SUPPLEMENTARY MATERIAL

Phenolic compounds from *Glycyrrhiza pallidiflora* Maxim. and their cytotoxic activity

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Twenty one phenolic compounds (**1-21**) including dihydrocinnamic acid, isoflavonoids, flavonoids, coumestans, pterocarpanes, chalcones, isoflavan and isoflaven were isolated from the roots of *Glycyrrhiza pallidiflora* Maxim. Phloretinic acid (**1**), chrysin (**6**), 9-methoxycoumestan (**8**), isoglycyrol (**9**), 6''-O-acetylanonin (**19**) and 6''-O-acetylwistin (**21**) were isolated from *G. pallidiflora* for the first time. Isoflavonoid acetylglycosides **19**, **21** might be artifacts which could be produced during the EtOAc fractionation process of whole extract. Compounds **2-4**, **10**, **11**, **19** and **21** were evaluated for their cytotoxic activity with respect to model cancer cell lines (CEM-13, MT-4, U-937) using the conventional MTT assays. Isoflavonoid calycosin (**4**) showed the best potency against human T-cell leukemia cells MT-4 (CTD₅₀, 2.9 μ M). Pterocarpanes medicarpin (**10**) and homopterocarpin (**11**) exhibit anticancer activity in micromolar range with selectivity on the human monocyte cells U-937. The isoflavan (3*R*)-vestitol (**16**) was highly selective on the lymphoblastoid leukemia cells CEM-13 and was more active than the drug Doxorubicin.

Keywords: *Glycyrrhiza pallidiflora*; flavonoids; pterocarpanes; isoflavonoids; cytotoxic activity

3. Experimental section

3.1. General experimental procedures

NMR spectra were acquired on Bruker AV-400 (¹H: 400.13 MHz, ¹³C: 100.78 MHz) or Bruker AV-600 (¹H: 600.30 MHz, ¹³C: 150.95 MHz) (Bruker BioSpin GmbH, Rheinstetten, Germany) instruments, using tetramethylsilane (TMS) as an internal standart. The NMR signals were assigned by using various ¹H-¹H and ¹H-¹³C correlation spectroscopy experiments (COSY, COLOC, COXH). The IR spectra were recorded by means of the KBr pellet technique on a Bruker Vector-22 spectrometer. The UV spectrum were obtained on an HP 8453 UV Vis Spectrometer. Optical rotations were measured with a PolAAr3005 polarimeter. The melting points were determined on a Stuart SMF-38 melting point apparatus and are uncorrected. The mass spectra were recorded on a DFS spectrometer (Thermo Scientific, evaporator temperature 200-270°C). C,H- analysis was carried out on a Carlo-Erba 1106 elemental analysis instrument. The GC/MS was performed in a Hewlett-Packard instrument with a HP 5890 Series II gas chromatograph and HP 5971 (EI, 70 eV) mass-selective detector using a HP-5ms cap. column (30 m $\times$ 0.25 mm i.d.; film thickness, 0.25 μ m); carrier gas was He at flow rate of 1 ml/min; programmed column temp. was at 50°C for 2 min, from 50°C to 300°C at 4°/min, and 300°C for 30 min; vaporizer temp., 280°; ion-source temp., 175°; and scan rate, 1.2 scans/s in mass range 30-650 amu. The contents (%) of compounds were determined from peak areas in chromatograms without using correction coefficients. Spectral and analytical investigations were carried out at Collective Chemical Service center of Siberian Branch of the Russian Academy of

Sciences. Extractive compounds were isolated by column chromatography on silica gel (0.063-0.200 mm, Acros) or Kieselgel 60 (0.063-0.200 mm, Merck KGaA, Darmstadt, Germany) and eluted with chloroform, chloroform-ethanol (v/v=100:0; to 0:1); chloroform-methanol (v/v=80:0; to 1:10); benzene-EA (v/v=10:1; to 1:10), petroleum ether-acetone (v/v=5:1; to 0:1). Preparative TLC was carried out on glass plates (30×30 cm with 2 mm thick sorbent layer; silica gel F₂₅₄ 35- 70µm, Across-Organics) or on Silica gel 60 F₂₅₄ plates (Qingdao Marine Chemical, Inc.) (CHCl₃-EtOH, 10:1 mobile phase). The purity of separation was monitored by TLC on Silufol UV₂₅₄ plates using various solvent systems, and spots were visualized by detection under UV light or by treatment with iodine vapor.

3.2. Plant material

The roots of *Glycyrrhiza pallidiflora* Maxim. were collected from Russian Far East, Primorsky Territory, Khorolsky District, in the vicinity of Sivakovka village in November 2008 and in April 2009. The plants were collected and identified by Prof. Petr Gorovoy. A voucher specimen № 103395 deposited at the Herbarium of G.B. Elyakov Pacific Institute of Bioorganic Chemistry, Far Eastern Branch of the Russian Academy of Sciences (Vladivostok, Russia)

3.3. Extraction and isolation

The plant materials (250 g) were air-dried, powdered and successively extracted with hexane, diethyl ether, *tert*-butyl methyl ether, ethyl acetate and 95% ethanol (4×500 mL, with heating on a water bath at 45°C for 6 h), respectively. The combined extracts were concentrated under reduced pressure to the crude extracts. The ethanol extract (76 g) was suspended in distilled water and then partitioned sequentially with equal volume of ethyl acetate (EA). Evaporation of EA layer *in vacuo* gave 3.44 g (1.37%, 9% from ethanol extract weight) of EA soluble fraction.

The hexane extract (1.6 g, 0.64%) was separated and analyzed by standard literature method. NMR and GC-MS data (of methylated fraction) were used to identify free sterols and their esters [β -sitosterol (M^+ 414), β -stigmasterol (M^+ 412), brassicasterol (M^+ 398), stigmast-4-en-3-one (M^+ 410), methyl ursolate (M^+ 470) total content -11%]. Fatty acids (-21%) are represented mainly by palmitic (C_{16:0}) (4.7%), stearic (C_{18:0}) (2.6%), linoleic (C_{18:2}) (10.3%), oleic (C_{18:1}) (8.1%) and 9,12,15-octadecatrienic (C_{18:3}) (1.8%) acids.

3.3.1. Isolation of flavonoids

Separation of ether extract.

The ether extract (3.25 g, 1.3%) was subjected to silica gel column chromatography (0.063-0.200 mm, 4×330 cm, 120 g) eluted with CHCl₃-EtOH (v/v=100:0, 100:1, 50:1, 20:1, 10:1, 5:1, and 0:1) to afford fractions A–I. Fraction B (700 mg) was subjected to silica gel column chromatography (200–300 mesh, eluent CHCl₃-EtOH) to afforded 5 subfractions. Phloretinic acid (**1**) (210 mg) was isolated from subfractions 1,2. Chromatography of residue of combined subfractions 3-5 (500 mg) (benzene-EA, 10:0.5→1:10), produced five subfractions, crystallization of which from ether gave isoflavonoids **2** (32 mg), **3** (45 mg), and **5** (11 mg). Repeat chromatography of fractions C (750 mg), D (600 mg), E (500 mg), F (400 mg), and I (250 mg) (CHCl₃-EtOH, 100:0.5→0:1), produced five-seven subfractions, which after NMR analysis were crystallized from a mixture of ether-EA, 1:1. Additionally compounds **2** (70 mg), **3** (33 mg), **4** (104 mg), **5** (28 mg), and **6** (33 mg) were isolated.

Separation of TBME extract.

The TBME extract (0.95 g, 0.38%) was subjected to silica gel column chromatography (0.063-0.200 mm, 2×330 cm, 50 g) eluted with CHCl₃-EtOH (v/v=100:1, 80:1, 50:1, 20:1, 10:1, 5:1, and 0:1) to afford fractions A–F. Repeat chromatography of fraction A (200 mg) (petroleum ether-acetone (v/v=5:1→0:1) gave 4 subfractions. Phloretinic acid (**1**) (28 mg) was isolated by TLC plate chromatography of the 1-th subfraction. By treatment of SF₂₋₄, with ether compounds **2** (11 mg) and **10** (18 mg) were isolated. After repeat chromatography of fraction B (220 mg) (benzene:EA, 10:0.5→10:1) (-)-medicarpin (**10**) (20 mg), (-)-homopterocarpin (**11**) (9 mg), and (-)-maackiain (**12**) (5 mg) were obtained. Fraction C (60 mg) was applied to a silica gel plate and eluted with a gradient of CHCl₃-EtOH to give after crystallization from ether-EA (v/v=1:1) compounds **9** (15 mg), **10** (8 mg), and **12** (9 mg). Fraction D (180 mg) was purified repeatedly by silica gel column chromatography (eluted with a gradient of CHCl₃-EtOH (100:1, 80:1, 50:1, 10:1, v/v) produced four subfractions, which finally, after trituration with ether gave compounds **8** (20 mg), **10** (18 mg), **11** (6 mg), and **12** (5 mg). Column chromatography of fraction E (180 mg) (eluted with a gradient of CHCl₃-EtOH (100:1, 80:1, 50:1, 10:1, 5:1) afforded 5 subfractions. These subfractions were triturated with ether; compounds **7** (17 mg), **11** (7 mg), and **12** (6 mg) were isolated. Column chromatography of fraction F (110 mg) gave compounds **8** (12 mg), **9** (10 mg), **11** (15 mg), and **12** (15 mg). Repeatedly chromatography from the residues of all MTBE fractions (~0.5 g) on silica gel column (eluted with a gradient of CHCl₃-EtOH (80:1, 50:1, 10:1, 5:1, 0:1), afforded 5 subfractions. Purification by TLC on glass plates additionally gave compounds **8** (8 mg), **9** (8 mg), **10** (13 mg), **11** (10 mg), and **12** (5 mg).

Separation of EA extract

The EA soluble fraction (3.44 g, 0.39%) was subjected to silica gel column chromatography, eluted with CHCl₃-EtOH (v/v=100:0, 100:1, 50:1, 10:1, 5:1, and 1:10) to give six fractions (A: 550 mg, B: 490 mg, C: 850 mg, D: 550 mg, E: 650 mg, F: 350 mg). Chromatography of EA extract (0.98 g) gave additional six fractions (A: 210 mg, B: 200 mg, C: 250 mg, D: 120 mg, E: 100 mg, F: 100 mg). Column chromatography on silica gel of combined fraction A (760 mg) (eluent CHCl₃-EtOH) gave additionally phloretinic acid **1** (60 mg). Repeat chromatography of the residue from this fraction (690 g) (benzene:EA, 10:0.5→10:1), produced 5 subfractions, crystallization of which from ether-EA mixture gave compounds **10** (65 mg), **16** (24 mg), **17** (18 mg), and **18** (12 mg). By column chromatography on silica gel of combined fraction B (690 mg) (eluted with a gradient of CHCl₃-EtOH (80:1, 50:1, 10:1, 5:1, 1:5) followed by TLC plates purification of all 5 subfractions compounds **16** (33 mg), **17** (28 mg), and **18** (25 mg) were additionally isolated. Column chromatography on silica gel of combined fractions C (1100 mg) (eluted with a gradient of CHCl₃-EtOH (80:1, 60:1, 40:1, 30:1, 20:1, 10:1, 7:1, 5:1, 1:5) gave 9 subfractions, triturated of which with ether afforded compounds **10** (40 mg), **13** (41 mg), **14** (18 mg), **15** (15 mg), **16** (52 mg), **17** (20 mg), **18** (32 mg), and **20** (23 mg). Further separation of the combined residues (850 mg) (eluted with a gradient of CHCl₃-MeOH (100:1, 80:1, 50:1, 10:1, 5:1, 1:5) and triturated of the obtained 6 subfractions with ether gave additionally **13** (28 mg), **14** (12 mg), **15** (15 mg), **17** (23 mg), **18** (20 mg), and **20** (82 mg). Column chromatography on silica gel of combined fractions D (670 mg) [eluted with a gradient of CHCl₃-MeOH (80:1, 50:1, 10:1, 5:1, 2:1, 1:5, 1:10] gave 8 subfractions crystallization of which from respective solvents gave compounds **13** (24 mg), **14** (15 mg), **15** (22 mg), **16** (30 mg), **17** (23 mg), **19** (15 mg), **20** (35 mg), and **21** (24 mg). Column chromatography on silica gel of combined fractions E (750 mg) [eluted with a gradient of CHCl₃-MeOH (80:1, 50:1, 10:1, 5:1, 1:5, 1:10], repeatedly purification of obtained subfractions by TLC plate and crystallization of the combined subfractions (after NMR ¹H analysis) from ether-EA mixture gave **13** (16 mg), **14** (35 mg), **15** (40 mg), **18** (45 mg), **19** (11 mg), **20** (52 mg), and **21** (18 mg). Column chromatography on silica gel of combined fractions F (450 mg) [eluted with a gradient of CHCl₃-MeOH (80:1, 50:1, 10:1, 5:1, 1:5, 1:10]

gave 6 subfractions, triturated of which with ether additionally afforded compounds **15** (15 mg), **16** (12 mg), **18** (49 mg), **19** (13 mg), **20** (35 mg), and **21** (10 mg).

6''-O-Acetylwistin (21) was obtained as a yellow powder; m.p. 205-208°C (EtOH). $[\alpha]_D^{25} -73$ (c 1.1, MeOH); IR: $\nu = 3390, 3280, 2930, 1735, 1708, 1686, 1633, 1603, 1506, 1447, 1360, 1261, 1220, 1113, 1080, 1051, 1013, 980, 844$ and 810 cm^{-1} . UV (EtOH) λ_{max} (lg ϵ): 236 (4.02), 262 (4.19), 324 nm (3.99). ^1H NMR (600 MHz, CD_3OD , J in Hz) δ 1.99 (3H, s, CH_3), 3.38 (1H, m, H-5''), 3.49 (1H, dd, $J = 9.2, 7.0$, H-4''), 3.58 (1H, dd, $J = 8.5, 7.2$, H-2''), 3.68 (1H, dd, $J = 9.2, 8.5$, H-3''), 3.79 (3H, s, 4'- OCH_3), 3.86 (3H, s, 6'- OCH_3), 4.17 (1H, dd, $J_{a-b} = 12, J_{a-x} = 6$, H-6''a), 4.36 (1H, dd, $J_{a-b} = 12, J_{b-x} = 2$, H-6''b), 4.92 (1H, br d, $J = 7.2$, H-1''), 6.89 (2H, brd, $J = 7.6$, H-3',5'), 7.34 (1H, s, H-8), 7.65 (1H, s, H-5), 7.75 (2H, brd, $J = 7.6$, H-2',6'), 8.31 (s, 1H, H-2); ^{13}C NMR (150.95 MHz, CD_3OD): $\delta = 19.8$ (CH_3), 55.2 (CH_3), 55.9 (CH_3), 63.3 (C-6''), 70.4 (C-4''), 73.3 (C-2''), 74.7 (C-5''), 76.8 (C-3''), 101.1 (C-1''), 103.5 (C-8), 104.7 (C-5), 113.7 (C-3',5'), 117.9 (C-10), 122.8 (C-3), 124.3 (C-1'), 130.7 (C-2',6'), 147.6 (C-6), 151.3 (C-9), 151.8 (C-7), 159.1 (C-4'), 153.4 (C-2), 172.2 (C=O), 174.4 (C-4); MS (EI, 70eV) m/z (%): 502 (M) (15), 299 (25), 298 (100), 297 (14), 132 (25), 131 (12), 91 (19), 55 (38), 43 (21), 41 (43); calcd for $\text{C}_{25}\text{H}_{26}\text{O}_{11}$: 502.1621, found: 502.1629; Anal. calcd for $\text{C}_{25}\text{H}_{26}\text{O}_{11}$: C 56.76, H 5.27, found: C 59.88, H 5.56.

3.4. Cell Culture and determination of cytotoxicity

The human cancer cells of the MT-4, CEM-13 (the cells of T-cellular human leucosis), and U-937 (human monocytes) were used in this study. The cells were cultured in the RPMI-1640 medium that contained 10% embryonic calf serum, L-glutamine (2 mmol/L), gentamicin (80 lg/ml), and lincomycin (30 mg/ml) in a CO_2 incubator at 37°C. The tested compounds were dissolved in DMSO and added to the cellular culture at the required concentrations. Three wells were used for each concentration. The cells which were incubated without the compounds were used as a control. Cells were placed on 96-well microliter plates and cultivated at 37°C in 5% CO_2 /95% air for 72 h. The cell viability was assessed through an MTT [3-(4,5-dimethylthiazol-2-yl)-2,5-phenyl-2H-tetrazolium bromide] conversion assay. 1% MTT was added to each well. Four hours later DMSO was added and mixed for 15 min. Optical density (D) of the samples was measured on a BioRad 680 multi-well spectrophotometer (USA) at the wavelength of 570 nm. The 50% cytotoxic dose (CTD_{50}) of each compound (i.e., the compound concentration that causes the death of 50% of cells in a culture, or decreases the optical density twice as compared to the control wells) was calculated from the data obtained. Statistical processing of the results was performed using the Microsoft Excel-2007, STATISTICA 6.0, and GraphPad Prism 5.0 programs. The results are given as an average value $\pm$ a deviation from the average. Reliability of differences (p) was estimated using the Student t test. The differences with $p < 0.05$ were considered as reliable. The experimental results are given as the data average values obtained from three independently conducted experiments. Doxorubicin was used as the positive control.