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Isolation of a Potential Antitumor Fraction from *Rumex hymenosepalus*

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Extracts of *Rumex hymenosepalus* have shown antitumor activity against the Sarcoma 180 and Walker 256 test systems of the Cancer Chemotherapy National Service Center, Bethesda, Md. The extracts have been fractionated by means of solvent extraction and paper chromatography into two distinct fractions. The partial characterization of these fractions is described, including the characterization of leucocyanidin as one of the constituents and the identification of benzoic acid as a hydrolysis product of a polyphenolic ester.

RUMEX HYMENOSEPALUS, a dicotyledon of the family *Polygonaceae*, also known by the common name canaigre, is a plant native to the southwestern United States. In tests performed on the various extracts of the plant by the Cancer Chemotherapy National Service Center, it was discovered that the tannin-containing fraction of the plant exhibited antitumor activity. An investigation of this fraction was begun in order to attempt to identify the precise chemical character of the antitumor substance. It should be noted at this point that the response was irregular. Some fractions would show activity in a series of tests; however, upon repetition, the activity would be lost.

A literature search revealed the existence of several methods of extracting tannins from the plant as employed by leather chemists. Chemical compounds already discovered in the plant include substances such as chrysophanic acid, physcion (1), and possibly emodin (2). Polyphenols are said to predominate in canaigre tannins (3). Members of this classification of compounds vary little in phenolic reactivity and therefore are very difficult to separate chemically (3). Polyphenols are capable of a great deal of mutual solubilization, resulting in solid solutions which tend to behave as if they were single substances (4). The roots, the major tannin-containing part of the plant, also contain sugars and starches, which make the usual methods of tannin removal more complicated (4). Therefore, a special method of tannin extraction was developed to fill the requirements of this investigation.

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EXPERIMENTAL

Extraction.—Six hundred grams of the frozen tuber-like roots of the plant were ground into a damp reddish-brown meal-like material in a Wiley mill equipped with a 4-mm. size screen. The ground material was then washed with petroleum ether and ethyl ether. The residues obtained from these extractions were set aside for possible future investigation.

The plant marc was air dried for a 24-hr. period. It was then extracted with 4 L. of a 95% ethanol–methanol (1:1) mixture for 120 hr. The resulting reddish-brown solution was separated from the marc by filtration and allowed to evaporate to dryness. The plant marc was discarded.

The amorphous brown residue of the methanol–ethanol extraction was dissolved in approximately 1 L. of distilled water. The solution was then washed repeatedly with a total of 2 L. of chloroform.

The washed water extract was then frozen and lyophilized at −50° to dryness. Approximately 120 Gm. of a light orange-brown semicrystalline powder was obtained. The lyophilized extract was submitted to the Cancer Chemotherapy National Service Center for antitumor testing. Testing in Walker 256 test system (5) gave a value of 35% t/c (test/control) at 60 mg./Kg. Also against the Sarcoma 180 test system (5) a value of 14% t/c at a dose of 90 mg./Kg. was obtained. These results indicate definite activity.

Physical and Chemical Characteristics.—The amorphous orange-brown powder obtained was subjected to a series of tests to determine functional composition. Elementary analysis by sodium fusion showed the absence of nitrogen, sulfur, or halogens. Solubility tests indicated the presence of weakly acidic materials. Aqueous solutions of the extract demonstrated indicator-like properties by color changes with change of pH. Ferric chloride, 5% aqueous solution, gave a blue-black color reaction,

indicating the presence of phenols. Gelatin and alkaloidal precipitation tests for tannins were positive. An aqueous solution of the extract, when treated with sodium hydroxide aqueous solution, concentrated sulfuric acid, and magnesium-hydrochloric acid, gave color tests which indicate the presence of flavonoidal materials. A sharp melting point was not attainable. The material darkened at 250° but did not melt.

The infrared spectra (KBr pellet) showed broad absorption at 2.9 to 3 μ, a short band at exactly 6 μ, a long band at 6.3 μ, medium band 6.65 μ, a long band at 7 μ. In the region from 8 to 15 μ short broad absorption occurred at 8.3, 9.15, 9.8, 12.2, and 13.15 μ. The spectra showed correlation in all areas with a spectrogram for tannins and catechins of purified Quebracho extract tannins (6). The weak plateau in the carbonyl region has been reported by Jones (7) in both Quebracho and redwood tannins. The infrared spectra verified the tannin nature of the material; aromatic hydroxyl, aromatic absorption, and the lack of strong carbonyl absorption being the outstanding characteristics. A Perkin-Elmer Infracord instrument was used to obtain spectra.

Ultraviolet spectra of the extract shows a maximum absorption at 285 mμ, with a minimum at 245 mμ and a leveling off at 350 mμ. This is the characteristic absorption of leucoanthocyanidins, flavan-3,4-diols, and catechins (8).

The qualitative organic analysis, infrared, and ultraviolet spectra would indicate that the material should be characterized as a polyphenolic, flavonoidal tannin.

Paper Chromatography.—The amorphous tannin fraction was then subjected to paper chromatography. The paper used was Whatman No. 1. The solvent system employed consisted of butanol–acetic acid–water (4:1:5). The upper phase only was used. Ethanolic-vanillin acidified with hydrochloric acid, 5% aqueous ferric chloride, and *p*-toluene sulfonic acid were utilized as chromogenic spraying agents. Tank saturation and descending chromatography were used. A yellow streak extended to an R_f value of 0.64. The streak turned various shades of red and pink on spraying with ethanolic-vanillin HCl and heating in an oven. A light yellow visible spot at R_f 0.54 appeared lavender in ultraviolet light. A spot that was colorless in visible light appeared light blue in ultraviolet light at R_f 0.64. A light yellowish-brown visible spot at R_f 0.72 appeared lavender in ultraviolet light. A yellowish visible spot at R_f 0.94 very close to the solvent front became blue when observed under ultraviolet light. Only the streak responded to the ethanolic-vanillin HCl chromogenic spray. All spots and the streak became blue on spraying with 5% ferric chloride aqueous solution and heating in an oven.

Comparison paper chromatography utilizing D-catechin and the lyophilized extract and spraying with *p*-toluene sulfonic acid indicated that catechins were not present since the color developed by catechins did not appear in the extract.

The tannin fraction was then subjected to hydrolysis with 10% aqueous sodium hydroxide solution and dilute hydrochloric acid. Both short- and long-term hydrolysis was carried out. Both methods of hydrolysis yielded only a white crystalline solid, m.p. 122°; neutralization equivalent, 123. An infrared spectrum of the crystalline material corresponded with a commercial sample of benzoic acid over all wavelengths.

Initial paper chromatography of the ethanolic–methanolic aqueous lyophilized extract in butanol–acetic acid–water (4:1:5) upper phase, indicated the possible presence of polymeric and monomeric molecules. In order to characterize the chemical nature of this material, an attempt to separate the polymers from the monomers was carried out.

Since the lyophilized extract was very soluble in water, solvents that were immiscible with water were utilized in fractionation separation. It was found that ethyl acetate would extract the portion of the tannin that corresponded to the spots of high R_f values detected on paper (fraction A), and the material that showed streaking from the origin in the initial chromatography remains in the aqueous solution (fraction B).

Fractions A and B were then subjected to paper chromatography separately using the same solvent system indicated above. The R_f values and ultraviolet fluoresence corresponded in each case to that of the mixture described above. Fraction A showed the four spots only, whereas fraction B showed only the streaking. The streaking material responded to the ethanolic-vanillin HCl test for converting leucoanthocyanidins to anthocyanidins as the streak turned pink-red when sprayed with this reagent and heating the paper in the oven. The single spots did not respond to this test.

Fractions A and B were submitted for antitumor testing. Fraction A was too toxic at the dose levels tested, and testing is being continued (at lower dose levels). Fraction B was active initially, but activity was not reproducible. Investigation of fraction B was undertaken to determine its chemical nature as originally it showed activity.

A golden-brown powder obtained on evaporation of fraction B, when boiled for 15 min. in ethanolic concentrated HCl, becomes orange, then turns deep red. This would indicate the conversion of leucoanthocyanidins to anthocyanidins.

The powder obtained from the evaporation of fraction B was dissolved in water and boiled for 15 min. in ethanolic concentrated HCl. The resulting red solution was then extracted with amyl alcohol. The amyl alcohol extracted an orange-red material, and the aqueous solution remained yellow in color.

Upon evaporation of the amyl alcohol extract, a orange-red powder resulted. This orange-red powder was subjected to paper chromatography on Whatman No. 3 paper in the three solvent systems listed in Table I since they have proved effective for identifying anthocyanidins (9, 10). In addition to the pink-red spot, an unidentifiable orange pigment appeared at higher R_f values in all systems.

TABLE I.—SOLVENT SYSTEMS

Solvent System	R_f Value	Color of Spot
I, Formic acid–3 *N* HCl (1:1)	0.20	Pink-red
II, Water–acetic acid–conc. HCl (10:30:3)	0.435	Pink-red
III, Acetic acid–conc. HCl–water (5:1:5)	0.34	Pink-red

After drying the papers obtained above, the pink-red spots were exposed to ammonia fumes. In each case, the spots turned blue. Upon exposure to concentrated HCl fumes, the blue spots became red again. This is also indicative of anthocyanidins. On spraying with 5% aqueous ferric chloride, the red spots turned blue. The blue color with $FeCl_3$ indicates the hydroxylation pattern found in protocatechuic or gallic acids.

Since the R_f values obtained corresponded to those in the literature reported for the red pigment, cyanidin, a commercial sample of cyanidin was obtained and the chromatography repeated with the commercial material. The R_f values in all three systems corresponded to that of cyanidin when the natural product was resolved with the commercial sample.

An attempt was then made to isolate the red pigment by preparative paper chromatography utilizing solvent system I. Several sheets of Whatman No. 3 were spotted and resolved with this solvent system. Strips corresponding to the red-pink material were cut from the paper. This material was then extracted by methanol 0.1 *N* HCl. This extraction resulted in a pink-red liquid. Visible spectral analysis of this solution showed a maximum absorption at 535 mμ. Other spectral features in the visible region matched that of the commercial sample of cyanidin.

This solution was evaporated to dryness and subjected to infrared analysis using KBr pellets. For comparison purposes, a commercial sample of leucocyanidin was converted to cyanidin, and the spectra obtained from the natural source and the commercial source were compared. The two spectra showed correlation in all regions of the infrared. An interesting spectral feature in the infrared spectra of the anthocyanidins indicated above is the weak multiplet absorption between 5.8 and 6.0 μ.

Hydrolysis of Cyanidin.—The amyl alcohol extracted powder was dissolved in an ethanol–water mixture and refluxed with 5 *N* sulfuric acid for 24 hr. The mixture was then treated with sodium hydroxide solution until it was just alkaline. It was then made neutral with dilute hydrochloric acid solution and extracted with chloroform. The chloroform extract was then treated with a saturated solution of sodium bicarbonate. This would remove the acids and leave the phenolic material in the chloroform. The sodium bicarbonate solution was acidified with dilute hydrochloric acid and again extracted with chloroform. The chloroform solution was evaporated and chromatographed on paper. Using a known sample of protocatechuic acid, it was possible to identify this material as a hydrolysis product of the cyanidin isolated from the plant. The solvent systems employed were BAW (4:1:5) and 5% glacial acetic acid.

SUMMARY

The defatted ethanol–methanol aqueous extract of the dried roots and tubers of *R. hymenosepalus* demonstrates antitumor activity in Walker 256 and Sarcoma 180 test systems in mice.

A phytochemical analysis shows the presence of polyphenolic flavonoidal tannin material. This material has been separated into two fractions, a water-soluble ethyl acetate extractable fraction (A) and a water-soluble nonethyl acetate extractable fraction (B). Fraction B has been shown to consist of leucoanthocyanidin and other flavonoidal units. Proof of the presence of leucoanthocyanidins has been demonstrated by conversion of the leuco- to the anthocyanidin pigment cyanidin. Paper chromatography, visible, and infrared spectral analysis were used in the identification of the pigment.

Hydrolysis of the original extract resulted in the isolation of benzoic acid. The benzoic acid is believed to result from the cleavage of flavonols in fraction B or the hydrolysis of polyphenolic benzoate esters in fraction A.

Fractions A and B were subjected to further antitumor trials. Initial testing of fraction B showed antitumor activity. Subsequent testing resulted in a loss of this activity. New tests are being carried out on this fraction. Fraction A has been shown to be too toxic in the doses tested. It is being investigated at lower doses. The results of the continued investigation will be reported in subsequent reports.

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