Viopudial, a Hypotensive and Smooth Muscle Antispasmodic from *Viburnum opulus* (36479)

JOHN A. NICHOLSON, THOMAS D. DARBY, AND CHARLES H. JARBOE

Department of Pharmacology, University of Louisville School of Medicine, Louisville, Kentucky 40201

The American variety of *Viburnum opulus,* native to the northern and eastern United States, is commonly referred to as the "Cranberry Bush" or "High Bush Cranberry" and is currently used as a decorative plant. In the past, extracts of the bark and leaves were used by the North American Indians as a diuretic, a tobacco substitute, for the treatment of swollen glands and mumps, and for eye disorders (1, 2). Questionable reports of favorable therapeutic results obtained from the use of *Viburnum* tinctures in certain uterine disorders date back to the mid-1800's (3), the results being related to an alleged antispasmodic effect. Recently we demonstrated that alcoholic and aqueous bark extracts of *V. opulus* were indeed capable of relaxing the *in vitro* barium stimulated rat uterus (4); scopoletin was shown to be partially responsible for this effect (5).

At this time we wish to report a second active constituent of *V. opulus,* a new nonalkaloidal material isolated from plant bark. This substance has also shown striking cardiovascular effects on the rat, cat and dog, in addition to antispasmodic effects on *in vitro* rat uteri.

Materials and Methods. Fifty pounds of authentic *V. opulus* bark was extracted with water in an Eppenbach stirrer and the aqueous solution back-extracted with methylene chloride. The methylene chloride was vacuum evaporated, leaving a highly aromatic, dark brown, viscous oil (93.0 g). The crude oil was chromatographed on a 3.5 × 7.5 cm neutral alumina (Woelm; activity grade 1) column in 20 g lots. Each column was eluted with 450 ml of chloroform which was removed under water-pump vacuum, leaving a dark brown, viscous oil (87.5 g). The oil was mixed with 6 vol of sand and the resulting mixture continuously extracted with petroleum ether (40–60°) for 2 weeks. The solvent was evaporated to 300 ml and 200 ml ethyl ether was added to solubilize the petroleum ether extractables. The cooled solution was extracted with cold 1 *N* NaOH (5 × 75 ml), followed by cold 1 *N* HCl (2 × 75 ml). After drying over $MgSO_4$ the solvent was removed under water-pump vacuum to leave an aromatic oil (15.11 g) which was taken up in methanol, cooled in a dry ice–acetone bath, and filtered to remove the precipitated fats and waxes. Removal of the methanol left an oil (12.0 g) which was chromatographed in 4 g aliquots on a 3 × 16 cm silicic acid column. Each column was eluted with a mixture of ethyl ether:petroleum ether (1:4). Sufficient solvent (approximately 200 ml) was used to elute a bright yellow band. This band was immediately preceded by a broad, light brown band, the first colored material to be eluted from the column. Following the elution of the yellow band, the column was eluted with an additional 250 ml of the same solvent mixture and the solvent removed from this second fraction by evaporation. Thin-layer chromatographic (tlc) analysis of the residual oil on Silica Gel H (Brinkman) and elution with ethyl ether, showed a major component at R_f 0.77 when developed with iodine. This component was also observed upon gas–liquid chromatographic (glc) analysis using a 5 ft × ¼ in. 10% SE-30/60–80 mesh Chromosorb W column, with a carrier gas flow of 100 ml/min and a column temperature of 220°; the retention time was 15 min. Final purifica-

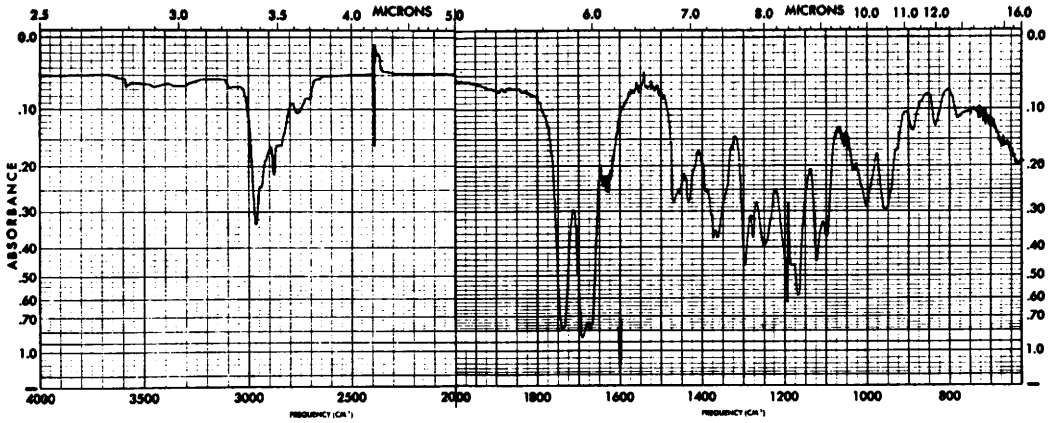

FIG. 1. Infrared spectrum of viopudial. Thin-film spectrum taken on NaCl plates with air as reference. Instrument: Perkin-Elmer 237 Grating Infrared Spectrophotometer.

tion of the compound was accomplished by rechromatographing the oil on one or two additional silicic acid columns, as needed, and monitoring the fractions by tlc and glc. After elimination of the solvent, the appropriately combined fractions gave 2.03 g of a pale yellow liquid, for an overall yield of 0.009%. Spectroscopic and elemental analyses showed this material to be a sesquiterpene dialdehyde, and it was thus termed "viopudial." The infrared spectrum of viopudial is seen in Fig. 1.

The antispasmodic activity of viopudial was studied on the *in vitro* barium stimulated rat uterus using a previously described method (4). Cardiovascular effects on the pentobarbital anesthetized rat, cat and dog were observed upon iv administration of viopudial. Blood pressure measurements were taken from the right femoral artery using a Stathum pressure transducer and myocardial contractility was determined using a Walton–Brodie strain-gauge arch attached to the right or left ventricle. The results were recorded on a Grass Polygraph.

Results and Discussion. During the initial stages of the isolation of viopudial, the various steps were followed by bioassay on the *in vitro* barium stimulated rat uterus. As purification progressed, it was determined by tlc that the fractions were becoming less complex. Continued monitoring by tlc and glc showed the fractions also becoming more concentrated in one of the major components. Using the glc operating parameters previously described, a 10 mg sample of this major component was obtained by preparative glc on the SE-30 column. Verification that this ma-

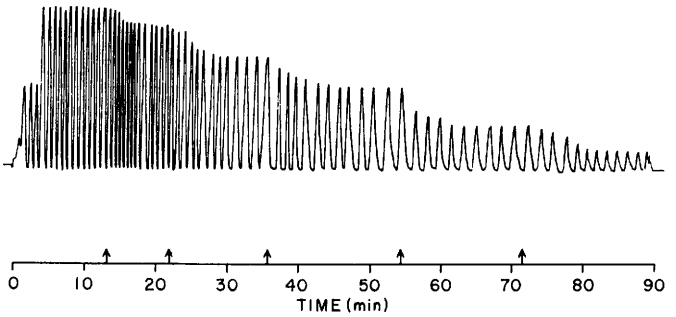

FIG. 2. Viopudial inhibition of barium stimulated rat uterus. Uterus was stimulated with $BaCl_2$ at a bath concentration of 6.4×10^{-2} mg/ml. Addition of 12.5 μg viopudial in 0.05 ml propyleneglycol was made at times indicated by arrows. Previous control experiments showed no appreciable effect upon addition of the vehicle.

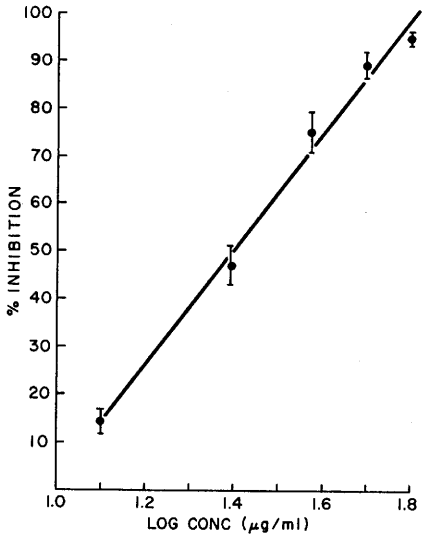

FIG. 3. Cumulative dose–response of viopudial on *in vitro* rat uterus. Each point consists of the mean response from bioassay of 12 uterine horns prepared from 6 rats of the same age, sex, weight, and strain. The straight line was determined by the method of least squares.

terial was greater than 99% pure was obtained by: 1. glc analysis on the SE-30 column. 2. glc analysis on a 5 ft × ¼ in. 5% XE-60/60–80 mesh Chromosorb W column at 155° with a carrier gas flow of 100 ml/min N_2. 3. tlc analysis with six different solvent systems (ethyl acetate, benzene, acetone, methanol, dioxane, and chloroform), all of which showed a single spot when developed in an iodine atmosphere. This purified substance was shown to possess strong antispasmodic effects on the *in vitro* rat uterus.

When the bath concentration of the barium stimulated rat uterus preparation was increased by five 12.5 μg increments of viopudial in propylene glycol over a 90 min period, a series of stable inhibited contractions was obtained (Fig. 2). The ED_{50} for the spasmolytic effect of viopudial was 24 μg/ml as determined from the log dose–response curve (Fig. 3). The stability of the inhibited contractions indicated the active component, whether the parent compound or a metabolite thereof, to be relatively stable in this *in vitro* preparation. Prior administration of propranolol, a β-adrenergic blocking agent, did not inhibit the antispasmodic effect of viopudial. Tolazoline, an α-adrenergic blocking agent, also gave no inhibition. These data, plus the fact that atropine was likewise found to have no blocking effect on the viopudial activity, indicated that at least on the rat uterus, viopudial acts as a musculotropic agent.

In vivo rat experiments showed hypotensive effects at an iv dose of 250 μg/kg. At 1 mg/kg these effects became somewhat sustained and the decrease in blood pressure was accompanied by a decrease in heart rate. Further experiments in both cats and dogs showed

TABLE I. Cardiovascular Effects of Viopudial in the Dog.[a]

	Control (± SE)	iv Viopudial (± SE)	% Change	p (t)	Time (± SE)
Systolic BP (mm Hg)	123.9 (± 6.8)	93.9 (± 10.2)	− 24.2	< 0.05	
Diastolic BP (mm Hg)	92.2 (± 4.9)	58.9 (± 8.3)	− 36.1	< 0.01	
Heart Rate (beats/min)	128.0 (± 8.2)	102.0 (± 7.2)	− 20.3	< 0.05	
IST	10.9 (± 1.0)	7.9 (± 0.9)	− 27.5	< 0.10	
Onset of action (sec)					20.9 (± 0.2)
Maximum response (sec)					38.0 (± 1.4)
Duration of action (min)					2.1 (± 0.3)

[a] Values represent means obtained from three iv doses of viopudial (2 mg/kg) in each of three dogs anesthetized with pentobarbital (30 mg/kg). Isometric systolic tension (IST) was recorded as mm pen deflection on the chart paper. All values were recorded at the point of maximum change from the control. Values for onset of action, maximum response, and duration of action are with respect to viopudial's effect on the blood pressure. Viopudial was administered in 35% EtOH at a concentration of 1.4%. Statistical level of significance was obtained from paired *t*-test.

TABLE II. Viopudial Inhibition of Acetylcholinesterase in the Dog.[a]

Sample	Arterial blood pressure (mm Hg)	% Change
Control	145/85	
ACh		
Saline	120/50	— 17/41
Control	145/85	
ACh		
Serum	135/65	— 7/23
Saline		
Control	150/80	
Viopudial		
Saline	145/80	— 3/0
Control	155/80	
ACh		
Viopudial	100/25	— 35/69
Serum		
Control	155/75	
ACh		
Viopudial	105/30	— 32/60
Saline		

[a] Sample composition consisted of 1 ml of each component except saline, which was used to bring total sample volume to 3 ml. ACh (acetylcholinesterase), 10 μg/ml; viopudial, 500 μg/ml (35% EtOH). Total volume of each injected sample was 3 ml.

the same results, but at somewhat higher doses.

Table I shows the cardiovascular effects of iv viopudial (2 mg/kg) in pentobarbital anesthetized dogs. Normal reflex stimulation of the heart, an expected result of the hypotensive effect, was effectively inhibited as noted by the significant decrease in both myocardial contractility and heart rate. These cardiovascular effects were not blocked by a prior 1 mg/kg dose of atropine.

Similar cardiovascular effects were obtained in cats, and in both cats and dogs the characteristic increase in arterial blood pressure resulting from temporary bilateral carotid artery occlusion was not affected by prior administration of a 2 mg/kg dose of viopudial. Thus the inhibition of sympathetic effects was ruled out as a possible mechanism of action. In addition, the hypotensive effect of viopudial was shown to be uninhibited by the prior administration of diphenhydramine, an antihistaminic agent. Thus the liberation of histamine by viopudial was also eliminated as a possible mechanism of action.

Due to the cardiac inhibitory effects of viopudial, the inhibition of cholinesterase and consequent potentiation of acetylcholine activity was considered a possible mechanism of action. Although it had previously been shown that atropine did not block the cardiovascular effects of viopudial, it was thought this could be due to a strong inhibition of cholinesterase, thereby resulting in levels of acetylcholine sufficient to competitively overcome the atropine blockade.

When dog serum was mixed with acetylcholine, both in the absence and presence of a pharmacologically insignificant dose of viopudial, and the sample bioassayed for acetylcholine by injecting it iv into the dog, it was found that viopudial potentiated the action of acetylcholine. This is shown in Table

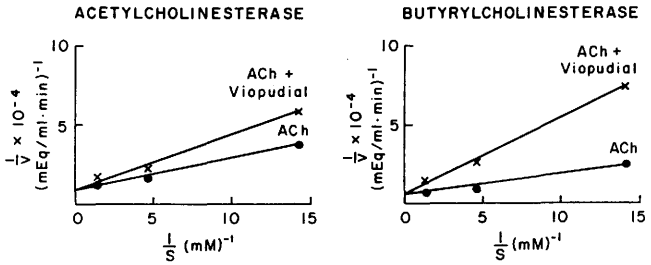

FIG. 4. Viopudial inhibition of cholinesterase. Acetylcholinesterase was from bovine erythrocytes. Butyrylcholinesterase was from horse serum.

TABLE III. Comparison of Viopudial and Physostigmine Inhibitory Constants with Acetylcholine as Substrate.

Agent	Enzyme	Source	$-\log K_i$
Physostigmine	Acetylcholinesterase	Electric eel	7.2
Viopudial	Acetylcholinesterase	Bovine erythrocytes	3.3
Viopudial	Butyrylcholinesterase	Horse serum	3.7

II. The decrease in arterial blood pressure due to a dose of acetylcholine was appreciably diminished upon the addition of serum to the sample immediately prior to the bioassay. After demonstrating that a 500 μg dose of viopudial was pharmacologically insignificant, a mixture of viopudial, serum and acetylcholine was similarly bioassayed; the effect of acetylcholine was appreciably greater than that of a sample containing no viopudial. Thus, viopudial appeared to be inhibiting the action of cholinesterase, resulting in the potentiation of acetylcholine. That this proposed enzymatic blockade is essentially complete was shown by the final sample which consisted of saline, acetylcholine, viopudial, and no serum. In the presence and absence of injected serum cholinesterase, the decrease in arterial blood pressure was of the same magnitude, thus demonstrating the effectiveness of viopudial in potentiating the effect of acetylcholine.

Rather than repeat the above procedure in an attempt to substantiate the anticholinesterase activity of viopudial, an *in vitro* experiment was used to obtain evidence of this activity. Using a Radiometer Automatic Titrator, the rate of acetylcholine hydrolysis was ascertained by determining the rate at which standard NaOH had to be added to maintain the pH of the reaction at 7.4. Figure 4 shows that when the rate of hydrolysis of acetylcholine was determined at various substrate concentrations in the presence and absence of viopudial, and Lineweaver–Burk plots constructed, viopudial acted as a competitive inhibitor of acetylcholinesterase and butyrylcholinesterase. The calculated inhibitory constants are shown in Table III and compared with the known value for physostigmine (6).

Summary. The administration of viopudial, a *Viburnum opulus* component, produces bradycardia, hypotension, and some decrease in myocardial contractility. Experimental evidence indicates that viopudial's mechanism of action is partly due to its effects on cholinesterase. *In vitro* demonstrations of a competitive inhibitory effect on both acetylcholinesterase and butyrylcholinesterase showed viopudial to be relatively weak when compared to the known potent inhibitor, physostigmine. Additional mechanistic effects, such as a direct musculotrophic action, may also be responsible for the overall activity.

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