Phytochemical and Pharmacological Investigation of *Anemopsis californica*

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Anemopsis californica was the subject of a phytochemical and pharmacological investigation to determine the plant principle responsible for its reported antispasmodic activity. The extraction procedure yielded among various fractions, leucoanthocyanidins, an unidentified crystalline fraction, and 4-allylveratrole, which was the most active antispasmodic agent. The possibility of antitumor activity attributed to this plant could not be verified by the screens employed.

An infusion of the rhizomes and roots of *Anemopsis californica* (Nutt.) Hook and Arn. (Yerba del Mansa) was used by the Spanish Americans and Pima Indians to cure a variety of ailments for many years (1); in 1916, it was considered the favorite antispasmodic of the Mexicans (2). Found in the southwestern United States and northern Mexico, this plant is the only species of the genus *Anemopsis* and is the type species for the *Saururaceae* family.

In 1957, Horton and Paul (3) identified methyleugenol (4-allylveratrole) in this plant, but no further work with anemopsis has been reported. The 1959 extracts of the plant were screened for possible antitumor activity; although preliminary tests were favorable, improvements in the testing program establishing higher criteria for activity resulted in this plant being dropped from further testing.

The purpose of this study then became the characterization of the constituent responsible for the antispasmodic activity. Preliminary extracts were prepared in the same manner as the native preparations and tested for antispasmodic activity. Later studies were made with purified fractions.

EXPERIMENTAL

Materials.—All chemicals, except technical petroleum ether, were of reagent quality. Infrared spectra were obtained with a Perkin-Elmer Infracord spectrophotometer. Chromatographic eluates were collected in 10-ml. amounts by an automatic fraction collector (Research Specialties Co.). All melting points were obtained using a Reichert Co. Kofler hot stage. Isolated intestine antispasmodic evaluations were performed with a Statham strain gauge (GL-4-250) connected to an amplifier (Brush Electronics Co.) and dual pen recorder (RD-2321-00). The roots and rhizomes of *A. californica* used in the investigation were marketed by La Nacional Mexican Products, Tucson, Ariz., and authenticated by personnel of the University of Arizona Herbarium.

General Plant Extraction Procedure.—Approximately 2 Kg. of dried anemopsis was ground in a Wiley mill fitted with a 2-mm. mesh sieve. Within 0.5 hr. after grinding, the powder was placed in a 5-gal. container and successively macerated for 24-hr. periods in the following solvents for the number of replications indicated: petroleum ether ×2, dichloromethane ×0, 95% ethanol ×1, and 93% ethanol acidulated with 2% HCl ×0. Following each maceration period, the slurry was filtered and the residual marc remacerated with fresh solvent. Each filtrate obtained was concentrated to a paste under vacuum.

The extraction procedure above was modified slightly by the addition of two methanol maceration steps preceding the acidulated ethanol extraction. Methanol, a selective solvent for leucoanthocyanidins, should have removed these plant constituents before the acidulated ethanol converted them to anthocyanidins (4).

Plant Extract Purification Procedures.—Portions of the crude methanol extract produced a positive leucoanthocyanidin color test when heated after being treated with bis-2-(2-methoxyethoxy) ethyl ether[1] and HCl. To isolate the leucoanthocyanidin portion of the methanol extract, the concentrate was successively extracted with petroleum ether, *n*-butanol, toluene, acetone, and water. The residual water-insoluble powder produced the most pronounced positive leucoanthocyanidin color reaction. The tan powder proved to be ineffective against p-1534 leukemia[2] and was a weak antispasmodic (Table II, Sample *A*).

Purification of the Acidulated Ethanol Concentrate.—A 20-Gm. portion of the concentrate was thoroughly mixed with 25 Gm. of aluminum oxide, and the mixture was washed with three 50-ml. portions of benzene, followed by two 50-ml. portions of water. The remaining 7 Gm. of purple powder and associated aluminum oxide was placed on a 600 × 50-mm. chromatographic column packed with an aluminum oxide–petroleum ether slurry. The column was developed with the following solvents: 1.0 L. of benzene, 1.0 L. of *n*-butanol, and 2.0 L. of acidulated ethanol. The dried purple powder from the *n*-butanol soluble eluate was found to possess no antitumor activity.

Purification of Unknown Crystals Removed from the Petroleum Ether Concentrate.—A small amount (0.05%) of crude green crystals was removed from the concentrate. After the crystalline needles were purified by refluxing with successive 25-ml. portions of heptane, followed by recrystallization from methanol, a 0.012% portion remained. The needles melted between 119.5–120.5° and possessed

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[1] B.p. 120–122° at 3 mm. Marketed by Matheson Coleman and Bell, Norwood, Ohio.
[2] Tested by Eli Lilly Co., Indianapolis, Ind.

TABLE I.—RANKING SYSTEM USED TO MEASURE SPASMODIC PROTECTION

Spasmodic Values, %[a]	Arbitrary Ranking No.
0–25	1
26–50	2
51–66	3
67–75	4
76–80	5
81–85	6
86–90	7
91–95	8
96–100	9
Relaxation of muscle tension	10

[a] The plant extract spasmodic protection values were obtained by calculating the percentage decrease in methacholine chloride intestine stimulation *versus* the preceding standard.

no antispasmodic activity. Further studies on the structure of this compound are being conducted.

Purification of a Yellow Oil from the Petroleum Ether Concentrate.—A portion of the concentrate was freed of insolubles by filtering at −20°. Twenty-five grams of the filtered oil was placed on a chromatographic column composed of 680 Gm. of aluminum oxide, 200 ml. of 0.25 *M* aqueous Na_2HPO_4, and petroleum ether. The first colored band (yellow) eluted from the column was collected in tubes 1–21. Tube 2 contained 1.1 Gm., and 21 contained 0.4 Gm. Portions of the yellow oil from each of the 21 tubes were dissolved in heptane and shaken with an equal quantity of methanol. The clear heptane layer was removed and purified by steam distillation. The antispasmodic test results were entered in Table II, Sample *D*.

The distilled oil possessed an enhanced antispasmodic activity and a very bitter taste; a few milligrams of oil placed on the tongue elicited a persistent mouth-watering saline taste for 7 days. The purified oil possessed an infrared spectrogram characteristic of a published spectrum of 4-allylveratrole,[3] a synthesized sample, and an authentic sample.[4]

PHARMACOLOGY

Experimental.—The Magnus isolated intestine longitudinal muscle technique (5), including modifications indicated below, satisfied the need for a rapid antispasmodic screening test. An adult guinea pig was fasted for 24 hr. Its excised intestines were immediately chilled in an ice-jacketed aerated container of Sollmann-Radamaeker's solution (S.R. solution) (5). A cleaned intestinal segment was pierced at both ends by a hook affixed *via* a thread to an arm of a strain gauge. The remainder of the segment was looped under a fixed hook sealed in a movable glass aerating tube. After the segment was lowered into an 80-ml. smooth muscle bath, a constant drug-free muscle tension of 15 Gm. was reapplied as needed throughout the experiments. The 15-Gm. base-line tension was recorded at the 10-mm. height on the strip chart when the amplifier Chart Multiplier (Brush Electronic Co.) was set at position 5.

After the isolated intestinal segment became acclimated to its artificial muscle bath environment, it was subjected to an alternating series of 3.12 mcg./ml. of methacholine chloride[5] in 37° S. R. solution, followed by two 80-ml. 37° S.R. solution washes. This procedure was repeated until the methacholine chloride-induced smooth muscle stimulation, *i.e.*, that stimulation appearing immediately after the methacholine chloride addition, increased to a maximum contraction two or more times. The maximum contraction obtained was considered the standard, or 100% level, for only that plant extract or known drug tested immediately following the standard and two smooth muscle bath washings. A methacholine chloride stimulation and two S.R. solution washes constitute one trial.

Each plant extract or drug was permitted to contact the intestinal segment for 2.5 min. before the second series of 3.12 mcg./ml. quantities of methacholine chloride and S.R. solution washings were added to the muscle bath. The test sample's antispasmodic intensity, or spasmodic protection, was recorded as the percentage decrease in its isolated intestine-stimulation intensity from the preceding standard. After the first spasmodic protection percentage had been obtained, the intestinal segment was washed with two 80-ml. portions of S.R. solution, and another portion of methacholine chloride was added. After recording the second spasmodic protection percentage, the methacholine chloride additions and washings were repeated until the antispasmodic effect of the test sample disappeared, and a new standard was obtained. This technique furnished several spasmodic protection values for each of the 5 to 20 test samples from one intestinal segment. The protection values were subsequently grouped into arbitrary ranks (Table I).

Water-insoluble plant extracts or drugs were suspended in aqueous methylcellulose U.S.P. solutions[6] before they were added to the smooth muscle bath. Phenolic plant extracts or chemicals were incompatible with aqueous methylcellulose, but these test samples and others were soluble in bis-2-(2-methoxyethoxy) ethyl ether (Table II, Sample *F*). At low smooth muscle bath concentrations (3 mg./ml.), the solvent was well tolerated by the isolated tissue and produced negligible antispasmodic activity. The Cancer Chemotherapy National Service Center, however, found this solvent too toxic for injection into intact animals.

Data and Results.—Several anemopsis extracts, such as 10% aqueous percolate, protected the isolated intestine against $BaCl_2$ stimulation but not against acetyl-β-methacholine. Plant samples that afforded spasmodic protection against methacholine chloride proved also to be even more active against $BaCl_2$ stimulation.

The results in Table II indicate that the more potent antispasmodics also possess a longer duration of action. An extension of the drug-intestinal segment contact time beyond 2.5 min. (test sample *C*) also prolonged the antispasmodic activity. Test Sample *H* was a more potent antispasmodic than any plant extract tested. When plant extracts were dissolved in 0.25 ml. (3125 mcg./ml.) of bis-2-(2-methoxyethoxy) ethyl ether, the antispasmodic

[3] Sadtler Research Laboratories, infrared spectrogram No. 17520.

[4] Eugenol Methyl Ether. Marketed by K and K Laboratories, Jamaica, N. Y.

[5] Marketed as Mecholyl by Merck, Sharp and Dohme, West Point, Pa.

[6] A biological technique used by Eli Lilly Co., Indianapolis, Ind.

TABLE II.—PROTECTION AGAINST METHACHOLINE CHLORIDE-INDUCED SPASMS

	Test Sample[a]	Concn., mcg./ml.	Methacholine Chloride Concn., mcg./ml.	Ranking Each Trial, No.[b] 1st	2nd	3rd	4th
A	Water-insoluble portion of methanol extract	125	0.78	2	↑	—	—
B	Petroleum ether extract	125	3.12	6	3	—	—
		375	3.12	8	2	2	—
		375	3.12	10	—	1	—
		375	3.12	9	—	3	2
		375	3.12	9	—	3	2
	Chromatographed petroleum ether extract, center of band, tube No. 8	62	3.12	6	—	1	—
		125	3.12	6	2	1	—
		125	3.12	7	3	↑	—
		250	3.12	9	5	3	2
		500	3.12	10	8	7	6
	Bath contact time, 30 min.	500	3.12	8	7	10	10
D	Steam distilled oil from tubes 1–21	42	3.12	3	2	—	—
		125	0.78	9	4	—	—
E	Synthesized 4-allylveratrole	62	3.12	7	1	1	—
		125	3.12	8	↑	—	—
		250	3.12	8	5	1	↑
		500	3.12	8	7	2	1
F	Bis-2-(2-methoxyethoxy) ethyl ether	6,250	3.12	1	↑	—	—
		10,000	0.78	2	—	—	—
		12,500	3.12	2	1	—	—
		12,500	3.12	3	1	—	—
G	Steam distilled pine oil	125	0.78	7	2	1	—
		125	3.12	7	3	1	—
		250	3.12	10	9	2	—
H	Bentyl[c]	125	3.12	9	9	9	8

[a] *A–F* refers to products in the text. [b] —, not tested; ↑, an increase of intestinal muscle contraction force over that of the preceding methacholine chloride standard. [c] β-Diethylaminoethyl-1-cyclohexyl-cyclohexane-carboxylate, provided by William S. Merrell Co., Cincinnati, Ohio. (Required seven trials to re-establish the methacholine chloride standards.) Trials 5, 6, and 7 gave rankings of 5, 3, and 1, respectively.

contribution by the solvent was negligible. In the crude petroleum ether concentrate, 4-allylveratrole constituted about 70% of the weight. All other fractions from the concentrate possessed less antispasmodic activity than the latter.

SUMMARY

The medicinal history of *A. californica* indicated that its crude extracts possessed antispasmodic properties. This investigation of the plant extracts indicated that the potent antispasmodic principle was 4-allylveratrole. However, pharmacological test results ranked a commercial product,[7] far above that of 4-allylveratrole.

A modification of the Magnus isolated intestine longitudinal muscle method for antispasmodic evaluation was used. The modifications permitted the use of replicate pharmacological tests on one test sample, and a single intestinal segment was successfully used for as many as 20 different plant samples.

Substantially higher smooth muscle bath concentrations of some plant extracts produced an apparent methacholine chloride reversal; the observations were recorded, and no attempt was made to substantiate this finding.

Many water-insoluble plant extracts were found to be soluble in bis-2-(2-methoxyethoxy) ethyl ether. The use of this solvent at low concentrations (3 mcg./ml.) in the antispasmodic bioassay was successful.

Selected plant fractions subjected to antitumor screening first gave positive results, but later the extracts failed to pass confirmatory tests.

[7] Bentyl. Marketed by the William S. Merrell Co., Cincinnati, Ohio.

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