as high as 4×10^{-3} *M* did not depress the slope to a negative value, an indication that the system in the initial state was aggregated fully under its experimental environment.

That a simple relationship between micelle formation and inhibition of deaggregation does not exist in these systems is apparent in these experiments. It has been reported previously (1) that the critical micelle concentration of the AOT fraction used in these studies is approximately 2.5×10^{-3} *M*. The system at a total AOT concentration of approximately 3×10^{-3} *M* did show some slight deaggregation tendency. Complete inhibition was noted at a total AOT concentration of approximately 3.5×10^{-3} *M* (addition of 1.5×10^{-3} moles per liter AOT to the standard emulsion). Slight deaggregation was also observed in an experiment in which an emulsion containing 6×10^{-3} *M* AOT total concentration (deaggregation completely inhibited for 15 days) was diluted to a final concentration of 1% hexadecane and 3×10^{-3} *M* AOT. Thus, in these systems some deaggregation was apparent, even at AOT concentrations apparently above the critical micelle concentration.

It is interesting to compare the results of the inhibition of deaggregation by AOT to those obtained with NaCl in the previous study (1). In a similar 2% hexadecane in water emulsion containing 0.09% AOT prepared in an identical manner, a concentration of approximately 3×10^{-3} *M* NaCl was required to inhibit deaggregation completely. In AOT, a concentration between 1×10^{-3} and 1.5×10^{-3} *M* additional AOT was effective in completely preventing deaggregation. If this inhibition is related to the decrease in the zeta potential only, it would be expected that a concentration of 3×10^{-3} *M* AOT would be necessary to inhibit the deaggregation, because AOT should make the same contribution to ionic strength as NaCl, assuming complete dissociation. However, the observed inhibition with AOT is greater, and the difference can be explained only by the existence of some other mechanism operating in the system, either alone or along with a drop in the zeta potential. It seems logical to relate this difference in the inhibition observed to a film–film attraction mechanism, perhaps rendered more effective by multilayer adsorption on the particle at higher AOT concentrations. The results also show that addition of 1×10^{-3} moles per liter AOT results in a greater depression of deaggregation rate than the addition of *n*-octyl alcohol at the same concentration. This would be in general agreement with the concept that increased molecular size of the film components enhances the attractive forces between particles which cause the aggregation.

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Phytochemical Investigation of *Acacia angustissima*

By RICHARD H. HAMMER and JACK R. COLE

Preliminary pharmacological screening of the ethanol and aqueous extracts of *Acacia angustissima* has revealed sedative and antitumor effects. A phytochemical study has established the presence in the defatted ethanol extract of the leucoanthocyanidin moiety, 7, 3′, 4′-trihydroxyflavan-3,4 diol (I). The intractable amorphous ethanol extract, after partial solvent fractionation, was separated into seven fractions with a 100-stage countercurrent distribution. Three of these seven fractions contained the same leucoanthocyanidin unit (I) A fourth countercurrent fraction has been characterized tentatively as a flavone or flavonol compound. Further studies are being conducted to determine the extent of polymerization of the three fractions and to isolate the monomer leucoanthocyanidin.

ACACIA ANGUSTISSIMA (Mill) Kuntze, *Leguminosae*, grows as a small shrub or bush at elevations above 3000 ft. and can be found in Arizona, Texas, Missouri, southern Florida, and South America. In the past, the *Acacia* genus has served as a source of condensed tannins for the tanning industry (1) and as a source of the dried gummy exudate used as a demulcent, emulsifying agent, and vehicle in the preparation of emulsions and troches.

Various concentrations of acacia were tested by Pollia (2) against sarcoma tumors in rats, but no inhibition of the tumor growth was observed. Antitumor properties have been demonstrated in the aqueous, chloroform–ethanol, and ethanol extracts of the pulverized leaves, stems, and

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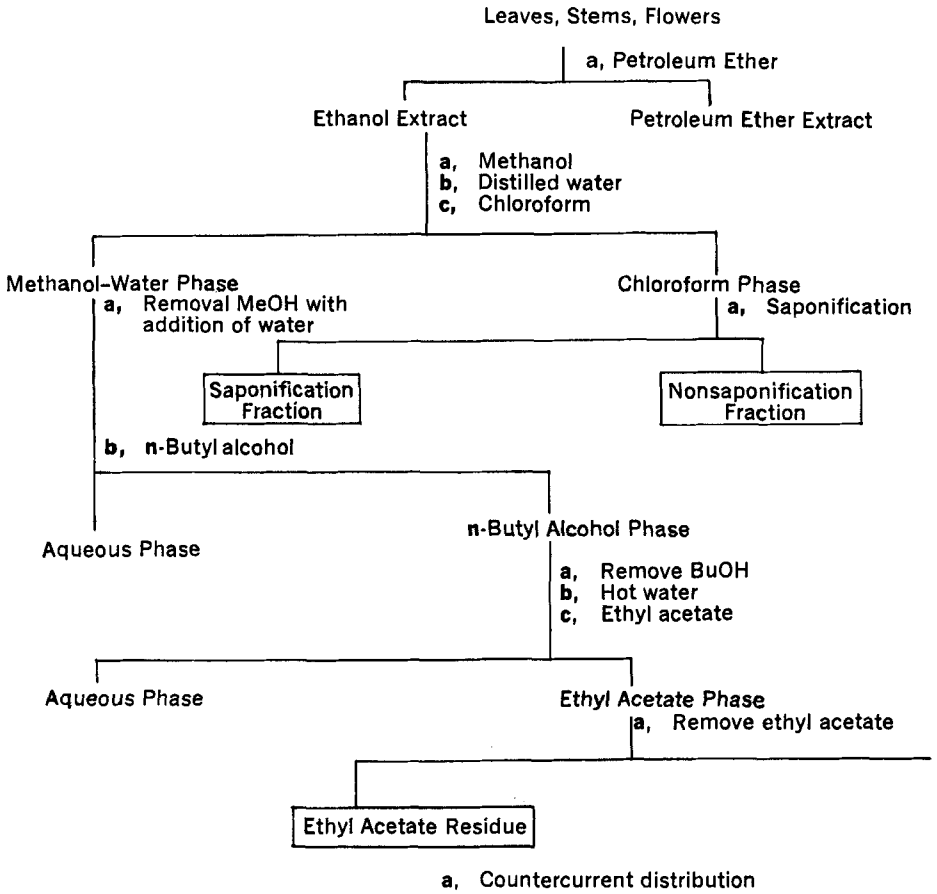


Fig. 1.—Final fractionation procedure of the ethanol extract.

flowering parts of *A. angustissima*.[1] A sedative effect was also observed in animals with the aqueous and ethanol extracts. Fractionation studies were initiated to isolate and identify the antitumor and sedative constituent(s).

EXPERIMENTAL

The plant was collected near Tucson, Ariz., and stored in polyethylene bags at −10° until the fractionation was undertaken. The preliminary extracts of *A. angustissima* were prepared and submitted to the screening center by Dr. Mary Caldwell and co-workers (College of Pharmacy, University of Arizona, and supported in part by CCNSC research contract SA-43-ph3754) in a project designed to screen plants indigenous to the southwestern United States and Mexico for possible antitumor activity.

General Extraction and Purification.—Nine-hundred grams of pulverized leaves, stems, and flowering parts were extracted in a 1-L. Soxhlet with 2.50 L. of petroleum ether (Baker's analyzed reagent, b.p. 30–60°) for 51 hr. The marc was air-dried for 30 min. and extracted in the Soxhlet with 2.50 L. of ethanol U.S.P. After 42 hr., the Soxhlet was recharged with 2.50 L. of fresh solvent and extracted a second time for 48 hr. The combined ethanol extracts were concentrated *in vacuo* to a viscous olive-colored residue weighing 147.8 Gm. The defatted ethanol extract was extracted with 1.60 L. of hot anhydrous methanol (analytical reagent grade) (200 ml. × 8), and distilled water (1.70 L.) was added to the mixture to facilitate separation of the two layers when extracting with chloroform. The mixture was extracted in a 4-L. separator with chloroform (400 ml. × 2, 200 ml. × 5) to remove chlorophyll. The rust-colored methanol–water phase was distilled *in vacuo* with the addition of 0.40 L. of water periodically throughout the distillation. The aqueous mixture was filtered and extracted with *n*-butanol (analytical reagent grade) (400 ml. × 1, 300 ml. × 1, 200 ml. × 9), and the alcohol phase was distilled *in vacuo* to a dark brown residue. The *n*-butanol residue was extracted with hot distilled water (200 ml. × 8), filtered, and extracted with ethyl acetate (500 ml. × 1, 300 ml. × 5). The ethyl acetate was removed *in vacuo* leaving a tan amorphous residue weighing 3.70 Gm. (See Fig. 1.)

The residue gave the following positive qualitative tests: Wilson's boric acid test (3, 4), magnesium and hydrochloric acid (5), ferric chloride (6), and 1%

[1] Antitumor testing was conducted by the Cancer Chemotherapy National Service Center, National Institutes of Health, U. S. Public Health Service, Bethesda, Md.

TABLE I.—COUNTERCURRENT DISTRIBUTION BANDS

Upper Phase			Lower Phase		
Fraction	Wt. Dried Residue, Gm.	Tubes	Fraction	Wt. Dried Residue, Gm.	Tubes
A	0.123	17–20	E	0.854	0–20
B	0.037	60–62	F	0.174	60–99
C	1.574	0–16, 21–30	G	0.225	21–59
D	0.472	31–59, 63–99			

gelatin test (5). These tests indicated possible condensed tannins and/or flavan[2] compounds. All attempts to crystallize the amorphous tan residue ended in failure. The substance did not melt but turned dark brown as the temperature increased.

One-Hundred Stage Countercurrent Distribution.—A 100-stage automatic countercurrent instrument[3] was used for the countercurrent distribution. The tan amorphous residue (3.70 Gm.) was dissolved in 500 ml. of lower phase solvent (ethyl acetate–methanol–water, 6:1:5) after the two phases had been saturated for 24 hr. in a 6-L. separator. The balance of the lower phase tubes were filled manually (100 ml./tube), and the upper phase solvent was added to the large tank. The upper phase (100 ml./tube), after completion of 100 cycles, was withdrawn by the fraction collector into 100 tubes, each tube receiving 100 ml. The lower phase was withdrawn manually, and both the upper and lower phase tubes were analyzed at three ultraviolet wavelengths to determine what tubes contained the chemical components. The contents of the upper phase tubes were examined at 260, 300, and 360 mμ, and the lower phase contents at 275, 300, and 360 mμ. The wavelengths were chosen at areas in the ultraviolet where flavan compounds generally show large absorption. Fractions A, B, C, D, E, F, and G were located in this manner. The solvent from each fraction was removed *in vacuo*, and the residues were dried in an oven at 50° for 2 hr. (See Table I.)

Fractions A, B,[4] C, and D gave positive Wilson's boric acid tests, negative magnesium and hydrochloric acid tests, and olive to dark brown colors with ferric chloride. Fraction F gave a positive magnesium and hydrochloric acid test and an olive color with ferric chloride. Fractions E and F gave negative magnesium and hydrochloric acid tests and questionable color tests with ferric chloride. Fractions E, F, and G were not tested with Wilson's boric acid due to a lack of solubility in acetone. Fractions A, C, and D also gave positive color tests with vanillin (7). These qualitative tests indicate condensed tannins or leucoanthocyanidins for fractions A, B, C, and D and a flavone or flavonol for fraction F.

Preliminary Paper Chromatography.—The procedure followed was essentially that of Gage (8). Whatman No. 1 paper, 46 × 57 cm., was cut into strips 22 × 57 cm. unless otherwise indicated. Fractions A, C, and D were spotted on the papers in methanol and irrigated in *n*-butanol–glacial acetic acid–water (BAW, 4:1:5), Forestal (glacial acetic acid–concentrated hydrochloric acid–water, 30:3:10), and 5% acetic acid. The developed chromatograms were observed in visible and ultraviolet light, sprayed with 3% toluene-*p*-sulfonic acid (TSA) and heated in an oven at 80° for 10–15 min. (9). Elongated pink to red spots or streaks on the paper after TSA spray usually indicate leucoanthocyanidin units, either in the condensed tannin form or the monomer unit (9, 10). Fractions C (R_f 0.95) and D (R_f 0.66) demonstrated positive pink-red spots after TSA spray.

Ultraviolet and Infrared Analysis.—Infrared and ultraviolet spectra were obtained on a Perkin-Elmer Infracord and a model 202 ultraviolet-visible spectrophotometer. All determinations on the contents of the countercurrent tubes to isolate the fractions were made on a Beckman DU spectrophotometer. Ultraviolet analyses were made by dissolving a given amount of the fraction in methanol and examining the solutions in silica cells along with a solvent blank. The infrared curves were obtained by distributing 1.20–1.80 mg. of sample in 0.40 Gm. of potassium bromide and pressing 0.3 Gm. of this material *in vacuo* into a clear disk with a hydraulic press.

Microfusion of Fraction D.—Fraction D was degraded by fusing with potassium hydroxide according to the method of Roux (11). The degradation products were separated into phenolic acids and phenols by the sodium bicarbonate method. The degradation products were identified, after spraying with ferric chloride (2%), in four solvent systems—BAW, Forestal, phenol–water (PW, 9:1), and 5% acetic acid—as β-resorcylic (II) and protocatechuic acids (III). (See Table II.)[5]

Synthesis of Leucoanthocyanidins.—Leucocyanidin (VIII) was prepared by reducing quercetin to dihydroquercetin by the method of Geissman and Lischner (12), and second, reducing dihydroquercetin with sodium borohydride to leucocyanidin (13, 14). Synthesis of 7,3′,4′-trihydroxyflavan-3,4-diol (I) and 7,3′,4′,5′-tetrahydroxyflavan-3,4-diol (VII) from dihydrofisetin and dihydrorobinetin, respectively, was accomplished in a one-step reduction with sodium borohydride (13, 14).

Conversion to Anthocyanidins.—Fractions A, B, C, D, leucocyanidin (VIII), 7,3′,4′-trihydroxy-, and 7,3′,4′,5′-tetrahydroxyflavan-3,4-diols (I, VII) were converted to anthocyanidins according to the method of Bottomley (14). The anthocyanidins were spotted on Whatman No. 1 paper and irrigated in two solvent systems, Forestal and 90% formic acid–3 *N* hydrochloric acid (1:1). (See Table III.) The spots were observed in visible and ultraviolet light. Fractions B, C, and D were identical to fisetinidin in all three solvent systems, and all four spots emitted a dull orange color under ultraviolet light.

Visible Spectrophotometer Analysis of Anthocyanidin Spots on Paper Strips.—The anthocyanidin spots of fractions B, C, D—fisetinidin (IV), ro-

[2] Flavan compounds are considered to be flavonols, flavones, leucoanthocyanidins, catechins, dihydroflavonols, and flavan.

[3] H. O. Post Scientific Instrument Co., Inc.

[4] Due to the small quantity of fraction B isolated, tests conducted on this fraction were limited throughout the study.

[5] The fusion products were also determined in BAW (6:1:2) and developed by spraying with diazotized sulfanilic acid (24). For the unknown phenolic acids, spots were revealed at R_f 0.83 (light brown), 0.92 (brown), identical to β-resorcylic acid (R_f 0.93, brown) and protocatechuic acid (R_f 0.84, light brown).

TABLE II.—R_f VALUES OF THE MICROFUSION PRODUCTS OF FRACTION D AND THE AUTHENTICATED SAMPLES IN FOUR SOLVENT SYSTEMS

Sample	BAW[a]	Forestal[a]	PW[a]	5% Acetic Acid[a]
Unknown phenolic acids	0.86G 0.94V	0.94G 0.96V	0.49G 0.53V	0.54BG[b]
Unknown phenols	0.35V[c]	...	0.42PY[d]	...
2,4-Dihydroxybenzoic acid (β-resorcylic acid)	0.95V	0.97V	0.57V	0.59V
3,4-Dihydroxybenzoic acid (protocatechuic acid)	0.89G	0.94G	0.52G	0.61G

[a] Color code: V, violet; Y, yellow; GR, gray; B, blue; PY, pale yellow; G, green; OL, olive. [b] One spot only was observed with 5% acetic acid. [c] A large elliptical spot extended from the starting line to the designated R_f value. [d] A continuous streak from the starting line to the designated R_f value.

TABLE III.—R_f VALUES OF SELECTED SYNTHETIC ANTHOCYANIDINS COMPARED TO FRACTIONS A, B, C, AND D

Fraction	Forestal[a]		Formic Acid–HCl[a]	
	Visible	U.V.	Visible	U.V.
A	[b]	0.73PY 0.90DO[c] 0.97Y	[d]	
B	0.89F	0.89DO 0.95FY	0.45P	0.45DO 0.58FY
C	0.88P	0.88DO 0.96FY	0.44F	0.44DO
D	0.88F	0.88DO 0.95FY	0.44F	0.44DO
Fisetinidin	0.89F	0.89DO 0.96FY	0.45P	0.13FY
Robinetinidin	0.80R	[b]	0.31R	0.31R FRo
Cyanidin	0.76R	[b]	0.23R	0.23FRo

[a] Color code: P, pink; R, red; Y, yellow; PY, pale yellow; DO, dull orange; FY, fluorescent yellow; FRo, faint rose. [b] There were no spots observed. [c] The dull orange was only faintly observed under U.V. [d] R_f value was not obtained in this solvent system.

binetinidin (V), and cyanidin (VI)—were cut out of the paper in thin strips so that they would fit into a silica cell. A blank strip was cut and placed in a second cell to serve as a blank sample, and the spots were examined in visible light (15–17). The absorption maxima in visible light for fractions B (530 mμ), C (529 mμ), and D (530 mμ), were identical to fisetinidin (529 mμ). Robinetinidin (V) and cyanidin (VI) absorbed at 540 and 549 mμ, respectively. An attempt was made to complex the compounds with aluminum sulfate (5%), causing a bathochromic shift; however, the shift did not occur but remained the same as previously.

RESULTS AND DISCUSSION

Examination of the countercurrent fractions by paper chromatography in three solvent systems, after spraying with TSA, revealed possible condensed tannins comprised of leucoanthocyanidin units for fractions C and D.

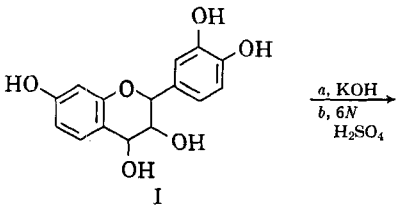
Ultraviolet absorption of fractions A, C, and D produced identical absorption maxima at 209–210 and 282–283 mμ. The latter was the general area of absorption for catechins (18), dihydroflavonols and leucoanthocyanidins (19), condensed tannins (10) and hydrolyzable tannins (20). Fraction F had a broad absorption band at 350 mμ in ultraviolet light, an indication of a flavone or flavonol compound (21). None of the other countercurrent fractions absorbed at the 350 mμ wavelength.

Infrared studies were made on fractions A, C, and D. The curves were similar, except A and C had one absorption peak at 9.80 μ that D did not have. The infrared absorption peaks might possibly indicate the following: 2.95 μ, hydroxyl OH group; 7.40, 7.90, 9.05 μ secondary alcohols; 7.40 and 8.72 μ, phenols; 12.0 μ, two adjacent aromatic CH absorption frequencies. Other investigators have demonstrated similar but not identical infrared spectra for condensed tannins and leucoanthocyanidin (10, 22, 23). Fractions A, C, and D were similar to the infrared curves found in the literature for condensed tannins and leucoanthocyanidins but not identical.

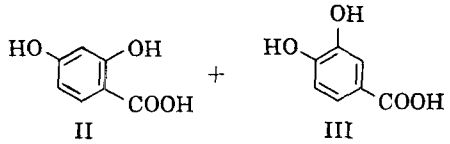
To establish further the identity of fractions B, C, and D, fraction D was degraded by fusing with potassium hydroxide, separated into possible aromatic phenols and aromatic phenolic acids, and identified by paper chromatography in four solvent systems. (See Table II.) The degradative products were identified as β-resorcylic (II) and protocatechuic acids (III). The identification of II and III as degradative products of fraction D would indicate that the structure is a 7,3′,4′-trihydroxyflavan-3,4-diol moiety (I). (See Scheme I.)

Leucoanthocyanidins and condensed tannins comprised of leucoanthocyanidins units are unique because they may be converted to anthocyanidins by heating in 2-propanol containing 5% hydrochloric acid.

An example of this would be the conversion of 7,3′,4′-trihydroxyflavan-3,4-diol (I) to the respec-

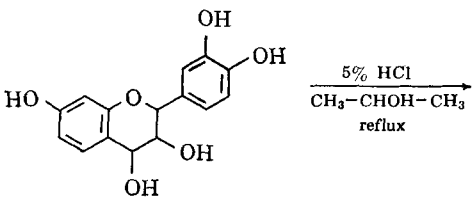


I



II III

Scheme I



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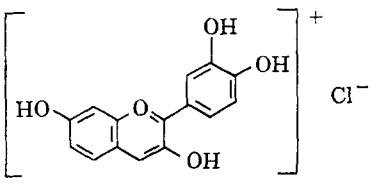
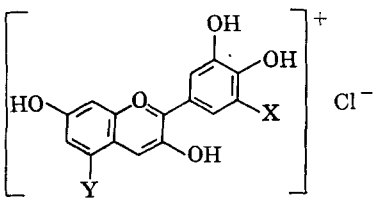
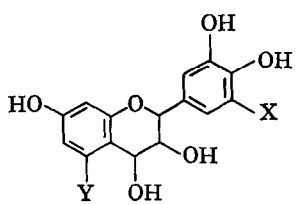


IV

Scheme II



(IV) X,Y = H fisetinidin
(V) X = OH, Y = H robinetinidin
(VI) X = H, Y = OH cyanidin



(VII) X = OH, Y = H leucorobinetin
(VIII) X = H, Y = OH leucocyanidin

tive anthocyanidin, fisetinidin (IV). (See Scheme II.) The anthocyanidins, after removal of the phlobatannins, were spotted on paper strips and compared to fisetinidin (IV), robinetinidin (V), and cyanidin (VI). Fractions B, C, and D were identical to fisetinidin in both solvent systems. (See Table III.) These results agree with those obtained from the potassium hydroxide fusion and indicate a 7,3′,4′-trihydroxyflavan-3,4-diol moiety (I) for fractions B, C, and D.

The amorphous nature of fractions B, C, and D, in addition to the different R_f values obtained when examined by paper chromatography, indicated that the leucoanthocyanidin moiety (I) was present in the condensed tannin form in these fractions. An explanation for their different R_f values would be that the extent of polymerization or molecular weight varies between fractions, yet each condensed tannin contains the same basic leucoanthocyanidin unit (I).

Antitumor results are inconclusive at this time. Further biological tests and isolation studies are currently in progress to determine if the antitumor and sedative activity resides in the condensed tannin-leucoanthocyanidin fractions.

SUMMARY

A phytochemical study of *A. angustissima* has established the identity of the leucoanthocyanidin unit 7,3′,4′-trihydroxyflavan-3,4-diol in three of the seven countercurrent fractions.

A fourth countercurrent fraction has been tentatively characterized as a flavone or flavonol.

Further studies are being undertaken to determine the extent of polymerization of the three fractions, isolate the monomer leucoanthocyanidin, and determine whether the antitumor and sedative properties reside in the condensed tannin-leucoanthocyanidin fractions or other flavan compounds present in *A. angustissima*.

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