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Phytochemical Investigation of *Arctostaphylos columbiana* Piper and *Arctostaphylos patula* Greene (*Ericaceae*)

By GEORGE H. CONSTANTINE, JR., PHILIP CATALFOMO, KIRTI SHETH, and LEO A. SCIUCHETTI

Improved methods of extraction and new techniques of isolation and identification of 11 components from the title plants were conducted. A solvent mixture of diverse polarity extracted four polycyclic compounds, a hydrocarbon, two flavonoids, and four phenols. Another phenolic, assumed to be widespread in the family, could not be shown to be present. Antibacterial and antifungal activity was found in the extracts obtained from both plants.

ETHNOBOTANICAL and early medical literature have often cited the usefulness of various *Arctostaphylos* species for a variety of medicinal uses. Members of the genus have been used by the Pacific Northwest Indians. These include their uses as healing poultices and as a curative for severe colds (1), as an eyewash and styptic (2), as a food (3–5), and as smoking tobacco (6). In fact, Reagan (7) reported drunken and erratic behavior of Indians who smoked the leaves of *A. uva-ursi* and stated that it was smoked as a medicine and in religious ceremonies.

The most widely known member of the genus, *A. uva-ursi*, reportedly was used by the early Greeks and Romans (8). Griffith (9), however, stated that DeHaen in the 18th century be given credit for its use as a remedy in kidney and bladder diseases. *A. uva-ursi* was official in the first U.S.P. in 1820, remained in the N.F. until 1946, and still is found in a few proprietary urinary tract remedies.

Previous phytochemical examination of the genus has been mainly restricted to *A. uva-ursi*. Arbutin was isolated in 1852 (10). Rosenthaler (11) isolated methylarbutin in 1927 and Britton and Haslam (12) have very recently identified three galloyl esters of arbutin. Two flavonoid compounds, isoquercitrin (13) and hyperin (14), have also been isolated. Hermann (14) reportedly found "*A. uva-ursi* tannin" to consist of gallic acid, ellagic acid, and glucose, whereas Britton and Haslam (12) have reported the tannin to consist of penta- to hexa-*o*-galloyl-β-D-glucose derivatives. Ibrahim (15) recently identified *o*-pyrocatechuic acid in *A. uva-ursi* as well as in other members of the family. The triterpenoid ursolic acid was first isolated from *A. uva-ursi* (10). The corresponding alcohol, uvaol, was also isolated from this plant (16).

Members of the genus have not been thoroughly investigated for biological activity. *A. uva-ursi* extracts were found to inhibit Ehrlich ascites tumor growth (17). Antibacterial activity has also been exhibited against *B. subtilis*, *E. coli*, and *S. aureus* (18). *A. patula*

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extracts were reported to inhibit cultures of *Mycobacterium tuberculosis* (19).

EXPERIMENTAL

Plant Collection and Treatment

A. columbiana and *A. patula* were gathered in Marion County, Oreg., during the fall of 1965.[1] The plants were allowed to air-dry on greenhouse benches for not less than 2 weeks. They were then divided into their morphological parts, *i.e.*, leaves, roots, and stems, and ground to a course powder in an Abbé mill. The powdered material was then stored in air-tight plastic bags in the dark until time for analysis.

Preliminary Investigation.—Five-gram samples of each plant part were subjected to selective solvent extraction using petroleum ether U.S.P., chloroform U.S.P., alcohol U.S.P., and distilled water, successively. The extracts were then chromatographed (ascending technique) on Whatman No. 1 paper with the solvent systems *n*-butanol–acetic acid–water (4:1:5, organic phase) and *n*-butanol–pyridine–water (2:1:1). Antimony trichloride (10% w/w in chloroform) detected positive triterpene/sterol compounds (pink to red) in all except the aqueous fractions of both plants. Dragendorff's spray reagent demonstrated negative results in all of the extracts. Ferric chloride (1% in ethanol) and phosphomolybdic acid (5% in methanol) detected the presence of phenolic compounds. The alcoholic and aqueous extracts of *A. patula* contained 11 phenolic compounds, whereas, seven phenolic compounds were found in *A. columbiana*.

Additional testing was conducted on all of the selective solvent fractions from above. Anisaldehyde and Liebermann-Burchard spot tests also indicated the presence of triterpene/sterols in all but the aqueous fractions. Dragendorff's, Mayer's, and Wagner's reagents indicated the absence of alkaloid substances. Ferric ammonium sulfate (3% in water) and vanillin (1% in HCl) reagents also indicated the presence of phenolic compounds in the alcoholic and aqueous fractions.

Extraction and Fractionation.—One kilogram of the leaves from each plant was extracted individually for 72 hr. in a Soxhlet apparatus with 6 L. of methanol–skelly B (1:1). The dark green residue obtained after concentration in a flash evaporator was dissolved in skelly B–water (1:1) and placed in a large separator. On standing, a copious green interphase developed which was removed along with the organic phase and washed with water (3 × 125 ml.). The aqueous phase and washings (fraction II) were set aside for later examination of phenolic constituents. The organic phase (fraction III) was examined for triterpenes and sterols.

Triterpenes and Sterols.—Samples of fraction III were saponified according to the method of Huneck and Snatzke (20). Refrigeration of the acidified mixture yielded a copious greenish-yellow precipitate (fraction IV). The mother liquor was subjected to liquid–liquid extraction for 48 hr. using hexane (analytical reagent) as the lighter solvent. The soluble components were designated fraction V. Fractionation steps are summarized in Scheme I.

[1] Identified by Dr. K. L. Chambers, Professor and Curator of the Oregon State University Herbarium. Voucher specimens of both plants are located at same.

Isolation of Ursolic Acid.—Fraction IV gave a positive Liebermann–Burchard test (red to immediate purple) and anisaldehyde test (red). Thin-layer chromatography (TLC)[2] of fraction IV employing chloroform–acetone (9:1) as a solvent system and antimony trichloride as a spray reagent indicated only one spot (R_f 0.14) identical to ursolic acid which was also chromatographed on the same plate. The crude material (fraction IV) after repeated washings with cold chloroform was crystallized from ethanol. Fine needles of ursolic acid were obtained, m.p. 277–280°.[3] Literature values for ursolic acid range from 224° (21) to 291° (22). Superimposable infrared spectra were obtained in comparing the isolate with ursolic acid.

Column Chromatography of Fraction V.—Fraction V, when chromatographically screened on a thin-layer plate (chloroform–acetone, 9:1), revealed the presence of three compounds when sprayed with antimony trichloride spray reagent (R_f 0.39, 0.49, and 0.58). An 8-Gm. sample of fraction V was mixed with 10 Gm. of activated alumina[4] and placed atop a 300-Gm. alumina column (50 × 240 mm.). The column was eluted with successive solvents of increasing polarity. The results are summarized in Table I.

Fraction A upon drying gave a white lustrous product (1.1 Gm.) which was recrystallized from acetone, melted sharply at 64°, and was tentatively identified as nonacosane, m.p. 64–65°. Fractions B and C were combined, treated as above, and yielded additional nonacosane (0.7 Gm.).

Thin-layer chromatography of fraction D revealed the presence of only one compound which had an R_f value comparable to that of β-amyrin. Evaporation *in vacuo* yielded a semisolid yellow residue (0.8 Gm.) which was recrystallized from petroleum ether and yielded β-amyrin, m.p. 194–196°. A mixed melting point with an authentic sample of β-amyrin,[5] m.p. 192–196°, showed no depression. The infrared spectra of the compound and authentic β-amyrin were identical.

Fraction E (0.7 Gm.) contained a mixture of three compounds which had R_f values comparable to those of β-amyrin, β-sitosterol, and uvaol. The dried fraction was deposited on top of a small column of alumina (100 Gm., 30 × 240 mm.) and carefully eluted. Fraction E′ [chloroform–ether (3:1)] eluted only β-sitosterol (as revealed by TLC) which was crystallized from chloroform–methanol and gave a m.p. of 135°. Mixed melting points with an authentic sample of β-sitosterol,[5] m.p. 133–135°, did not cause any depression. Superimposable infrared spectra were obtained in comparing the isolate with the authentic sample.

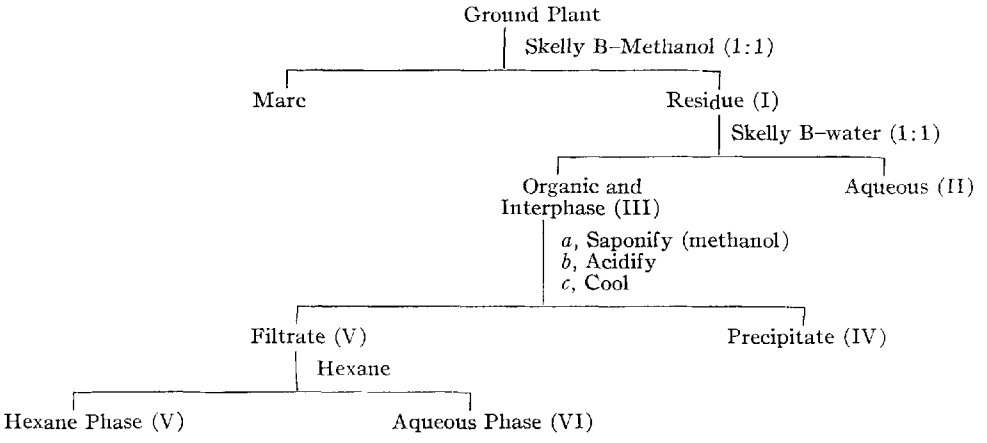
Fractions F and G contained only one component which had an R_f value comparable to that of uvaol. The dried fraction (0.8 Gm.) was recrystallized from ethanol and yielded fine white needles, m.p. 222–224°. Reference uvaol, m.p. 223–224°, was prepared by lithium aluminum hydride reduction of ursolic acid utilizing a modified procedure of Nystrom and Brown (23). Mixed melting points of

[2] TLC plates (20 × 20 cm.) prepared using Silica Gel G, according to Stahl. Apparatus: C. DeSaga, Heidelberg, Germany. U.S. Representative: Brinkman Instruments Co., Westbury, N. Y.

[3] Thomas-Hoover melting point apparatus, uncorrected.

[4] Alumina activated; Matheson, Coleman and Bell, East Rutherford, N. J.

[5] Nutritional Biochemicals, Cleveland, Ohio.



Extraction Scheme for Sterols and/or Triterpenes
Scheme I

TABLE I.—COLUMN CHROMATOGRAPHY OF FRACTION V FROM *A. patula*

Fraction	Eluant	Vol., ml.	*Rf* of Steroidal Component[a]
A	Hexane	1000	...
B	Hexane–benzene (1:1)	1000	...
C	Benzene	1000	...
D	Benzene–chloroform (7:3)	3000	0.58
E	Benzene–chloroform (1:1)	4000	0.58 0.49 0.39
F	Chloroform	2500	0.39
G	Chloroform–methanol (1:1)	2000	0.39

[a] Detected by TLC in chloroform–acetone (9:1); 10% w/w $SbCl_3$ in $CHCl_3$ as spray reagent and heating in an oven at 110° for 5 min.

isolated and reference uvaol showed no depression and the infrared spectra of the two were identical.

Co-Chromatography.—In these experiments the identity of β-amyrin, β-sitosterol, uvaol, and ursolic acid isolated from the extract was also verified by means of TLC. The isolated compound and the corresponding authentic sample were co-spotted. Controls of the isolated compound and the reference compound were spotted singly. These plates were then run in three different solvent systems and when developed showed that the co-spot which did not separate had the same R_f value as that of the two control spots. The results of co-spotting experiments are summarized in Table II.

Rather than repeating the entire procedure of isolation of compounds from *A. columbiana* a sample of fraction III of this plant was co-spotted along with standard β-amyrin, β-sitosterol, ursolic acid, and uvaol in three different solvent systems. The results are shown in Table III and indicate the presence of the same compounds in *A. columbiana*.

Phenolic and Related Compounds.—Portions of fraction II (Scheme I) of both plants were subjected to liquid–liquid extraction using ethyl acetate and ether, successively. Portions of fraction II were also subjected to acidic or alkaline hydrolysis and the hydrolysates were then extracted with ether. The extraction scheme is summarized in Scheme II. Samples were spotted in several channels on chromatoplates. Spots corresponding to reference compounds were removed from the plates with a vacuum-zone extractor (24) and the compounds were eluted from the extractor with hot methanol. Ultraviolet spectra of the methanolic solutions were run and the solutions were then co-spotted in three different solvent systems. Arbutin (hydroquinone-β-D-glucoside) was identified in fraction A by co-spotting and absorption maximum of 283 mμ. Ellagic acid was identified by co-spotting and absorption maximum of 253 mμ. Gallic acid was obtained from fraction D and identified by co-spotting and absorption maximum of 273 mμ. Hydroquinone was found both in the free state (fraction A) and in the acid hydrolyzed fraction (fraction D). Co-spotting identified its presence as did the absorption maxima, 289–290 mμ. Hyperin (quercetin-3-galac-

TABLE II.—CO-SPOTTING OF REFERENCE AND ISOLATED COMPOUNDS FROM *A. patula*[a]

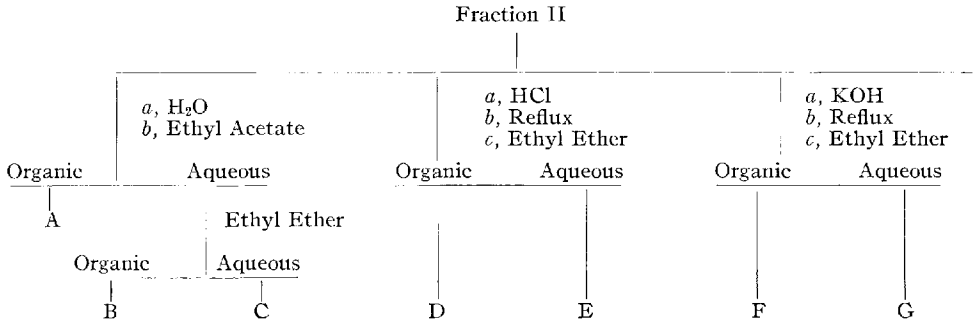
Compd.	*Rf* Values BE[b]	CA[b]	HBE[b]
1, Ursolic acid	0.14	0.13	0.04
2, Fraction IV	0.13	0.12	0.04
3, Co-spot (1 and 2)	0.13	0.12	0.04
4, β-Amyrin	0.50	0.58	0.30
5, Fraction D	0.51	0.58	0.30
6, Co-spot (4 and 5)	0.51	0.58	0.30
7, β-Sitosterol	0.41	0.49	0.22
8, Fraction E′	0.42	0.49	0.23
9, Co-spot (7 and 8)	0.42	0.49	0.23
10, Uvaol	0.32	0.38	0.07
11, Fractions F and G	0.33	0.39	0.08
12, Co-spot (10 and 11)	0.33	0.39	0.08

[a] Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110° for 5 min. [b] BE, benzene–ethyl acetate (4:1); CA, chloroform–acetone (9:1); HBE, heptane–benzene–ethanol (50:50:5).

TABLE III.—CO-SPOTTING OF REFERENCE COMPOUNDS AND *A. columbiana* FRACTION III

Solvent Systems	R_f Values[a] Fraction III[a]	β-Amyrin	Co-Spot, β-Amyrin and III	β-Sitosterol	Co-Spot, β-Sitosterol and III	Ursolic Acid	Co-Spot, Ursolic Acid and III	Uvaol	Co-Spot, Uvaol and III
BE[b]	0.14		0.14		0.14	0.14	0.14		0.14
	0.32		0.32		0.32		0.32	0.32	0.32
	0.38		0.38	0.38	0.38		0.38		0.38
	0.48	0.48	0.48		0.48		0.48		0.48
	0.08		0.08		0.08	0.08	0.08		0.08
CA[b]	0.33		0.33		0.33		0.33	0.33	0.33
	0.42		0.42	0.42	0.42		0.42		0.42
	0.54	0.54	0.54		0.54		0.54		0.54
	0.10		0.10		0.10	0.10	0.10		0.10
HBE[b]	0.18		0.18		0.18		0.18	0.18	0.18
	0.29		0.29	0.29	0.29		0.29		0.29
	0.43	0.43	0.43		0.43		0.43		0.43

[a] Detected with antimony trichloride (10% in chloroform, w/w) and heated at 110° for 5 min. [b] BE, benzene–ethyl acetate (4:1); CA, chloroform–acetone (9:1); HBE, heptane–benzene–ethanol (50:50:5).



Fractionation Scheme of Phenolic Compounds
Scheme II

toside) was also found in fraction A and identified by co-spotting and absorption maxima of 360 and 255 mμ. Free quercetin was also identified in fraction A and by its absorption maxima of 370 and 252 mμ. The results of the co-spotting experiments are found in Table IV.

Ibrahim (15) recently isolated *o*-pyrocatechuic acid from various members of the *Ericaceae*, including *A. uva-ursi*. A standard sample[6] was chromatographed in six different solvent systems along with all of the plant phenolic fractions (A–G), and no evidence could be obtained for its presence in *A. columbiana* or *A. patula*. The six solvent systems used were BAW, BMA, CEF, TEF (*cf.* Table IV), BPA (benzene–pyridine–acetic acid, 36:9:5) and BDA (benzene–dioxane–acetic acid, 90:25:4).

Antibacterial–Antifungal Screen.—The small tube method of Catalfomo and Schultz (25) was used for screening the crude plant extracts and the compounds found to exist in the plants. Crude extracts consisted of skelly B–methanol (1:1) and 70% ethanol fractions (5 Gm./150 ml.) from each plant. These fractions were then dried *in vacuo* and redissolved in 70% ethanol shortly before use. The known compounds were also dispersed in 70% ethanol. Controls, tubes innoculated with the organism only, and blanks, tubes with the solvent and the organism, showed adequate growth. The results are shown in Table V.

[6] Obtained through the courtesy of Dr. R. V. Ibrahim, Alexandria, Egypt, U.A.R.

RESULTS AND DISCUSSION

A new procedure was devised in order to remove as many components of interest in one extraction procedure. Miscible solvents of diverse polarity had to be selected and experimentation demonstrated that a skelly B–methanol (1:1) mixture was best suited for this need.

Five compounds were isolated from the sterol/triterpene fraction of *A. patula*. Ursolic acid and uvaol had been previously identified in the genus but β-amyrin, β-sitosterol, and nonacosane had not. All of the compounds were identified in *A. columbiana* by means of co-chromatography except for nonacosane which was also isolated.

The new extraction solvent also removed phenolic components which were fractionated from other components by physical and chemical methods and were separated from one another by TLC. Ultraviolet spectra and co-spotting verified the presence of two flavonoids, quercetin and hyperin; two quinonoids, arbutin and hydroquinone; and two phenolic acids, gallic and ellagic acid. *o*-Pyrocatechuic acid, previously assumed to be widespread in the family, could not be shown to be present in the two species investigated.

Antibacterial–antifungal screening was conducted on the crude plant extracts and the compounds found to be present in the plants. All of the crude extracts demonstrated fungicidal activity against *Trichophyton mentagrophytes* which could not be attributed to the known compounds. Further frac-

TABLE IV.—CO-SPOTTING OF PHENOLIC COMPONENTS AND REFERENCE COMPOUNDS[a,b]

	BMA[c]	CAW[c]	CEF[c]	TEF[c]	BAW[c]	BAW'[c]
Arbutin	0.11	0.68	...	0.04	...	...
Ellagic acid	0.04	...	...	...	0.03	0.02
Gallic acid	0.19	...	0.29	0.28	...	...
Hydroquinone	0.38	...	0.56	0.44	...	...
Hyperin	0.10	0.64	...	0.03	...	...
Quercetin	0.36	0.74	...	0.31	...	...

[a] R_f values consist of standards and eluted components (co-spot) from both species. [b] Detection: arbutin, Millon's spray reagent; ellagic acid, 1% ethanolic ferric chloride; gallic acid, hydroquinone, hyperin, and quercetin, 5% methanolic phosphomolybdic acid (PMA). [c] BMA, benzene–methanol–acetic acid (10:2:1); CAW, chloroform–acetic acid–water (35:50 17.5); CEF, chloroform–ethyl acetate–formic acid (5:4:1); TEF, toluene–ethyl formate–formic acid (5:4:1); BAW, butanol–acetic acid–water (4:1:1); BAW', butanol–acetic acid–water (4:1:5).

tionation of these extracts is in progress to determine wherein this activity may reside. The skelly B–methanol extract from *A. columbiana* also demonstrated fungicidal activity against *Candida albicans*, whereas neither of the *A. patula* extracts did. In contrast, the skelly B–methanol extract of *A. patula* was antibacterial against *Escherichia coli* but neither *A. columbiana* extract was so effective.

Hydroquinone was effective against both bacteria, *E. coli* and *Staphylococcus aureus*, whereas the glycoside arbutin was effective only in higher concentrations against *S. aureus*.

Quercetin demonstrated equivocal results against all organisms except *S. aureus*, whereas no such activity was noted with the galactoside, hyperin.

The triterpene, β-amyrin, demonstrated the widest spectrum of activity among the triterpene/sterols and the triterpene alcohol, uvaol, contained no activity.

Newer applications of techniques for the extraction, fractionation, and identification of compounds from two diverse chemical classes have been demonstrated. Eleven compounds from two previously uninvestigated plants have been identified and the microbiological activity of the crude plant extracts and the compounds have been recorded.

TABLE V.—ANTIBACTERIAL–ANTIFUNGAL STUDIES

Compd. and Concn.	*E. coli*	*S. aureus*	*C. albicans*	*T. mentagrophytes*
A. columbiana[a]				
1:100	±[c]	±	±	−
1:500	±	+	±	−
A. columbiana[b]				
1:100	+	+	−	−
1:500	+	+	−	−
A. patula[a]				
1:100	+	+	±	−
1:500	+	+	±	−
A. patula[b]				
1:100	−	+	±	−
1:500	−	+	±	−
Arbutin				
1:100	+	−	+	+
1:500	+	+	+	+
Hydroquinone				
1:100	±	−	+	+
1:500	±	−	+	+
Quercetin				
1:100	±	+	±	±
1:500	±	+	±	±
Hyperin				
1:100	+	+	+	+
1:500	+	+	+	+
Gallic acid				
1:100	±	−	+	±
1:500	+	±	+	±
Ellagic acid				
1:100	±	+	+	+
1:500	±	+	+	+
β-Amyrin				
1:100	±	±	±	+
1:500	±	±	±	+
β-Sitosterol				
1:100	+	±	+	±
1:500	+	±	+	±
Ursolic acid				
1:100	+	±	+	+
1:500	+	±	+	+
Uvaol				
1:100	+	+	+	+
1:500	+	+	+	+

[a] Dried 70% ethanol extract. [b] Dried skelly B–MeOH (1:1) extract. [c] +, growth; −, no growth; ±, equivocal growth.

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