce alkaloids, but the rarely occurring 4-phenylcoumarins (neoflavonoids).

Previous investigations resulted in the isolation of three cucurbitacin derivates, as well as 5-*O*-β-D-glucopyranosyl-3′,4′-dihydroxy-7-methoxy-4-phenylcoumarin (Mata et al. 1990a). In another work, the isolation of seven 4-phenylcoumarins from the stems was described, which showed *in vitro* activity against *Plasmodium falciparum* (Köhler et al. 2001). During our ongoing phytochemical research on antiplasmodial plant species from Central America, we analysed the leaves of *E. mexicanum*, which were not previously investigated and isolated one new compound (**2**) (Fig. 1) and three known 4-phenylcoumarin glycosides **1**, **3**, **4** together with the known aglycone **5**. In addition, two new flavonol glycosides **6** and **7** were obtained. The characterisation of these compounds was achieved by various chromatographic and spectroscopic methods (^{1}H NMR, ^{13}C NMR and MS).



1 R_1 = β-D-glucopyranosyl, R_2 = OH, R_3 = H
2 R_1 = β-D-glucopyranosyl, R_2 = H, R_3 = CH_3
3 R_1 = β-D-galactopyranosyl, R_2 = H, R_3 = CH_3
4 R_1 = β-D-galactopyranosyl, R_2 = OH, R_3 = CH_3



6 R_1 = E-p-coumaroyl, R_2 = R_3 = α-L-rhamnopyranosyl
7 R_1 = E-p-coumaroyl, R_2 = α-L-rhamnopyranosyl, R_3 = 2,4-diacetyl-α-L-rhamnopyranosyl

2. Investigations, results and discussion

Column chromatography of the dichloromethane extract of *E. mexicanum* leaves afforded 3′-hydroxy-4′,5,7-trimethoxy-4-phenylcoumarin (**5**), already known from the stems (Köhler et al. 2001). Interestingly, **5** is the only 4-phenylcoumarin aglycone detectable in the leaves, whereas in the stems several further derivatives are present.

The butanol extract was separated by column chromatography with Sephadex LH 20 material and after that purified by preparative TLC which resulted in the isolation of 5-*O*-β-D-glucopyranosyl-7,3′,4′-trihydroxy-4-phenylcoumarin (**1**). Astonishingly, compound **1**, which was previously obtained from the three related rubiacaeous species *Coutarea hexandra*, *Hintonia latiflora* and *Exostema caribaeum* (Aquino et al. 1988; Mata et al. 1990b; Calera et al. 1996) was isolated now for the first time from *E. mexicanum.*

The ethyl acetate extract was fractionated on RP-18 material. Further purification was achieved using preparative TLC and yielded the phenolic compounds **2**–**4** and **6**–**7**.

By comparison with spectroscopic data, **3** was identified as 5-*O*-β-D-galactopyranosyl-4′-hydroxy-7-methoxy-4-phenylcoumarin, previously isolated from *Hintonia latiflora* (Mata et al. 1990b), whereas **4** turned out to be 5-*O*-β-D-galactopyranosyl-3′,4′-dihydroxy-7-methoxy-4-phenylcoumarin, first isolated from *Exostema caribaeum* (Mata et al. 1987).

The (−) FAB mass spectrum of compound **2** showed a $[M-H]^-$ peak at *m/z* 445, corresponding to a molecular formula of $C_{22}H_{22}O_{10}$. The same molecular weight had been determined for compound **3**, thus both had to be isomers. Indeed, the ^{1}H NMR spectrum of **2** displayed again characteristic signals for a 4-phenylcoumarin glycoside. The position of the methoxy group was deduced from NOE experiments. Irradiation of the methoxy signal caused an enhancement of the signals for H-6 and H-8, thus the methoxy group had to be in position 7 as in compound **3**, revealing that both substances possessed the same aglycone. Comparison of the ^{1}H NMR chemical shifts of the aglycone moiety between **2** and **3** additionally showed that the hexose had to be attached to the C-5 hydroxy group. The known compounds **3** and **4**, though, displayed typical signals for a β-galactopyranosyl residue with an especially indicative H-4 signal at 3.79 ppm and 3.81 ppm, respectively, with a small coupling constant of J = 3.0 Hz, due to the equatorial position of H-4. This signal was missing in compound **1** and **2**, which instead displayed characteristic β-glucopyranosyl resonances (Aquino et al. 1988). Thus, compound **2** had to be 5-*O*-β-D-glucopyranosyl-4′-hydroxy-7-methoxy-4-phenylcoumarin, a new natural compound. This was supported by the ^{13}C NMR spectra of **1** and **2** which displayed typical signals for C-4″ of a glucopyranosyl residue at δ 70.8 and 71.2, respectively, whereas the β-galactopyranosyl residues of **3** and **4** were characterised by a signal between δ 68 and 69.

Compound **7** showed a $[M-H]^-$ peak at *m/z* 1115 in the (−) FAB-MS spectrum, corresponding to a molecular formula of $C_{52}H_{60}O_{27}$. The ^{1}H NMR spectrum (Table) displayed typical signals for a kaempferol moiety in the aromatic region. Further aromatic signals belonged to a *p*-coumaroyl residue. In addition, the resonances of four anomeric sugar protons at 4.44, 5.20, 5.55 and 5.64 ppm were observed, together with those for two acetyl groups at 2.02 and 2.20 ppm. Thus, **7** had to be an acylated kaempferol tetraglycoside. The small coupling constants of the anomeric protons at 4.44, 5.20 and 5.55 ppm hinted to three rhamnosyl moieties, which was supported by three methyl signals at 0.93, 1.04 and 1.26 ppm. The remaining sugar had to be a hexose. The downfield shift of the H-6 and H-8 signals of the kaempferol moiety compared to the aglycone indicated the attachment of a rhamnopyranosyl residue at the C-7 hydroxyl group, as in astrasikokioside I, a kaempferol tetraglycoside isolated from *Astragalus shikokianus* (Yahara et al. 2000). Indeed, comparison of the ^{1}H and ^{13}C NMR spectroscopic data of **7** with those of astrasikokioside I revealed a high degree of similarity between both compounds. Thus the hexose moiety of **7** had to be attached to the C-3 hydroxyl group of the kaempferol moiety and had to be further glycosylated in positions 2 and 6 due to the indicative downfield shifts of the corresponding carbons in the ^{13}C NMR spectrum. All proton and most carbon signals could be assigned from the H−H COSY and HSQC spectra, allowing the identification of the positions of the *p*-coumaroyl moiety and the acetyl groups from the characteristic downfield shifts of the corresponding protons. Finally, the hexose

Table: ^{1}H NMR data [400 MHz, CD_3OD, δ_H (ppm), J (Hz)] and ^{13}C NMR data [100.6 MHz, CD_3OD, δ_C (ppm)][a] of compound 7

Position	δ_H	δ_C
Kaempferol		
2		n.d.
3		n.d.
4		n.d.
4a		100.5
5		n.d.
6	6.48 d (2.0)	n.d.
7		n.d.
8	6.76 d (2.0)	95.7
8a		158.3
1′		122.9
2′	8.12 d (9.0)	132.5
3′	6.95 d (9.0)	116.0
4′		161.2
5′	6.95 d (9.0)	116.0
6′	8.12 d (9.0)	132.5
3-O-gal		
1	5.64 d (7.0)	101.1
2	3.94 t (7.0)	78.1
3	4.00 m	73.9
4	5.34 d (3.5)	71.9
5	3.48 m	72.2
6_a	3.52 m	64.6
6_b	3.58 m	64.6
2‴-O-rha		
1	5.20 br s	99.9
2	5.27 br s	74.1
3	4.20 br d	68.7
4	4.85 m	75.7
5	4.36 m	67.8
6	0.93 d (6.5)	17.1
6‴-O-rha		
1	4.44 br s	102.3
2	3.52 m	72.0
3	3.85 m	73.8
4	3.21 m	73.2
5	3.32 m	70.0
6	1.04 d (6.0)	17.9
7-O-rha		
1	5.55 br s	100.1
2	4.02 br s	71.8
3	3.82 m	72.1
4	3.60 m	71.0
5	3.62 m	73.9
6	1.26 d (6.0)	18.1
4‴-O-p-coumaroyl		
1		127.0
2	7.51 d (8.5)	131.6
3	6.84 d (8.5)	116.9
4		161.3
5	6.84 d (8.5)	116.9
6	7.51 d (8.5)	131.6
7	7.64 d (16.0)	114.9
8	6.35 d (16.0)	147.2
9		168.9
$CO\underline{C}H_3$	2.20 s	21.0
$\underline{C}OCH_3$		171.2
$CO\underline{C}H_3$	2.02 s	21.2
$\underline{C}OCH_3$		171.5

n.d. = not determined

[a] data from HSQC and HMBC. Due to limited amount not all carbons were observed.

residue could be unambiguously identified as galactopyranose from the small coupling constant of H-4 (δ 5.34, J 3.5 Hz) as well as the characteristic ^{13}C resonance of C-4 (δ 71.9) analogous to the 4-phenylcoumarin galactosides **3** and **4**. In conclusion, **7** represents an acylated derivative of the known kaempferol tetraglycoside astrasikokioside I.

Compound **6** showed a $[M-H]^-$ peak at *m/z* 1031 in the (−) FAB-MS spectrum, corresponding to a molecular formula of $C_{48}H_{56}O_{25}$. The ^1H NMR spectrum was very similar to that of **7** but displayed two signals less in the downfield region. In addition, the two acetyl signals were missing. Thus **6** had to be the didesacetyl derivative of **7**.

Both flavonol tetraglycosides are new natural compounds. Indeed, this is the first report of the occurrence of classical flavonoids from the genus *Exostema*. 4-Phenylcoumarin glycosides on the other hand seem to be a common chemotaxonomic marker of this genus, as well as the related genera *Hintonia* and *Coutarea*.

As 4-phenylcoumarin aglycones are known to be highly cytotoxic (Jenett-Siems et al. 2002; Ito et al. 2000), we evaluated the toxicity of the aglycone **5**, as well as the glycosides **3** and **4** against the bladder carcinoma cell line ECV-304 (Mosmann 1983; Onegi et al. 2002).

As expected, **5** revealed a high cytotoxicity with an IC_{50} value of 1.0 μg/ml whereas **3** and **4** proved to be non toxic (IC_{50} values > 50.0 μg/ml), maybe as a result of an insufficient membrane penetration due to their hydrophilic character. Similar effects were observed for the cytotoxic cucurbitacins from *E. mexicanum*: cucurbitacin F and cucurbitacin F-25-acetate displayed marked cytotoxicity, whereas 2-*O*-β-D-glucopyranosylcucurbitacin F-25-acetate proved to be inactive (Mata et al. 1990a).

3. Experimental

3.1. Equipment

NMR-spectra were recorded on a Bruker AVANCE DPX 400 or a Bruker DRX 500 NMR-spectrometer. EI-MS were performed on a Varian MAT CH_7A (70 eV). FAB-MS were recorded on a Varian MAT CH_5DF (CH_3OH/m-Nitrobenzylalkohol, Xenon, 3 kV). TLC was performed on silica gel 60 plates (F_{254}, 20 × 20 cm, Merck, Germany), solvent system: HCO_2H-H_2O-EtOAc (9 : 9 : 82), detection: 1% MeOH solution of diphenylboric acid-2 aminoethylester (= Naturstoffreagenz A). After drying the plates were examined under UV_{366}.

3.2. Plant material

Leaves of *Exostema mexicanum* were collected in the Dept. of Ahuachapán, El Salvador, at an altitude of 300 m. A voucher specimen (PBT 275) was deposited in the herbarium of the Jardin Botánico La Laguna (LAGU) in El Salvador.

3.3. Extraction and isolation

Air dried leaves (300 g) were ground and extracted three times with 1.0 l MeOH at room temperature. After evaporation of the solvent the residue (48.5 g) was acidified with a 2% (m/v) solution of tartaric acid. The aqueous layer was extracted with petrol ether (3 × 0.25 l), CH_2Cl_2 (3 × 0.25 l) and EtOAc (3 × 0.25 l) and finally with butanol (3 × 0.25 l). Solvents were evaporated at 40 °C under reduced pressure.

The EtOAc-extract (1.71 g) was subjected to column chromatography on LiChroprep® RP-18 material [Merck, 40–63 μm] (H_2O-MeOH 90 : 10 to MeOH). Fraction D_2 (H_2O−MeOH 60 : 40) yielded compound **4** (R_f = 0.35) upon prep. TLC (HCO_2H-H_2O-EtOAc 9 : 9 : 82), fraction E (H_2O−MeOH 50 : 50) allowed the isolation of **2** (R_f = 0.56), **3** (R_f = 0.47) and **6** (R_f = 0.11, 4 mg). From fraction F (H_2O−MeOH 30 : 70) **7** (R_f = 0.37, 8 mg) was obtained.

The butanol extract (2.61 g) was dissolved in 4 ml MeOH, and subjected to column chromatography on a glass column (2.5 cm diameter × 64 cm length) packed with Sephadex LH 20 (80 g) (eluated with 0.5 l MeOH into 200 fractions). The fractions 166–200 were purified by prep. TLC (HCO_2H-H_2O-EtOAc 9 : 9 : 82) and afforded compound **1** (R_f = 0.49, 35.2 mg) and again compound **4** (7.9 mg).

The CH_2Cl_2-extract (2.12 g) was subjected to column chromatography on silica gel 60 (Merck, 70–230 mesh) and sequentially eluted with CH_2Cl_2−MeOH 99 : 1 to MeOH. Fractions 25 and 26 (CH_2Cl_2−MeOH 98 : 2) were combined (230 mg) and subjected to column chromatography on LiChroprep® RP-18 material [Merck, 40–63 μm] (H_2O−MeOH 80 : 20 to MeOH). Pure **5** (12 mg) was obtained from the combined fractions 5a and 5b, eluted with H_2O−MeOH 30 : 70.

3.4. 5-O-β-D-Glucopyranosyl-4′-hydroxy-7-methoxy-4-phenylcoumarin (**2**)

Yellow oil; ^1H NMR (CD_3OD, 400 MHz): δ 2.68 (1 H, dd, J = 8.0, 9.5 Hz, H-2″), 3.45–3.65 (2 H, m, H-3″, H-4″), 3.50 (1 H, dd, J = 7.0, 11.0 Hz, H-6″a), 3.58 (1 H, dd, J = 5.0, 11.0 Hz, H-6″b), 3.88 (3 H, s, 4′OMe), 4.83 (1 H, d, J = 7.5 Hz, H-1″), 5.93 (1 H, s, H-3), 6.20 (1 H, d, J = 2.0 Hz, H-6), 6.39 (1 H, d, J = 2.0 Hz, H-8), 6.67 (2 H, s, H-6, H-8), 6.83 (2 H, d, J = 8.5 Hz, H-3′,5′), 7.22 (2 H, d, J = 8.5 Hz, H-2′,6′); ^{13}C NMR (CD_3OD, 100.6 MHz): δ 160.4 (C-2), 113.3 (C-3), 158.1 (C-4), 104.4 (C-4a), 156.2 (C-5), 99.8 (C-6), 163.4 (C-7), 96.6 (C-8), 156.6 (C-8a), 132.3 (C-1′), 127.3 (C-2′), 115.5 (C-3′), 157.6 (C-4′), 115.5 (C-5′), 127.3 (C-6′), 56.5 (7-OCH_3), 101.5 (C-1″), 74.5 (C-2″), 78.4 (C-3″), 71.2 (C-4″), 77.8 (C-5″), 62.6 (C-6″); (−) FAB-MS (m/z): 445 $[M-H]^-$.

3.5. Kaempferol-3-O-α-L-rhamnopyranosyl-(1 → 6)-[α-L-rhamnopyranosyl-(1 → 2)]-(4-coumaroyl-β-D-galactopyranosyl)-7-O-α-L-rhamnopyranoside (**6**)

Yellow oil; ^1H NMR (CD_3OD, 400 MHz): δ 1.02 (3 H, d, J = 6.5 Hz, H-6‴), 1.03 (3 H, d, J = 6.0 Hz, H-6⁗), 1.26 (3 H, d, J = 6.0 Hz, H-6″), 4.46 (1 H, br s, H-1⁗), 5.20 (1 H, br s, H-1‴), 5.34 (1 H, br s, H-4‴), 5.56 (1 H, br s, H-1″), 5.73 (1 H, d, J = 7.0 Hz, H-1‴), 6.34 (1 H, d, J = 16.0 Hz, H-8⁗⁗), 6.47 (1 H, d, J = 2.0 Hz, H-6), 6.75 (1 H, d, J = 2.0 Hz, H-8), 6.84 (2 H, d, J = 8.5 Hz, H-3⁗⁗, 5⁗⁗), 6.96 (2 H, d, J = 9.0 Hz, H-3′, 5′), 7.49 (2 H, d, J = 8.5 Hz, H-2⁗⁗, 6⁗⁗), 7.63 (1 H, d, J = 16.0 Hz, H-7⁗⁗), 8.16 (2 H, d, J = 9.0 Hz, H-2′,6′); (−) FAB-MS (m/z): 1031 $[M-H]^-$.

3.6. Kaempferol-3-O-α-L-rhamnopyranosyl-(1 → 6)-[2,4 diacetyl-α-L-rhamnopyranosyl-(1 → 2)]-(4-coumaroyl-β-D-galactopyranosyl)-7-O-α-L-rhamnopyranoside (**7**)

Yellow oil; ^1H NMR (CD_3OD, 400 MHz) and ^{13}C NMR (CD_3OD, 100.6 MHz): see Table; (−) FAB-MS (m/z): 1115 $[M-H]^-$.

3.7. In vitro cytotoxicity assay

The cytotoxicity of the substances **3**–**5** was estimated by a proliferation assay using the MTT-assay (Mosmann 1983). Test substances were dissolved in DMSO and diluted with medium to the desired concentrations. Human cancer cells with endothelial properties (ECV-304) were cultivated in Earle Medium 199 supplemented with 10% fetal calf serum in 96-well plates in an atmosphere of 5% CO_2 at 37 °C in a humidified environment. Cells were seeded at a density of approximately 1000 cells per well. After 24 h they were supplemented with 100 μl test substance in medium and cultivated for 4 further days. The cell viability was measured by the MMT-assay using DMSO to dissolve the formed purple formazan. The absorbance was quantified at 580 nm with an ELISA plate reader. The IC_{50} values were calculated by linear regression.

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