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Potential Antitumor Agents: A Cytotoxic Cardenolide from *Coronilla varia* L.

MARTIN WILLIAMS * and JOHN M. CASSADY ˣ

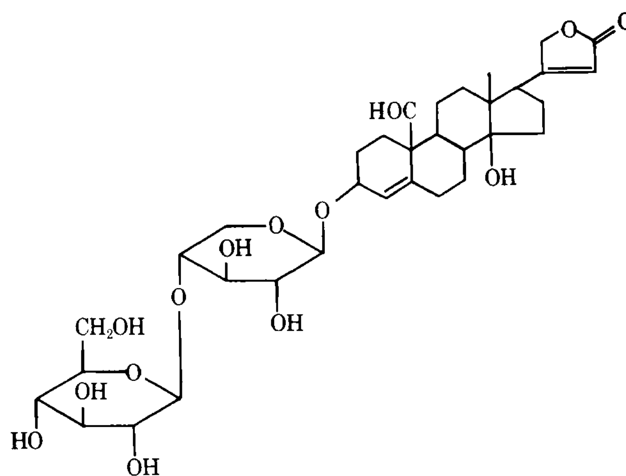
Abstract □ An alcoholic extract of the seeds of *Coronilla varia* L. showed inhibitory activity against KB cells in culture and was fractionated through a series of partitions, column chromatography, and preparative layer chromatography to yield hyrcanoside, daphnoretin, scopoletin, and umbelliferone. Hyrcanoside was also tested in the PS mouse leukemia assay and showed borderline activity.

Keyphrases □ *Coronilla varia*—seeds, alcoholic extract, cardenolide and three coumarins isolated and identified, cytotoxic and antileukemic activity screened □ Cardenolides—hyrcanoside isolated from *Coronilla varia* seeds, cytotoxic and antileukemic activity screened □ Coumarins—isolated from alcoholic extract of *Coronilla varia* seeds, cytotoxic and antileukemic activity screened □ Cytotoxic compounds—alcoholic extract of *Coronilla varia* seeds, cytotoxic and antileukemic activity screened □ Antitumor agents, potential—alcoholic extract of *Coronilla varia* seeds, cardenolide and three coumarins isolated and screened □ Antileukemic agents, potential—alcoholic extract of *Coronilla varia* seeds, cardenolide and three coumarins isolated and screened

An alcoholic extract of the seeds of *Coronilla varia* L. (var. Penngift) (family Leguminosae) was found to have inhibitory activity against cells derived from human carcinoma of the nasopharynx (KB)[1]. Systematic fractionation of the extract led to the isolation of two cardenolides, one of which was identified as hyrcanoside (I); three coumarins, daphnoretin (II), scopoletin (III), and umbelliferone (IV); and two unidentified compounds. Compounds I–IV have been reported to occur in Russian varieties of *C. varia* (1, 2), and III and IV have also been shown to occur in *C. varia* (var. Penngift) (3). There is considerable interest in varieties of *C. varia* as potential forage crops (4, 5) and in soil erosion control, and recent publications dealing with toxic constituents have appeared (6, 7).

DISCUSSION

An outline of the general fractionation and chromatographic procedures are given in the *Experimental* section, and the biological data



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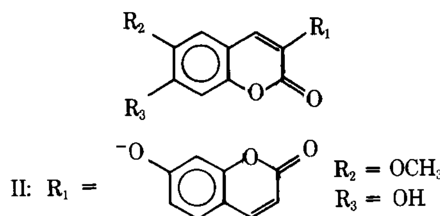

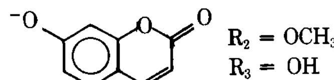

are presented in Table I. The concentrated alcoholic fraction (C) from percolation of the powdered seed material with ethanol was partitioned between chloroform and water, resulting in concentration of the cytotoxic activity in the aqueous phase (C). Further partitioning of the aqueous phase with mixtures of chloroform and ethanol in various proportions and with 1-butanol brought the activity into the organic phase. Chromatography of Fraction E on silicic acid yielded the coumarins and an unidentified cardenolide with an R_f of 0.56. Chromatography of Fractions G and H on silicic acid, followed by preparative layer chromatography on silica gel, gave hyrcanoside (I) from Fraction G and two unidentified compounds, R_f 0.49 and 0.39, from Fraction H.

Solid 1 (L) obtained from the chloroform fraction (E) was identified as daphnoretin (II) on the basis of a comparison of physical and spectral properties with literature values. Solid 2 (M) and Solid 3 (N) were identified as scopoletin (III) and umbelliferone (IV) by direct comparison of physical and spectral properties with those of authentic

[1] The cell culture and mouse leukemia assays were performed under the auspices of the Division of Cancer Treatment, Drug Research and Development, National Cancer Institute, according to the procedure given in *Cancer Chemother. Rep.*, **25**, 22(1962). In KB, a purified substance is considered active if it shows an $ED_{50} \leq 4$ µg/ml. In PS, a compound is considered active if it shows an increase in lifespan (ILS) of ≥25% relative to controls.

Table I—Cytotoxicity of Fractions from *C. varia* (11.0 kg, Seeds)

	Fraction	Weight, g	ED_{50} in 9KB, μg/ml
A	Hexane	500	100.00
B	Precipitate 1	36	45.0
C	Aqueous I		8.3
D	Precipitate 2	15	41.0
E	Chloroform	16	28.0
F	Organic I	17	3.1
G	Organic II	58	2.5
H	Organic III	131	2.6
I	Precipitate 3	36	2.6
J	Butanol	125	5.5
K	Aqueous II	950	100.0
L	Daphnoretin	0.263	43.0
M	Scopoletin	0.055	100.0
N	Umbelliferone	0.050	33.0
O	Unidentified cardenolide (R_f 0.56)	0.10	2.5
P	Hyrcanoside[a]	0.96	0.1, 0.7

[a] In the PS system, the percent T/C at 1.25 mg/kg was 133.

samples of these compounds. Solid 4 (O) was only tentatively identified as a cardenolide-type compound on the basis of its positive Kedde's reaction, characteristic NMR pattern, and IR spectrum.

Solid 5 (P), obtained from the organic Fraction II (G), was identified as hyrcanoside (I) on the basis of comparison of its physical and spectral properties with that of an authentic reference sample of hyrcanoside[2]. There was no depression in the melting point when a mixed melting point was determined with P and reference hyrcanoside, and the IR spectra of both P and the reference hyrcanoside were identical.

Organic Fraction III was not investigated further, even though active since a major component of the fraction was hyrcanoside. Precipitate 1 (B), obtained during the concentration of the ethanol extract, and Precipitate 2 (D), obtained during the concentration of the chloroform extract, were both found to be crude samples of daphnoretin (II). Precipitate 3 (I), obtained during the concentration of the 1-butanol extract, also was found to be a crude sample of hyrcanoside (I).

Biological activity in the KB system was determined for all fractions obtained through the general fractionation scheme and for all solids obtained during the chromatographic procedures. Since fractions containing the cardenolide glycosides were more active than the others, the cytotoxicity of the plant appears to be due to the presence of these glycosides in the seed. Hyrcanoside, the major glycoside present in the seeds, showed the maximum activity and probably accounted for most of the activity of the total seed extract.

EXPERIMENTAL[3]

Plant Material[4]—The seeds of *C. varia* L. (var. Penngift) (F.C. 40619) were powdered through an 80-mesh screen.

Extraction and Initial Separation—The powdered seed material (11.0 kg) was first extracted with *n*-hexane, and removal of the solvent left 500 g of hexane fraction. The defatted seed powder was then percolated at room temperature with 70% ethanol until the extract was almost colorless. Upon concentration of the ethanol extract under reduced pressure at about 80°, 36 g of Precipitate 1 (B) was obtained. The aqueous extract obtained after removal of the ethanol was then partitioned between chloroform and water. The aqueous phase was extracted three additional times with chloroform.

The combined chloroform extract, upon concentration, yielded 15 g of Precipitate 2 (D) and 16 g of chloroform Fraction E. The aqueous Fraction I (C) was then extracted four times with chloroform–ethanol (8.5:1.5). Upon concentration of the combined extract, it yielded 17 g of organic Fraction I (F). The aqueous phase was further extracted four times with chloroform–ethanol (2:1), which yielded 58 g of organic Fraction II (G). The aqueous phase was then extracted with chloroform–ethanol (1:1), which yielded 131 g of organic Fraction III (H). Finally, the aqueous phase was extracted four times with 1-butanol. The combined butanol extract, upon concentration, yielded 36 g of Precipitate 3 (I) and 125 g of the 1-butanol fraction (J). The aqueous phase was then freeze dried to yield 950 g of aqueous Fraction II (K).

Isolation of Daphnoretin (II), Scopoletin (III), and Umbelliferone (IV) from Fraction E—A sample of about 3 g of chloroform Fraction E was placed in a glass column previously packed with silicic acid (100 mesh). The elution was carried out with 1000 ml each of the following solvent mixtures: 5, 10, 15, 20, 25, 30, and 50% methanol in chloroform. A total of 425 tubes was collected and then combined into 10 fractions on the basis of TLC[5] results. Four of these fractions (1, 3, 5, and 8) were homogeneous and, upon concentration, yielded 52 mg of Solid 1 (L), 11 mg of Solid 2 (M), 10 mg of Solid 3 (N), and 26 mg of Solid 4 (O), respectively.

Solid 1 (L) and Daphnoretin (II)—Recrystallization from tetrahydrofuran–methanol yielded daphnoretin, mp 246–247°; IR ν_{max} (KBr): 3400 (OH), 3050 (aromatic CH), 2900 (aliphatic CH), 1715 (unsaturated lactone), ≈1600 (olefin), and 1275 (C—O, C=O stretch) cm^{-1}; NMR ($CDCl_3$): δ 3.90 (3H, singlet, CH_3O attached to aromatic system), 6.50 and 8.25 (each 1H, doublets, coupled vinyl protons), 7.25 and 7.35 (each 1H, doublets, coupled aromatic protons), and 7.05, 7.85, 7.95, and 8.10 (each 1H, singlets, aromatic protons); UV λ_{max} (CH_3OH) (log ε): 227 (4.17), 266 (3.85), 323 (4.28), and 345 (4.29) nm. The mass spectrum showed the molecular ion at *m/e* 352. Comparison (IR, UV, NMR, and mass spectra) of the isolated Solid 1 (L) with the literature values for daphnoretin showed that they were identical.

Solid 2 (M) and Scopoletin (III)—Recrystallization from acetone yielded scopoletin, mp 203–204°; IR ν_{max} (KBr): 3425 (OH), 3025 (aromatic CH), 2900 (aliphatic CH), 1710 (unsaturated lactone), ≈1600 (olefin), and 1275 (C—O, C=O stretch) cm^{-1}; NMR ($CDCl_3$) (dimethyl sulfoxide): δ 3.26 (1H, broad singlet, OH), 4.30 (3H, singlet, CH_3O attached to aromatic system), 6.30 and 8.10 (each 1H, doublets, coupled vinyl protons), and 7.00 and 7.70 (each 1H, singlets, aromatic protons); UV λ_{max} (CH_3OH) (log ε): 230 (4.23), 255 (2.76), 295 (2.81), and 345 (3.97) nm. The mass spectrum showed the molecular ion at *m/e* 192. Comparison (IR, UV, NMR, and mass spectra and mixed melting point) of the isolated Solid 2 (M) with authentic scopoletin showed that the two were indistinguishable.

Solid 3 (N) and Umbelliferone (IV)—Recrystallization from water yielded umbelliferone, mp 223–224°; IR ν_{max} (KBr): 3300 (OH), 1705 (unsaturated lactone), ≈ 1600 (olefin), and 1275 (C—O, C=O stretch) cm^{-1}; NMR ($CDCl_3$) (dimethyl sulfoxide): δ 3.22 (1H, broad singlet, OH), 6.21 and 8.15 (each 1H, doublets, coupled vinyl protons), 6.95 and 7.70 (each 1H, doublets, coupled aromatic protons), and 7.00 (1H, singlet, aromatic proton); UV λ_{max} (CH_3OH) (log ε): 244 (3.56), 257 (3.43), 322 (4.25), and 328 (4.27) nm. The mass spectrum showed the molecular ion at *m/e* 162. Comparison (IR, UV, NMR, and mass spectra and melting point) of the isolated Solid 3 (N) with authentic umbelliferone showed that the two were indistinguishable.

Isolation of Hyrcanoside (I) from Fraction G—A sample of about 10 g of the organic Fraction II (G) was placed in a glass column packed with silicic acid (100 mesh). Elution was first carried out with 1000 ml of chloroform followed by 1000 ml each of the following solvent mixtures: 10, 25, 50, and 75% ethanol in chloroform and then with 1000 ml each of 5 and 25% aqueous ethanol. A total of 540 tubes was collected and combined into 14 fractions on the basis of the TLC results. These fractions were then pooled into active (fractions 2–6) and inactive fractions on the basis of the biological testing data.

The combined active fraction was subjected to preparative layer chromatography. A sample of about 0.48 g was streaked on a precoated 2-mm thick preparative layer silica gel G plate with fluorescent indicator. The plate was developed in a chromatographic tank containing 100 ml of chloroform–ethanol (2:1). After development, the plate was examined under UV and four bands were marked. Each

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[2] The authors are grateful to Dr. N. F. Komissarenko, Karkov Chemical and Pharmaceutical Research Institute, Lenningrad, USSR, for an authentic sample of hyrcanoside. Comparison samples of the other compounds were available in collection at Purdue University.

[3] Melting points were determined on a Mel-Temp capillary melting-point apparatus and are uncorrected. The IR spectra were obtained as potassium bromide pellets on a Beckman IR-33 spectrophotometer. UV spectra were taken in methanol on a Perkin-Elmer model Coleman 125 spectrophotometer. NMR spectra were recorded on a Jeol 100-MHz spectrometer. Low-resolution mass spectra were obtained on a Hitachi RMU-6A spectrometer.

[4] Obtained from Dr. Paul R. Henson, Crops Research Division, Agricultural Research Service, U.S. Department of Agriculture, Beltsville, Md.

[5] Analytical TLC was done on Brinkmann precoated silica gel G plates (0.25 mm thick) without fluorescent indicator. The solvent system of chloroform–ethanol (2:1) was used as the mobile phase. After development, the plate was sprayed with 3,5-dinitrobenzoic acid reagent.

band was scraped separately, extracted with methanol, and concentrated. The extract from the second band yielded 160 mg of Solid 5 (P).

Solid 5 (P) and Hyrcanoside (I)—Recrystallization from methanol yielded hyrcanoside, mp 205–208°; IR ν_{max} (KBr): 3430 (OH), 2940 (aliphatic CH), 2870 (aldehyde CH), 1740 (aldehyde carbonyl), 1720 (unsaturated lactone), and 1620 (olefin) cm^{-1}. The NMR (dimethyl sulfoxide) spectrum was complicated due to the presence of numerous methylene and hydroxy protons. However, it clearly showed the presence of the methyl and vinyl protons and also the aldehyde proton; UV λ_{max} (CH_3OH) (log ϵ): 230 (4.10), 290 (1.65), and 322 (1.54) nm. Comparison (IR, UV, NMR, mixed melting point, and cochromatography in three different solvent systems[6]) of the isolated Solid 5 (P) with an authentic sample of hyrcanoside showed the two to be indistinguishable.

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[6] The solvent systems used were: 1, chloroform–ethanol (2:1); 2, chloroform–tetrahydrofuran–*N*-methylformamide–methanol (50:50:7:18); and 3, chloroform–acetone–methanol (6:2:2).

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Pharmacology of Malnutrition III: Binding of Digoxin to Normal and Kwashiorkor Serum

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Abstract ▫ Digoxin binding to normal and kwashiorkor serum was studied and found to be inferior in the latter. Digoxin should be used with care in hypoalbuminemic patients.

Keyphrases ▫ Digoxin—serum protein binding, normal and kwashiorkor serum ▫ Protein binding—digoxin, normal and kwashiorkor serum ▫ Malnutritic serum—digoxin binding, compared to normal serum ▫ Cardiotonic agents—digoxin, serum protein binding, normal and kwashiorkor serum

Circulating digoxin is known to be approximately 25% bound and 75% free in serum, the sole binding protein being albumin (1). Cardiac disease, associated with rheumatic fever, hypertension, and cardiomyopathy, is commonly seen in developing countries with concomitant poor nutrition. An *in vitro* study was thus performed to assess the effect of hypoalbuminemia on digoxin binding.

EXPERIMENTAL

Normal serum (albumin concentration of 3.75 g/100 ml) was obtained, with consent, from a patient hospitalized for an orthopedic disorder. Pooled kwashiorkor serum (albumin concentration of 2.4 g/100 ml) was obtained from several children admitted to a metabolic unit for various studies. Such serum was collected prior to the child's receiving therapy to avoid the presence of any competitive binders.

Tritiated digoxin[1] had a specific activity of 10,000 mCi/mmole and

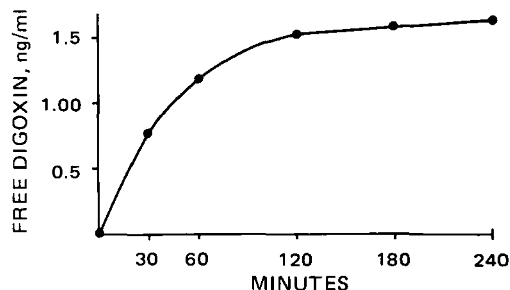

Figure 1—*Curve showing the equilibration time for ^{3}H-digoxin to be 180 min, there being no statistically significant increase in the free digoxin concentration between the 3- and 4-hr samples* ($p > 0.2$).

a mass concentration of 88,000 µg/ml. This solution was diluted with deionized water so that ultimately aliquots of the diluted solution could be added to 5-ml batches of normal and kwashiorkor serum, producing final digoxin concentrations of 0.1, 0.2, 0.3, 0.4, 0.5, 1.0, and 5 ng/ml.

The serum was then dialyzed by equilibrium dialysis (2) at 37°, using 0.4 ml of serum for each dialysis. The equilibration time for ^{3}H-digoxin was 180 min (Fig. 1), but in practice each run was extended to 240 min. Ten dialyses were performed at each concentration; the digoxin concentrations in the original serum, dialyzed serum, and dialyzate were assessed by liquid scintillation counting[2]. The efficiency for tritium was 38.6%, and each specimen was assayed to give a counting error of 0.7% or less. The amount of digoxin in each sample was calculated directly from these data.

[1] Radiochemical Centre, Amersham, United Kingdom.

[2] Packard Tri-Carb liquid scintillation counter.