Tumor Inhibitors VII. Podophyllotoxin, the Active Principle of *Juniperus virginiana*

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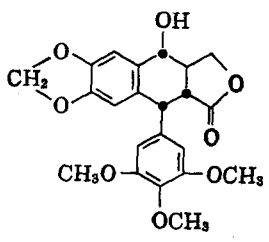
An alcohol extract of *Juniperus virginiana* L. was found to show tumor-inhibitory activity against Sarcoma 180 in mice and against human carcinoma of the nasopharynx carried in cell culture (KB). Systematic fractionation of the extract led to isolation and characterization of podophyllotoxin as the active principle.

IN THE COURSE of our continuing search for tumor inhibitors from plant sources, an alcoholic extract of *Juniperus virginiana* L.[1] was found to have significant inhibitory activity against Sarcoma 180 in mice.[2,3] An earlier study had shown that podophyllotoxin (I) was responsible for the inhibitory activity of the extract of *J. virginiana* L.



I

against the more sensitive Sarcoma 37 mouse tumor system (1). However, podophyllotoxin failed to show acceptable activity in earlier evaluations against Sarcoma 180 (2). Consequently, a systematic study aimed at isolation of the Sarcoma 180-inhibitory principle of *J. virginiana* L. was undertaken.

The preliminary fractionation of the alcohol extract is summarized in Fig. 1. Fraction E material was treated with 1:1 benzene–ethanol, and the soluble portion was subjected to a gross chromatographic separation on alumina. Further careful rechromatography on alumina, collecting fractions which were analyzed by thin-layer chromatography, led to isolation of podophyllotoxin (F) from fractions eluted by chloroform.

The Sarcoma 180 assay data for fractions obtained in a typical experiment are reported in Table I. The evaluation of assay results by the CCNSC on a statistical basis in sequential testing is such that a material is considered active if it causes reduction of tumor weight to 42% or less.[4] All fractions not containing podophyllotoxin were found to be inactive. The latter observations support the view that podophyllotoxin was principally responsible for the Sarcoma 180-inhibitory activity of the alcohol extract of *J. virginiana* L.

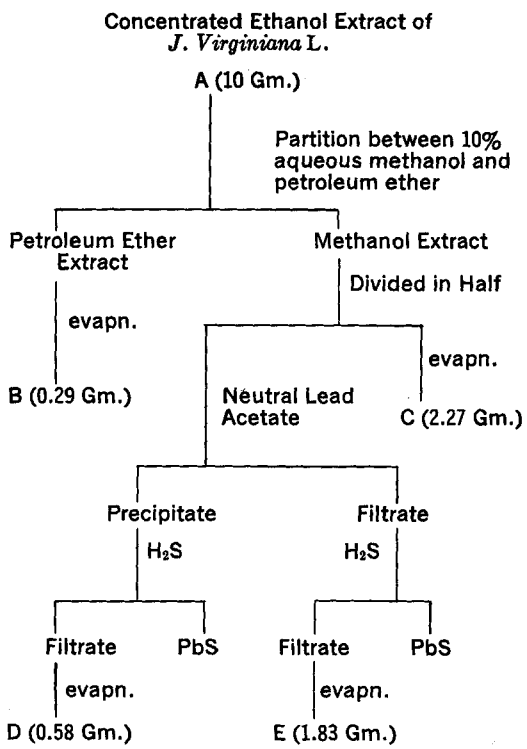


Fig. 1.—Flow sheet for fractionation of tumor-inhibitory extract from *J. virginiana* L.

The alcohol extract also showed significant inhibitory activity when tested *in vitro* against cells derived from human carcinoma of the nasopharynx (KB), and the assay data are reported in Table II. Evaluation of the tissue culture assay results by the CCNSC is such that a material is considered active if the ED_{50} (dose inhibiting growth to 50% of control growth) equals or is less than 4 mcg./ml.[4] The fractions containing podophyllotoxin were found to show the highest cytotoxicity. The results indicate that podophyllotoxin is the major cytotoxic principle of the extract of *J. virginiana* L.

EXPERIMENTAL

Melting points were determined on a Fisher-Johns melting point apparatus. Infrared spectra were determined in chloroform solution on a Beck-

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[1] Leaves and twigs, gathered in Maryland in September 1963. The authors acknowledge the receipt of the dried plant material from Dr. Robert E. Perdue, Jr., U. S. Department of Agriculture, Beltsville, Md., in accordance with the program developed with the U.S.D.A. by the Cancer Chemotherapy National Service Center.

[2] Assays were performed under the auspices of the Cancer Chemotherapy National Service Center. The procedures were those described in *Cancer Chemotherapy Rept.*, 25, 1(1962).

[3] Subsequent studies have demonstrated that alcoholic extracts of other *Juniperus* species (*e.g.*, *J. occidentalis* Hook from California, *J. scopulorum* Sarg. from New Mexico, *J. procera* Hochst. from Ethiopia) show similar activity. It is pertinent to note that the occurrence of podophyllotoxin in a number of *Juniperus* species has been reported previously (1, 3).

[4] For further details compare protocols described in the reference in *Footnote 2*.

TABLE I.—ACTIVITY OF FRACTIONS FROM *J. virginiana* L. AGAINST SARCOMA 180

Fraction	Dose, mg./Kg.	Survivors	Animal Wt. Change Diff. (T-C)	Tumor Wt., mg. (Test/Control)	T/C
A	600	2/4	−1.8	235/1130	Toxic
	400	4/4	+0.3	336/1130	29
	264	4/4	−1.5	679/1130	60
B	50	4/4	−0.6	755/1266	59
	25	4/4	−0.1	541/1266	42
	12.5	4/4	−0.1	1439/1266	113
C	550	1/4	−1.6	260/1266	Toxic
	225	4/4	−1.1	715/1266	56
	112	4/4	−1.3	916/1266	72
D	135	3/4	0.0	435/409	106
	67.5	4/4	+0.5	446/409	109
	33.8	4/4	+0.2	534/409	130
E	385	0/4	...	...	Toxic
	192	2/4	−3.6	643/1266	Toxic
	96.2	4/4	−1.5	801/1266	63
F	8	1/4	−6.0	100/1527	Toxic
	4	3/4	−5.1	397/1527	25
	2	4/4	−2.5	934/1527	61

TABLE II.—CYTOTOXICITY OF FRACTIONS FROM *J. virginiana* L.

Fraction	ED_{50}, mcg./ml.
A	0.23
B	2.2
C	0.25
D	2.7
E	0.17
F	0.022; 0.00095

man IR5A infrared spectrophotometer. The petroleum ether used was skellysolve B, b.p. 60–68°. Evaporations were carried out at temperatures less than 40°.

Extraction and Preliminary Fractionation of J. virginiana.—Coarsely ground leaves and twigs of *J. virginiana*[1] (1.0 Kg.) were twice extracted continuously with 95% ethanol for 5 hr., and the ethanol extract was concentrated under water pump-pressure to a thick dark syrup (A, 198 Gm.). A portion of the extract (10.0 Gm.) was partitioned between petroleum ether (200 ml.) and 10% aqueous methanol (200 ml.); after retaining half of the 10% aqueous methanol layer, the two solutions were evaporated under reduced pressure (B, 0.29 Gm., and C, 2.27 Gm., respectively). The remaining 10% aqueous methanol layer was treated with a saturated methanol solution of neutral lead acetate to complete precipitation. The precipitate was removed by centrifuging and washed with a little methanol before suspending in methanol and bubbling in hydrogen sulfide. The lead sulfide was filtered after precipitation had been completed, and the filtrate evaporated to a gummy residue (D, 0.58 Gm.). The supernatant liquid from lead acetate precipitation was also treated with lead sulfide, filtered, and evaporated (E, 1.83 Gm.).

Isolation of Podophyllotoxin.—A larger batch of fraction E (52.7 Gm.) was prepared from the crude extract (130 Gm.) and a portion (47.7 Gm.) was dissolved in ethanol (200 ml.) and treated with benzene (200 ml.). The bright yellow resinous precipitate (15.6 Gm.) was filtered, and the filtrate was added to a column of Merck alumina (1 Kg.) and washed with 1:1 benzene–ethanol until no further color was removed. Dilute acetic acid in methanol (0.5%) eluted only a small quantity (0.11 Gm.) of material, and the column was stripped with increasing concentrations of acetic acid in methanol (up to 20%), yielding a gummy residue (25.6 Gm.). The total benzene–ethanol eluted solid (3.15 Gm.) was dissolved in chloroform, added to a column of alumina (80 Gm.), and chromatographed with chloroform. Fractions were collected and examined by thin-layer chromatography on Silica Gel G using 35% acetone in chloroform as developing solvent and antimony pentachloride spray reagent. The fractions containing podophyllotoxin were combined, evaporated to dryness (0.102 Gm.), and crystallized from benzene. The colorless crystalline product (F, 19 mg.) was characterized as podophyllotoxin by mixed melting point, mixed thin-layer chromatography, and infrared spectral comparison with an authentic sample.[5] The solid recovered by evaporation of the mother liquor (81 mg.) was shown to contain some podophyllotoxin by thin-layer chromatography.

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[5] The authors thank Dr. J. L. Hartwell, Special Assistant to the Chief, CCNSC, for Natural Products, and the National Cancer Institute, for the authentic sample of podophyllotoxin.