(14) H. M. Pyle and H. F. Boyer, *Federation Proc.*, **21**, 164(1962).
(15) J. E. Lovelock and M. W. A. Bishop, *Nature*, **183**, 1394 (1959).
(16) H. C. Ansel and W. F. Leake, *J. Pharm. Sci.*, **55**, 687(1966).
(17) H. C. Ansel, *Am. J. Hosp. Pharm.*, **21**, 25(1964).
(18) H. C. Ansel and D. E. Cadwallader, *J. Pharm. Sci.*, **53**, 169(1964).
(19) D. E. Cadwallader and H. C. Ansel, *ibid.*, **53**, 169(1964).
(20) H. C. Ansel, *ibid.*, **54**, 1159(1965).
(21) H. C. Ansel and G. E. Cabre, (1967), unpublished data.
(22) F. Kavanagh, "Analytical Microbiology," Academic Press, New York, N. Y. 1963, pp. 141–159.
(23) D. H. Kay, "Techniques for Electron Microscopy," Davis, Philadelphia, Pa., 1965, pp. 166–212.
(24) F. B. Seibert, F. K. Farrelly, and C. C. Shepherd, *Ann. N. Y. Acad. Sci.*, **141**, 175(1968).
(25) G. E. Pottz, J. H. Rampey, and F. Benjamin, *ibid.*, **141**, 261(1968).
(26) A. V. Fowler and I. Zabin, *J. Bacteriol.*, **92**, 353(1966).
(27) L. F. Montes, J. L. Day, C. J. Wand, and L. Kennedy, *J. Invest. Dermatol.*, **48**, 184(1967).

ACKNOWLEDGMENTS AND ADDRESSES

Received October 24, 1968, from the *School of Pharmacy, University of Georgia, Athens, GA 30601*

Accepted for publication December 11, 1968.

Presented to the Basic Pharmaceutics Section, APhA Academy of Pharmaceutical Sciences, Montreal meeting, May 1969.

This investigation was supported by the Office of General Research, University of Georgia.

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Plant Antitumor Agents III: A Convenient Separation of Tannins from Other Plant Constituents

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Abstract □ Several methods were tested for the nondestructive separation of tannins from other plant constituents which might have antitumor activity. A comparison was made of precipitation of tannins by caffeine or lead acetate and chromatography on polyamide. The caffeine procedure was preferred over lead acetate; both tannins and nontannins were effectively separated and the caffeine conveniently removed. Polyamide was an effective tannin remover; nontannins were easily collected but tannins were irreversibly adsorbed. The application of the caffeine procedure to a variety of plants is described.

Keyphrases □ Tannin separation—antitumor plant constituents □ Caffeine—tannin precipitation □ Lead acetate, caffeine precipitation, chromatography—tannin separation comparison

Tannins are a ubiquitous plant constituent (1) and are quite soluble in ethanol, aqueous 50% ethanol, or water. For some years the Walker 256-carcinosarcoma tumor has been used by groups fractionating plants for antitumor agents under the Cancer Chemotherapy National Service Center program.[1] A variety of alcohol and water-soluble substances inhibit growth of this tumor in rats, including tannins, saponins, and certain cardiac glycosides, and other less well-defined substances. In some cases the initial activity may be due to mixtures of the above substances. Accordingly, a convenient, specific, and nondestructive method which would separate tannins from other constituents would be useful and is the subject of this report.

EXPERIMENTAL

Seven kilograms of air-dried whole plant[2] (root, stem, leaf, and flowers) was continuously extracted with 95% ethanol in a continuous, pilot-plant scale extractor (continuous hot-percolation type) for 48 hr. After concentration of the alcohol *in vacuo* to a syrup, the latter product was partitioned between chloroform and water (4 l. water extracted three times with 2 l. chloroform containing 10% ethanol). The aqueous fraction was freeze dried; the chloroform fraction was evaporated to a syrup *in vacuo*. Figure 1 gives the extraction data and 5WM activities of the various fractions.

Countercurrent Concentration—Two-hundred and fifty grams of the aqueous fraction (Fig. 1) was partitioned in a system consisting of ethyl acetate–methanol–water, 2:1:2; the phases were mutually saturated and equal volumes of upper and lower phase used in a 10-tube countercurrent distribution instrument.[3] A bank of ten, 4-l. separators set up containing 1,800 ml. of upper and lower phase, respectively. The freeze-dried water-soluble fraction from Fig. 1 was dissolved in the lower phase of this system, shaken, and after separation transferred to the next tube containing 1,800 ml. of lower phase. A similar quantity of upper phase was added to the first funnel and the partition continued for a total of 10 tubes. Volatile solvent was removed from upper and lower phases *in vacuo* at 40°; the residual upper-phase material in each tube was added to the corresponding lower phase and water removed by freeze drying. The results are shown in Table I. Fractions 6–9, Table I, all gave strong qualitative tannin tests [ferric chloride, salt-gelatin, method of Wall *et al.* (1)]. Fraction 7 was arbitrarily chosen for comparison of the various tannin-removal procedures.

Lead Acetate Precipitation—Ten grams of Fraction 7 in 100 ml. of water was treated with 30 ml. of neutral lead acetate aqueous solution (23%). The precipitate was centrifuged and suspended in methanol. The suspension was treated with a large excess of H_2S gas and the lead sulfide centrifuged. The residual solution was evaporated *in vacuo* and the residue taken up in water and freeze

[1] The procedure for this assay is described in *Cancer Chemotherapy Rept.*, **25**, 1(1962).

[2] The authors wish to thank Dr. Robert E. Perdue, Jr., New Crops Research Branch, ARS, USDA, Beltsville, Md., for all the plant material described in this paper.

[3] Craig CCD, H. O. Post Scientific Instruments Co.

Table I—10-Tube CCD of Crude Aqueous Fraction[a]

Tube No.	Weight Fraction, g.	T/C-5WM
0	4.8	>50% at 75–400 mg./kg.
1	5.9	>50% at 75–400 mg./kg.
2	10.5	>50% at 75–400 mg./kg.
3	13.1	>50% at 75–300 mg./kg.
4	12.5	>50% at 75–450 mg./kg.
5	13.0	42% at 450 mg./kg.
6	21.0	21% at 350 mg./kg.
7	56.6	5% at 350 mg./kg.
8	77.1	7% at 350 mg./kg.
9	46.4	26% at 200 mg./kg.

[a] System ethyl acetate–methanol–water, 2:1:2.

dried. The aqueous filtrate from the lead tannin precipitate was treated with H_2S in a similar manner, the lead sulfide removed, and the residual aqueous solution freeze dried. By this procedure 3.3 g. of material giving a strong tannin test was obtained from the lead precipitate, and 3.8 g. from the unprecipitated fraction. Some losses in total recovery were probably due to trapping of some products in the lead sulfide.

Caffeine Procedure—To 10 g. of Fraction 7 in 100 ml. of water was slowly added a 1.5% caffeine monohydrate aqueous solution until no further precipitation was noted. Often it was necessary to centrifuge and add additional caffeine solution to the residual centrifugate. The gummy caffeine precipitate was dissolved in a minimal quantity of methanol, diluted to 250 ml. with water, and extracted three times with equal volumes of chloroform. The residual aqueous solution was then placed in a liquid-liquid extractor and continuously extracted with chloroform for 20 hr. The aqueous layer was freeze dried. The filtrate from the caffeine precipitate was freed from excess caffeine by extraction three times with equal volumes of chloroform, and the aqueous layer freeze dried. In this example 2.4 g. was found in the caffeine precipitate and 4.9 g. in the caffeine filtrate fractions.

Polyamide—A column 66-mm. diameter was packed with 500 g. of polyamide powder[4] which had been allowed to soak in water overnight. Ten grams of Fraction 7 was dissolved in a minimum quantity of water. A gradient-elution system consisting of 2 l. H_2O, 2 l. 50% methanol, and 2 l. absolute methanol was set up and allowed to pass through the column. After this volume had passed through, the column was washed with an additional 2 l. of methanol, and then 2.1 l. of 5% acetic acid was run through the column. Finally the column was extruded and washed with 1,500 ml. of 0.1 *N* NaOH. The alkaline solution was acidified to pH 5.6. This latter alkaline extracted and acid-neutralized product was the only fraction which gave tannin tests. The treatment destroyed all biological activity, *cf.* Table II. All of the earlier fractions were also inactive.

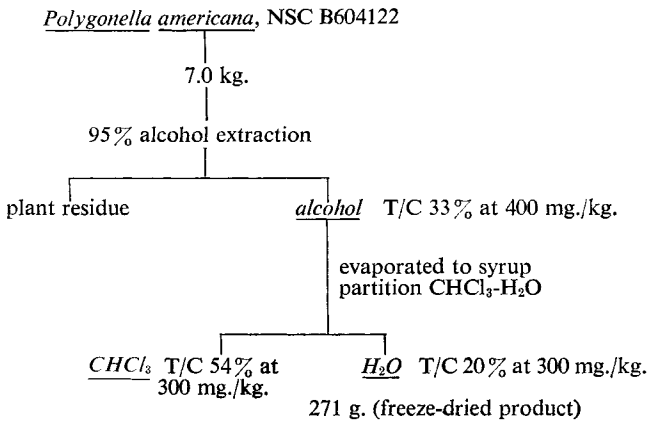

Figure 1—*Flowsheet for extraction of tannins from plants.*

[4] Ultramidpulver, Badische Analin und Soda Fabrik A. G.

Table II—Effect of Lead Tetraacetate, Caffeine, and Polyamide on Removal of Tannin From Plant Extracts[a]

Procedure	Dose, mg./kg.	T/C (WM)
Lead Acetate		
Precipitate, 3.3 g.	200	34
	100	45
Filtrate, 3.8 g.	350	103
	250	84
	100	86
Polyamide		
Eluate, 6.2 g.	Inactive all concentrations 250–100 mg./kg.	
Caffeine		
Precipitate, 2.4 g.	350	0/4 survival
	250	1/4
	150	15
	50	62
Filtrate, 4.9 g.	350	84
	250	89
	150	86

[a] Protocol 10 g. of Fraction 7, Table I, was used for all tests.

RESULTS AND DISCUSSION

The studies of the various tannin-separation procedures were conducted initially with an extract prepared from *Polygonella americana*, NSC B604122 (whole plant: root, stem, leaves). The plant originally gave a strongly positive qualitative test for tannins by the methods presented by Wall and co-workers (1). An ethanol extract was fractionated by the method shown in Fig. 1. As can be seen from this figure, growth of the WM-256 tumor in rats was strongly inhibited by the aqueous fraction. This fraction also gave a strong tannin test. A 10-tube countercurrent distribution of the above aqueous fraction was carried out as shown in Table I (*cf. Experimental*). A major fraction from this CCD extract (No. 7) was selected as a stock preparation which was used to compare the various tannin-purification methods. Three methods were tested: (*a*) precipitation of tannins with lead acetate; (*b*) precipitation of tannins with caffeine; and (*c*) removal of tannins by passage through polyamide columns. The use of lead acetate for clarification of sugar solutions dates prior to the 20th century. A recent example of its use in an antitumor saponin purification is given by Kupchan *et al.* (2) and the procedure used in this study is almost identical. The caffeine procedure has been described by Bell *et al.* (3, 4) and was modified somewhat in the present study. Polyamide has been recommended as a selective adsorbent for phenols, and to the authors' knowledge the present communication represents the first attempt to use it as a tannin remover. The results of the comparison of three methods are shown in Table II. In the lead procedure (Method I), tannins, and other compounds such as proteins, phenols, and certain acids are precipitated as the insoluble lead salt. Both the precipitate and filtrate are "deleaded" by treatment with hydrogen sulfide, after which both fractions are centrifuged to remove PbS, and the lead-free filtrate freeze dried and submitted to bioassay in the 5WM system with results as shown in Table II. Caffeine precipitates tannins from water solution quite specifically giving a water-insoluble precipitate. After centrifugation or filtration, the tannin-free filtrate is extracted with chloroform to remove excess caffeine (it will be noted that chloroform-soluble plant substances had already been removed, *cf.* Fig. 1). The water layer, after removal of the chloroform, is freeze dried. The caffeine precipitate is suspended in water and is freed from caffeine by continuous chloroform extraction; the tannins thus liberated go into aqueous solution, and after removal of the chloroform and any residual solids, the aqueous tannin solution is freeze dried. Both filtrate and precipitate fractions were tested in 5WM. In the polyamide method, the aqueous tannin solution was percolated through a column of polyamide using a gradient-elution system of water with increasing methanol content, followed by pure methanol and methanol-acetic acid. Fractions were removed and checked for tannin. No tannin was removed from the column, although about 62% of the total weight was recovered. Finally, material giving a tannin test could be extracted from the extruded column with 0.1 *N* NaOH, followed by acidification. Although this latter material gave a positive tannin test, it was totally inactive. Comparing the three procedures in Table II, it is apparent that the

Table III—Antitumor Activity of Caffeine Precipitates From Various Plants

NSC No.	Plant	Inhibition of WM256 T/C[a] Caffeine ppt.	Inhibition of WM256 % Filtrate[b]
B611369	*Polygonum paronychia*	49 at 60	Inactive 500 → 200
B611500	*Cedrus atlantica*	Toxic at 100; 47 at 50	
B612961	*Salix exigua*	6 at 90	Inactive 240 → 30
B631069	*Combretum molle*	12 at 135	Inactive 525 → 200
B633585	*Oenothera clavaeformis*	Toxic at 50; inactive at 25	
B634475	*Amelanchier stolonifera*	Toxic at 200; 38 at 100	
B652261	*Krameria triandra*	Toxic at 200; inactive at 135	Inactive 500 → 200
B604115	*Ludwigia alternifolia*	37 at 200	Inactive at 200
B604122	*Polygonella americana*	15 at 150	Inactive 350 → 50
B604199	*Cornus racemosa*	42 at 135	Inactive 500 → 200
B604269	*Cyrilla parvifolia*	Toxic at 100; inactive at 50	
B604461	*Tamarix gallica*	26 at 90	Inactive 500 → 200
B654556	*Ulmus americana*	Toxic at 100; inactive at 50	
B605373	*Cocus nucifera*	Toxic at 100; inactive at 50	
B605669	*Carpodiptera ameliae*	Toxic at 100; inactive at 50	
B605860	*Rosa abyssinica*	32 at 100	Inactive 300 → 100
B655855	*Rumex nervosus*	Toxic at 100; inactive at 50	
B655984	*Alectryon subcnereum*	38 at 100	Inactive 330 → 100
B620371	*Pinus lambertiana*	Toxic at 100; inactive at 50	Inactive 400 → 200
B620851	*Quercus kelloggii*	2 at 100	
B670051	*Cornus nutallii*	Toxic at 75	Inactive 400 → 250
B670064	*Cornus stolonifera*	Toxic at 100; inactive at 50	Inactive 300 → 50
B670156	*Fagus grandifolia*	Toxic at 150; inactive at 100	Inactive 200 → 50

[a] T/C of 42 or less is considered significant inhibition. [b] All doses expressed in mg./kg.

lead acetate and caffeine procedures give results which, considering the variability of the biological test system, are virtually identical. Both the lead precipitate and caffeine-precipitate fractions contain all the antitumor activity. Furthermore, the material is significantly concentrated, *i.e.*, lower doses are required to give activity (activity must be a T/C of 42% or less). The filtrate fractions from each of the two procedures were totally inactive. The polyamide column permitted separation of nonphenolic and simpler phenolic substances but did not permit recovery of tannins which contained antitumor activity. In terms of evaluating the methods there is a clear-cut preference for the caffeine procedure over the lead acetate in the sense that the former method is more specific and convenient. In particular when it is necessary to process large samples, the use of sizable quantities of the obnoxious and toxic gas H_2S is avoided. Moreover since the caffeine procedure is more specific, a purer tannin can be obtained in those instances in which isolation of the tannin constituent is desired. The caffeine can be recovered if desired and can be reused many times. In those cases in which the antitumor activity of a tannin-containing plant were due to a nontannin substance, the use of polyamide would offer many advantages, particularly if the compound were a nontannin phenol.

Although the comparative tests were conducted on an aqueous fraction which had been further purified by a countercurrent distribution procedure, further experience with the caffeine procedure has indicated that crude aqueous samples prepared as in Fig. 1 may be used. The authors' experience to date has shown that in every instance in which a strong qualitative tannin test was given by a chloroform extracted crude aqueous fraction, the Walker-256 carcinosarcoma inhibition exhibited by such extracts was due to tannin and was always found in the caffeine-precipitate fraction. The present data on a number of plants are shown in Table III. It will be seen that in every case tested, the caffeine filtrate was nontoxic and inactive. The caffeine precipitates were either active by accepted standards, *i.e.*, T/C of 42% or less, or toxic at one concentration and then inactive at half this toxic dose. Thus there can be little doubt of the association of this activity with tannins. Saponins, another ubiquitous plant constituent, are apparently found in plants which are low in tannin content. Although the authors have isolated a number of saponins with activity against the Walker-256 carcinosarcoma (5) such plants do not give a significant tannin test.

REFERENCES

(1) M. E. Wall, M. M. Krider, C. F. Krewson, C. R. Eddy, J. J. Willaman, D. S. Correll, and H. S. Gentry, *J. Am. Pharm. Assoc., Sci. Ed.*, **43**, 1(1954).

(2) S. M. Kupchan, R. J. Hemingway, J. R. Knox, S. J. Barboutis, D. Werner, and M. A. Barboutis, *J. Pharm. Sci.*, **56**, 603(1967).

(3) T. A. Bell, J. L. Etchells, C. F. Williams, and W. L. Porter, *Botan. Gaz.*, **123**, 220(1962).

(4) T. A. Bell, J. L. Etchells, and W. W. G. Smart, *ibid.*, **126**, 40 (1965).

(5) M. E. Wall, H. Taylor, and L. Ambrosio, in preparation.

ACKNOWLEDGMENTS AND ADDRESSES

Received December 19, 1968, from the *Chemistry and Life Sciences Laboratory, Research Triangle Institute, P. O. Box 12194, Research Triangle Park, NC 27709*

Accepted for publication March 5, 1969.

This investigation was supported by contract No. SA-43-ph-4322, with the Cancer Chemotherapy National Service Center, National Institutes of Health.

Previous paper: M. C. Wani and M. E. Wall, *J. Org. Chem.*, in press.