Cytotoxic Activities of Flavonoid Glycoside Acetates from *Consolida oliveriana*

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Key words
- *Consolida oliveriana*
- Ranunculaceae
- flavonoids
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- cytoxicity

Abstract

The flavonoids kaempferol, quercetin, trifolin, hyperoside 2″- and 6″- acetates, 7-glucotrifolin, biorobin and robinin were isolated from the aerial parts of *Consolida oliveriana*. Their derivatives kaempferol tetraacetate, quercetin pentaacetate, trifolin heptaacetate and hyperoside octaacetate exhibited significant cytotoxicity *in vitro* against three human cell lines HL-60, U937 and SK-MEL-1 while hyperoside 2″-acetate, hyperoside-6″-acetate, glucotrifolin decaacetate and heptamethyltrifolin were inactive.

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Introduction

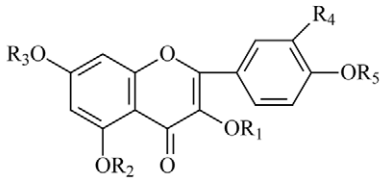
Consolida, a highly specialized genus of Ranunculaceae closely allied to *Delphinium* with its center of diversity in Anatolia, is like its relatives a rich source of alkaloids. Previous studies of the aerial parts of *Consolida oliveriana* (DC) Schrod., a species used medicinally in parts of Anatolia, have concentrated on the isolation of its constituent alkaloids [1], [2]. A large number of publications has dealt with the diterpenoid alkaloids of other *Consolida* species but reports on the flavonoid content of members of this genus are sparse [3], [4]. As part of our ongoing research on flavonoids in the genus *Delphinium* and related genera [5], [6] we have investigated the most polar fraction of the ethanol extract of the aerial parts of *C. oliveriana*. We now report isolation of the flavonoids kaempferol (**1**), quercetin (**2**), trifolin (**3**) [7], hyperoside (**4**) [7], 2″-acetylhyperoside (**5**) [8], 6″-acetylhyperoside (**6**) [7], 7-glucotrifolin (**7**) [9], biorobin (**8**) [10], [11] and robinin (**9**) [12] from this fraction. Compounds were identified by HR-MS as well as ^{1}H- and ^{13}C-NMR spectrometry.

The availability of these relatively polar flavonoids allowed us to follow up earlier work by the Las Palmas de Gran Canaria group on substituent effects governing cytotoxicities with flavonoids [15]. It had been shown that acetylation of certain polyhydroxyflavonoids, among which were quercetin derivatives containing a methoxy group on C-3, increased the antiproliferative activities of the parent compounds against HL-60 and other cell lines. While quercetin itself was modestly active against the HL-60 cell line the earlier results indicated that the increased activities of the acetate derivatives were not due to hydrolysis of the parent compounds. Methylation of kaempferol at the 3-position also appeared to bestow a modest degree of activity since earlier work [14] had shown that kaempferol itself only slightly affects HL-60 cells. It was therefore of interest to determine how peracetylation of not only kaempferol and quercetin but also peracetylation of some of the more highly substituted glycosides from *Consolida oliveriana* affects cytotoxicity. Compounds **1 – 4** and **7 – 9** were therefore converted to the corresponding polyacetates **1a – 4a** and **7a – 9a** and trifolin (**3**) was also converted to heptamethyltrifolin (**3b**). Known compounds **1, 1a, 2, 2a, 3 – 9** were identified by HR-MS as well as ^{1}H- and ^{13}C-NMR spectrometry. The ^{1}H- and ^{13}C-NMR spectra of the new compounds **3a**, **3b**, **4a**, **7a – 9a** as well as the previously unreported ^{13}C-NMR spectra of compounds **7**, **8** and **9** are available as Supporting Information.

Materials and Methods

General

The ^{1}H- and ^{13}C-NMR spectra were measured using a Bruker AMX-400 or a Bruker MAX-500 in-



1a	Kaempferol tetraacetate: R_1, R_2, R_3, R_5 = Ac, R_4 = H
2a	Quercetin pentaacetate: R_1, R_2, R_3, R_5 = Ac, R_4 = OAc
3	Trifolin: R_1 = Gal, R_2, R_3, R_4, R_5 = H
3a	Trifolin heptaacetate: R_1 = Gal Ac, R_2, R_3, R_5 = Ac, R_4 = H
3b	Heptamethyltrifolin: R_1 = Gal OMe, R_2, R_3, R_5 = Me, R_4 = H
4	Hyperoside: R_1 = Gal, R_2, R_3, R_5 = H, R_4 = OH
4a	Hyperoside acetate: R_1 = Gal Ac, R_2, R_3, R_5 = Ac, R_4 = OAc
5	2"-Acetylhyperoside: R_1 = Gal-2"-Ac, R_2, R_3, R_5 = H, R_4 = OH
6	6"-Acetylhyperoside: R_1 = Gal-6"-Ac, R_2, R_3, R_5 = H, R_4 = OH
7	7-Glucotrifolin: R_1 = Gal, R_3 = Glu, R_2, R_4, R_5 = H
7a	Glucotrifolin decaacetate: R_1 = GalAc, R_2, R_5 = Ac, R_3 = GluAc, R_4 = H
8	Biorobin: R_1 = rbinoside, R_2, R_3, R_4, R_5 = H
8a	Biorobin acetate: R_1 = rbinoside Ac, R_2, R_3, R_5 = Ac, R_4 = H
9	Robinin: R_1 = Robinoside, R_3 = Rha, R_2, R_4, R_5 = H
9a	Robinin acetate: R_1 = rbinoside Ac; R_3 = Rha Ac, R_2, R_5 = Ac, R_4 = H

Gal = β-galactopyranosyl; Glu = β-glucopyranosyl; Rha = α-rhamnopyranosyl; rbinoside = α-rhamnopyranosyl-(1→6)-β-galactopyranoside.

strument. FAB5 and exact mass measurements were determined using a Micromass Autospec instrument at 70 eV. ESI-IT mass spectral data were obtained by tandem electrospray ion trap mass spectrometry (ESI-IT-MS) (LCQ Deca XP Plus; ThermoFinnigan; San Jose, CA, USA). Column chromatography was performed over Sephadex LH-20 Pharmacia (ref. 17-0090-01; Upsala, Sweden); silica gel 60 (Merck 230–400 mesh; Darmstadt, Germany) and analytical TLC, Merck Kieselgel 60 F 254. HPLC separations were performed on a JASCO Pu-980 series pumping system equipped with a JASCO UV-975 ultraviolet detector and with a Waters Kromasil 5 (5 mm × 250 mm) column. A Macherey-Nagel VP 250/10 nucleodur Sphinx RP 5 mm column (Düren, Germany) was used for HPLC-RP chromatography; chromatograms were visualized under UV light at 255 and 366 nm and/or sprayed with oleum followed by heating. All solvents were distilled before use; the purity of all compounds was 99.0% as judged by HPLC. Stock solutions of 10 mM flavonoids were made in DMSO and aliquots were frozen at –20 °C. Tissue culture media and standard analyzed grade laboratory reagents were obtained from Sigma (St. Louis, MO, USA).

Plant material

Aerial parts of *Consolida oliveriana* (DC) Schrod. were collected and identified near Pazarkik in eastern Turkey at an altitude of 980 m by Prof. Julian Molero Briones, Department of Botany, Faculty of Pharmacy, University of Barcelona where a voucher specimen (BCF-37 810) has been deposited.

Extraction and isolation

Dried and powdered aerial parts of *Consolida oliveriana* (2.23 kg) were defatted with hexane (6 L) during one month and subsequently extracted repeatedly with 80% EtOH (7 L) at room temperature for two weeks. The extract was filtered and concentrated at reduced pressure. The remaining aqueous layer was exhaustively extracted with *n*-BuOH to give, after removal of the solvent, 36 g of a brown viscous residue. The aqueous layer was concentrated and filtered through a column of Amberlite XAD-2 resin (8 × 40 cm) to remove the polar compounds while the flavonoids remaining on the column were eluted with MeOH (see below).

10 g of the viscous *n*-BuOH extract were fractionated on a 50 × 8 cm column packed with Sephadex LH-20 and eluted with hexane-CH_2Cl_2-MeOH, 1 : 1 : 2, 15 500-mL fractions (S_1 – S_{15}) being collected. Frs S_1 – S_{15} contained mainly alkaloids contaminated by material exhibiting no UV absorption and frs S_4 – S_8 contained mixtures of glycosides. Frs S_9 and S_{10} and frs S_{11} – S_{13} gave after recrystallization from MeOH-EtAc quercetin (**2**, 556 mg), respectively, kaempferol (**1**, 472 mg). Frs S_4 – S_8 were chromatographed over a 40 × 4 cm Sephadex LH-20 column using hexane-CH_2Cl_2-MeOH (1 : 1 : 1), the elution being monitored by TLC analysis. This resulted in three fractions A (430 mg) B (70 mg) and C (55 mg). Fr B afforded after recrystallization from MeOH 90 mg of trifolin (**3**) and fr C 49 mg of hyperoside (**4**). Rechromatography of fr A over silica gel using 40 mL hexane-EtOAc mixtures of increasing polarity afforded from frs 64–77 (hexane-EtOAc 2 : 8) 24 mg of 6″-acetylhyperoside (**6**) after further purification over Sephadex LH-20 (hexane-MeOH-CH_2Cl_2, 2 : 1 : 1). Frs 86–90 (hexane-EtOHc, 1 : 9) yielded 21 mg of 2″-acetylhyperoside (**5**).

The material eluted from the Amberlite XAD-2 column with methanol was further purified over a 50×8 cm column packed with Sephadex LH-20 and eluted with CH_2Cl_2-MeOH (1 : 1), six frs (J_1 – J_6) of 500 mL each being collected. Frs J_1 – J_3 containing mainly alkaloids and other components exhibiting no UV absorption were combined with frs S_1 – S_3. Frs J_4 – J_6 (2 g) were subjected to gel filtration on Sephadex LH-20 using 42 200-mL frs of H_2O-MeOH (1 : 1). Frs 14 – 22 afforded after recrystallization from MeOH 260 mg of 7-glucotrifolin (**7**). Frs 28 – 31 yielded 45 mg of trifolin (**3**), frs 33 – 38 18 mg of hyperoside (**4**). Frs. 5 – 12 on RP-HPLC on a C-18 Sphinx column (100×10 mm, flow rate 1.4 mL min^{-1}) using MeOH-H_2O (1 : 1) afforded 7 mg of robinin (**9**, t_r 12 min) and 5 mg at biorobin (**8**, t_r 16 min).

General method for acetylation

Dry phenolic material was dissolved in the minimum volume of pyridine. Twice the amount of acetic anhydride was added and the solution was allowed to stand overnight at ambient temperature. The mixture was diluted with H_2O and extracted three times with EtOAc. The extract was evaporated under vacuum and the residue containing the polyacetate was further purified by column chromatography over silica gel using hexane-EtOAc as eluent. Mass spectra of the polyacetylated compounds, all gums, are listed below.

Kaempferol tetraacetate (**1a**): EI-MS: *m/z* = 412 (M^+ – $C_2H_3O_2$, 23), 370 (M^+ – 2 $C_2H_3O_2$, 57) 286 (M^+ - 4 $C_2H_3O_2$, 100).

Quercetin pentaacetate (**2a**): HR-EI-MS: *m/z* = 513.0997, calcd. for $C_{25}H_{20}O_2 + H^+$: 513.1033.

Trifolin heptaacetate (**3a**): HR-FAB-MS: *m/z* = 743.1784, calcd. for $C_{35}H_{34}O_{18} + H^+$: 743.1823.

Hyperoside octaacetate (**4a**): HR-FAB-MS: *m/z* = 823.1687, calcd. for $C_{37}H_{36}O_{20}Na$: 823.1698.

Glucotrifolin decaacetate (**7a**): HR-FAB-MS: *m/z* = 1030.2592, calcd. for $C_{47}H_{50}O_{26}$: 1030.2590.

Biorobin nonaacetate (**8a**): HR-FAB-MS: *m/z* = 972.2583, calcd. for $C_{45}H_{48}O_{24}$: 972.2536.

Robinin undecaacetate (**9a**): HR-FAB-MS: *m/z* = 1226.3344, calcd. for $C_{55}H_{63}O_{30}Na$: 1226.3302, m/z = 1202.3344, calcd. for $C_{55}H_{62}O_{30}$: 1202.3326.

Methylation of trifolin

To a solution of **3** (14 mg) in DMSO (2 mL) was added aqueous 50% NaOH (2 mL) and 2 mL of methyl iodide. The mixture was stirred at room temperature overnight, poured into H_2O and extracted with ethyl acetate. The organic extract was dried over Na_2SO_4, filtered and evaporated at reduced pressure. The residue was purified by HPLC (SiO2, EtOAc : hexane 3 : 2, flow rate 2 mL/min) to afford 10 mg of heptamethoxytrifolin **3b** (TR 62 min) as a gum. HR-FAB-MS: *m/z* = 569.1981, 547.2155 calcd. for $C_{28}H_{34}O_{11}Na$: 569.1999, calcd. for $C_{28}H_{35}O_{11}$: 547.2179.

Tumor cell growth assay

Human HL-60 and U 937 cells were obtained from the European Collection of Cell Cultures (Salisbury, UK) and human SK-MEL-1 melanoma cells (DSMZ No ACC 303) from the German Collection of Microorganisms and Cell Cultures, Braunschweig, Germany. HL-60 and U 937 were grown as described [15]. HL-60, U 937 and SK-MEL-1 cells were cultured suspended in RPMI 1640 (Sigma) with L-glutamine cell culture medium supplemented with 10% heat-inactivated fetal bovine serum (FBS) medium and antibiotics (100 units/mL of penicillin and 100 μg/mL of streptomycin) in 250 cm^3 culture vessels at 37 °C in a humidified atmosphere containing 5% CO_2. The cells were routinely kept in the logarithmic growth phase at 0.1 – 0.9×10^6 cells/mL by dilution with fresh medium at least every third day. Cells were counted by a hematocytometer; the viability was always greater than 95% as assayed by the 0.025% trypan blue exclusion method. Stock solutions of the flavonoids were further diluted with culture medium just before use. In all experiments the final concentration of DMSO did not exceed 0.5% (v/v), a concentration which is non-toxic to the cells.

Cytotoxicity assays were performed using a colorimetric 3-[4,5-dimethylthiazol-2-yl-]-2,5 diphenyl-2*H*-tetrazolium bromide (MTT) assay as described [15]. Briefly, 10^4 exponentially growing cells were seeded on 96-well microculture plates with various flavonoid concentrations (0.1 – 10 μM) in a 200 μL volume for 72 h. Surviving cells were detected based on their ability to metabolize 3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyltetrazolium bromide (Sigma) into formazan crystals. The optical density at 570 nm was used as a measure of cell viability. Cell survival was calculated as fraction of cells relative to control for each point: Cell survival (%) = mean absorbance in treated cells/mean absorbance in control wells × 100. Concentrations inducing 50% inhibition of cell growth (IC_{50}) were determined graphically for each experiment using the curve fitting routine of the computer software Prism 2.0™. (GraphPad) and the equation derived by DeLean et al. Etoposide (Sigma) was used as a positive control (IC_{50} = 0.32 ± 0.02 mM in HL-60 cells, IC_{50} = 1.25 ± 0.25 mM in U937 cells, not determined in SK-MEL-1 cells).

Supporting information

^{1}H- and ^{13}C-NMR spectra of the new compounds **3a**, **3b**, **4a**, **7a-9a** as well as the previously unreported ^{13}C-NMR spectra of compounds **7**, **8** and **9** are available as Supporting Information.

Results and Discussion

Kaempferol tetraacetate (**1a**), quercetin pentaacetate (**2a**), the polyacetates **3a**, **4a**, the monoacetates **5** and **6** as well as the methylated derivative **3b** were screened for *in vitro* cytotoxicity against the human myeloid leukemia HL-60 and U937 cell lines and against the human melanoma SK-MEL-1 cell line. The results, given in concentrations causing 50% growth inhibition (IC_{50}), are summarized in **Table 1**. Polyacetates **1a**, **2a**, **3a** and **4a** inhibited growth and viability of human HL-60 and U937 cells in a dose-dependent manner as determined by the 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT dye re-

Table 1 Effect of flavonoid acetates on the growth of three human cell lines

Compound	IC_{50} (μM)		
	HL-60	U937	SK-MEL-1
1a	45 ± 3	48 ± 17	37 ± 8
2a	38 ± 6	25 ± 11	58 ± 7
3a	21 ± 8	10 ± 2	15 ± 2
3b	88 ± 9	> 100	> 100
4a	15 ± 1	19 ± 2	23 ± 2
5	> 100	> 100	> 100
6	> 100	> 100	> 100
7a	> 100	> 100	> 100

Cells were cultured for 72 h. IC_{50} values were calculated as described in the Materials and Merthods section. Data shown represent the mean ± SEM of 2 – 3 independent experiments with three determinations in each.

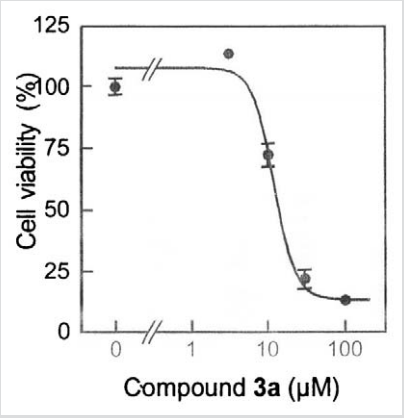


Fig. 1 The effect of trifolin heptaacetate on human U 937 cell viability. Cells were cultured in the presence of the indicated doses of **3a** for 72 h; thereafter cell viability was determined by the MIT assay as described. Results of a representative experiment are shown. Each point represents the average of triplicate determinations.

duction) assay. An example of a dose-response curve is shown in ◉ **Fig. 1**. Similar results were obtained with the human melanoma cell line. Glycoside polyacetates **3a** and **4a** were most effective against all lines, although not spectacularly so. Quercetin pentaacetate (**2a**) was also modestly active but no more so that quercetin itself [13] in agreement with the earlier suggestion that antiproliferative activity may actually decrease with increasing installation of acetate functions on C-5 and C-4′ of the flavone nucleus. Kaempferol tetraacetate (**2a)**, on the other hand, displayed increased, but still only very modest, activity compared with kaempferol itself [14]. Finally, as permethylation of betuletol 3-methyl ether, the most active naturally occurring flavonoid studied by our group earlier, had eliminated the compound's activity we were also interested in the effect of permethylation of **3** whose peracetate **3a** was the most active of our compounds. Indeed, the relatively non-polar heptamethyltrifolin (**3b**) containing no acetate function was inactive as were the isomeric monoacetylhyperosides **5** and **6** and, somewhat surprisingly, glucotrifolin acetate. However, it needs to be pointed out that none of the flavonol derivatives included in the present study were 3-methyl ethers which served as the basis for the generalizations in the earlier study [13].

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