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Technical Report R 439

MECHANISM OF RODENTICIDAL ACTIVITY OF *GLIRICIDIA SEPIUM*

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BUREAU OF YARDS AND DOCKS

U. S. NAVAL CIVIL ENGINEERING LABORATORY

Port Hueneme, California

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MECHANISM OF RODENTICIDAL ACTIVITY OF *GLIRICIDIA SEPIUM*

Technical Report R-439

Y-R011-01-01-055

by

Harry Hochman, Ph D

ABSTRACT

A study was made of the mechanism by which Gliricidia sepium (Yaite) exerts its rodenticidal properties. Extraction of the leaves of this plant, followed by physical and chemical fractionation, revealed the presence of coumarin as a constituent of the phenolic fraction. Consideration of the conditions under which these leaves are used as rodenticides, the known bacterial conversion of coumarin into the hemorrhagic agent dicoumerol, and pathological evidence in rats fed on incubated leaves point to coumarin as the basis for the rodenticidal properties of this plant.

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The Laboratory invites comment on this report, particularly on the results obtained by those who have applied the information.

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INTRODUCTION

When one considers the almost ideal conditions for growth and reproduction in a tropical climate, the proliferation of animal and plant species and even subspecies is readily understandable. To survive under these conditions, a plant must either grow so profusely as to outgrow its competitors and predators or contain chemical substances that are repellant or toxic to most insects and animals. Many plant species do produce toxic chemical substances.

Thousands of species of tropical plants have been tested for toxicity to insects, and a number have been found which are sufficiently toxic to some insects to be of potential commercial value. No species contains components that are toxic to all insects.

Among the many wood species that have been tested, one stands out as being unique. In addition to being toxic to some insects, it is also toxic to rats. This plant, Gliricidia sepium, is also known in Central America as Mata Ratas (rat killer) or by its Indian name, Yaite, and is commonly used by the natives of Central America as both a rodenticide and an insecticide.

Gliricidia sepium has two additional uses which one would not normally associate with a toxic plant. First, the tender young shoots are nontoxic to humans and are considered to be a delicacy in some parts of Central America. Second, silage composed of two-thirds corn and one-third Gliricidia sepium leaves is more acceptable to and shows greater weight gains for Red Sindhi cattle[1] than either plant alone.

In a letter to the editor of the journal "Pest Control," Williams[2] reported on the use of Gliricidia sepium leaves, which he called Yaite, as a rodenticide in southern Mexico and emphasized its lack of toxicity to children and pets. Since the Bureau of Yards and Docks has cognizance over all housing units belonging to the Navy and since a safe rodenticide and insecticide is desirable, the elucidation of the chemical structure of the component responsible for the peculiar properties of Gliricidia sepium was undertaken at the Naval Civil Engineering Laboratory.

EXPERIMENTAL PROCEDURES

The Gliricidia sepium leaves used in the experimental work at this Laboratory were obtained from two sources. One batch of dried leaves and stems was obtained from the United States Department of Agriculture, Agricultural Research Service,

Corps Research Division, Beltsville, Maryland. This sample was left over from a previous experiment. The other batch of leaves was obtained from Mr. Charles R. Southwell and Mr. Charles W. Hummer, Jr., who were with the U. S. Naval Research Laboratory, Canal Zone Corrosion Laboratory, Canal Zone.*

Processing of Gliricidia Sepium

The leaves and small stems in the two samples of Gliricidia sepium were separated and ground separately in a Wiley mill to pass a 20-mesh screen. Weighed quantities of the ground material were then extracted with organic solvents by percolation or by steeping.

Solvent extraction also removed large quantities of pigments, waxes, and other undesirable plant components. After removal of the solvent, the extracted material was separated into steam-distillable and nonsteam-distillable fractions. It can readily be seen from Table I that extraction by acetone or ethyl ether followed by steam distillation is more productive than direct steam distillation. This can be seen in detail in the following description of the three methods used to process Gliricidia sepium:

1. One thousand six hundred and thirteen grams of leaves was extracted with ethyl ether by percolation. Twenty-six liters of extract yielded 65.9 grams of residue (4.06%). Percolation was continued with methanol. A portion of the extract was lost when hose clamp leaked. Yield was 173 grams of residue (10.7%). Steam distillation of the residue of the ethyl ether extraction yielded 4.8 grams of product (0.297%).

2. Four hundred and eighty grams of stems was steam distilled directly, and 3.5 liters of distillate was collected. The steam distillate was extracted first with ethyl ether, and then with chloroform. Yield was 0.8 grams of mobile, dark brown oil (0.166%).

3. Seven hundred and twenty-one grams of stems was covered with acetone. After steeping four times with this solvent and decanting periodically, 15.8 grams (2.19%) of extract was obtained. Steam distillation yielded 2.5 grams (0.346%) of product.

A total of 8.1 grams of steam distillation product was obtained and was then fractionated into a neutral, acidic, and phenolic fraction by solvent extraction of an etherial solution. The flow diagram in Figure 1 describes the process in detail.

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* The present addresses of these men are, for Mr. Southwell, U. S. Naval Research Laboratory, Washington, D. C., and for Mr. Hummer, U. S. Naval Station, Rodman, Canal Zone.

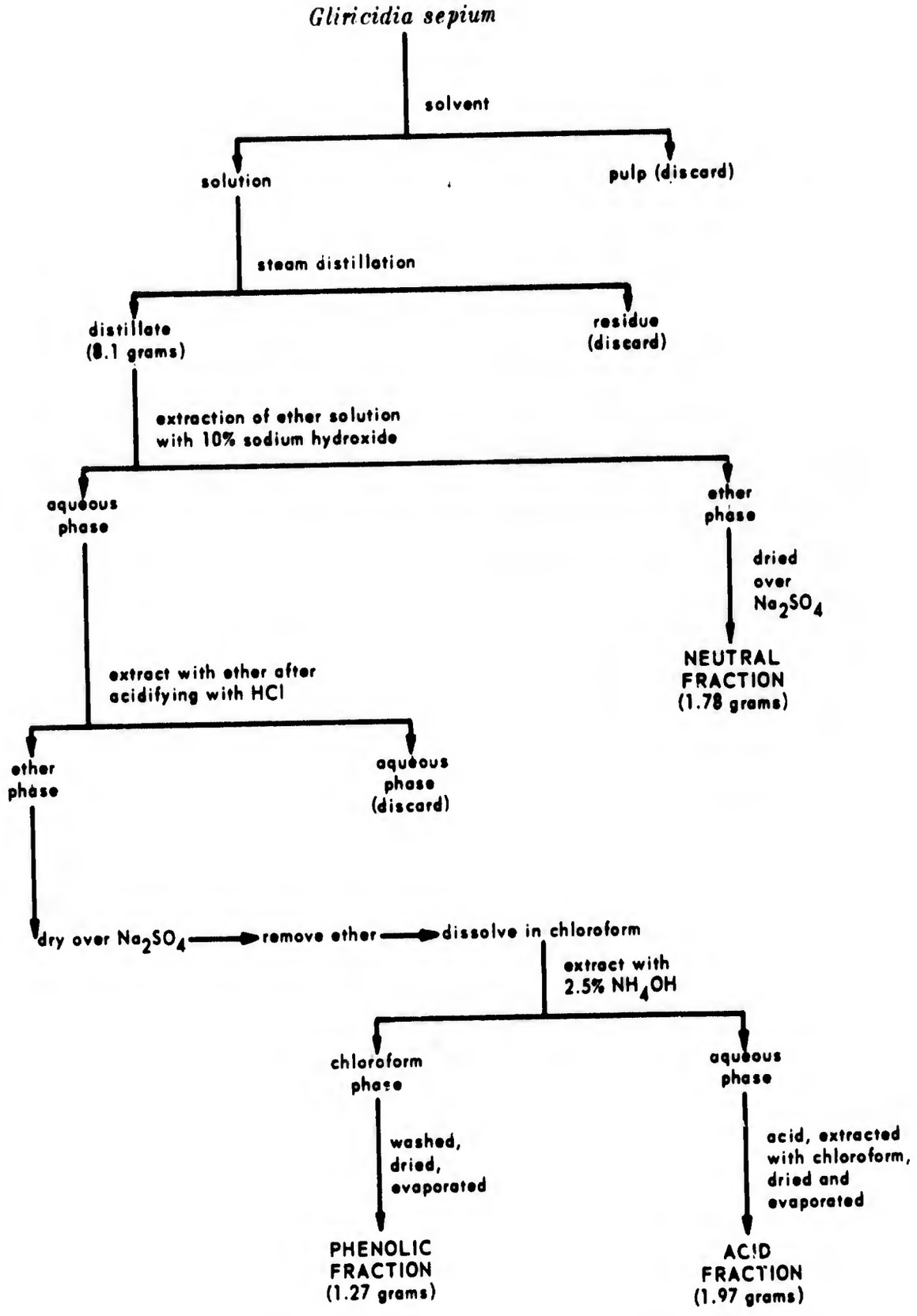


Figure 1. Flow diagram of separation of extracts of Gliricidia sepium into fractions.

Table I. Extraction of Gliricidia sepium

Solvent	Method of Extraction	Weight (gm)		Extract Residue		Steam Distillation Product	
		Leaves	Stems	Grams	Percent	Grams	Percent
Ethyl ether	percolation	1,613	—	65.9	4.06	4.8	0.30
Methanol	percolation	—	—	173	10.7	—	—
Acetone	steeping	—	721	15.8	2.19	2.5	0.35
—	direct steam distillation	—	480	—	—	0.8	0.17

Toxicity Testing

It would have been desirable to follow the progress of the separation by testing each fraction for rodenticidal activity, but neither the amounts of material nor the proper test facilities were available. Therefore, the toxicity of the several fractions to the marine wood boring organisms Teredo diegensis larvae and adult Limnoria tripunctata was determined instead. A toxicity screening test for these latter organisms had been developed as a part of another study.[3] The toxicity results are shown in Table II.

Table II. Toxicity of Fractions of Gliricidia sepium to Marine Borers

Marine Borers	Toxicity of Fractions (ppm)*		
	Phenolic	Acidic	Neutral
Limnoria tripunctata	25	100	50
Teredo diegensis larvae	25	6.25	25

* The toxicity to Limnoria tripunctata is the concentration which kills 50% of the organisms. The toxicity to Teredo diegensis larvae is the lowest concentration that will kill all of the organisms.

Isolation of Phenolic Substance

On the basis of the toxicity tests and the well-known physiological activity of many phenolic compounds, the phenolic fraction was chosen as the most promising fraction for further study.

The phenolic fraction was purified by solvent extraction and steam distillation. During the distillation a solid material collected in the condenser. The slightly turbid distillate was saturated with sodium chloride and extracted with ethyl ether. The solid in the condenser was washed out with ether, and the combined ether extracts were washed with a saturated sodium chloride solution and evaporated to dryness. The residue was dissolved in benzene, evaporated to dry the residue, and redissolved in benzene. Light petroleum ether was added to the benzene solution until there was a slight turbidity, and then the solution was placed in the deep-freeze for 1 hour. When the resultant crystalline mass was warmed to room temperature to melt the benzene, crystals remained which were filtered. The crystallization was repeated, and the combined yield of crystals was about 100 mg. These were dried in vacuum over paraffin and phosphorus pentoxide.

The mother liquors were evaporated, and the whole process was repeated on a benzene solution of the residue. Two additional batches of crystals were obtained, the first weighing 130 mg and the other 20 mg. These too were dried in vacuum over paraffin and phosphorous pentoxide. The melting point of the purest crystals, the first batch, was 67° to 68°C.

Identification of Phenolic Substance

The ultraviolet spectrum was obtained from a solution of the crystals in absolute ethanol (Figure 2). Two broad absorption maxima were obtained at 273 and 311 mμ. This suggested a benzenoid compound containing unsaturation conjugated with the ring.

The infrared absorption spectrum was rich in information (Figure 3). The precise positions of the absorption peaks were determined by a spectrophotometer with a higher resolution than that used to obtain Figure 3. The absorption peak at 3059 cm^{-1} must be due to either the aromatic or unsaturated C-H stretch. The absence of any absorption peaks just below 3000 cm^{-1} precludes the presence of saturated aliphatic methyl or methylene groups in the molecule. The 1031 cm^{-1}, 1263 cm^{-1}, and 1278 cm^{-1} peaks are suggestive of the alkyl-aryl ether, C-O-C, group. The last peak, at 1278 cm^{-1}, also suggests that the C-O-C group is in a ring. The twin peaks at 1744 cm^{-1} and 1757 cm^{-1} are typical of α, β-unsaturated carbonyl groups, and the strong peak at 1706 cm^{-1} is typical of α, β-unsaturated δ lactones.

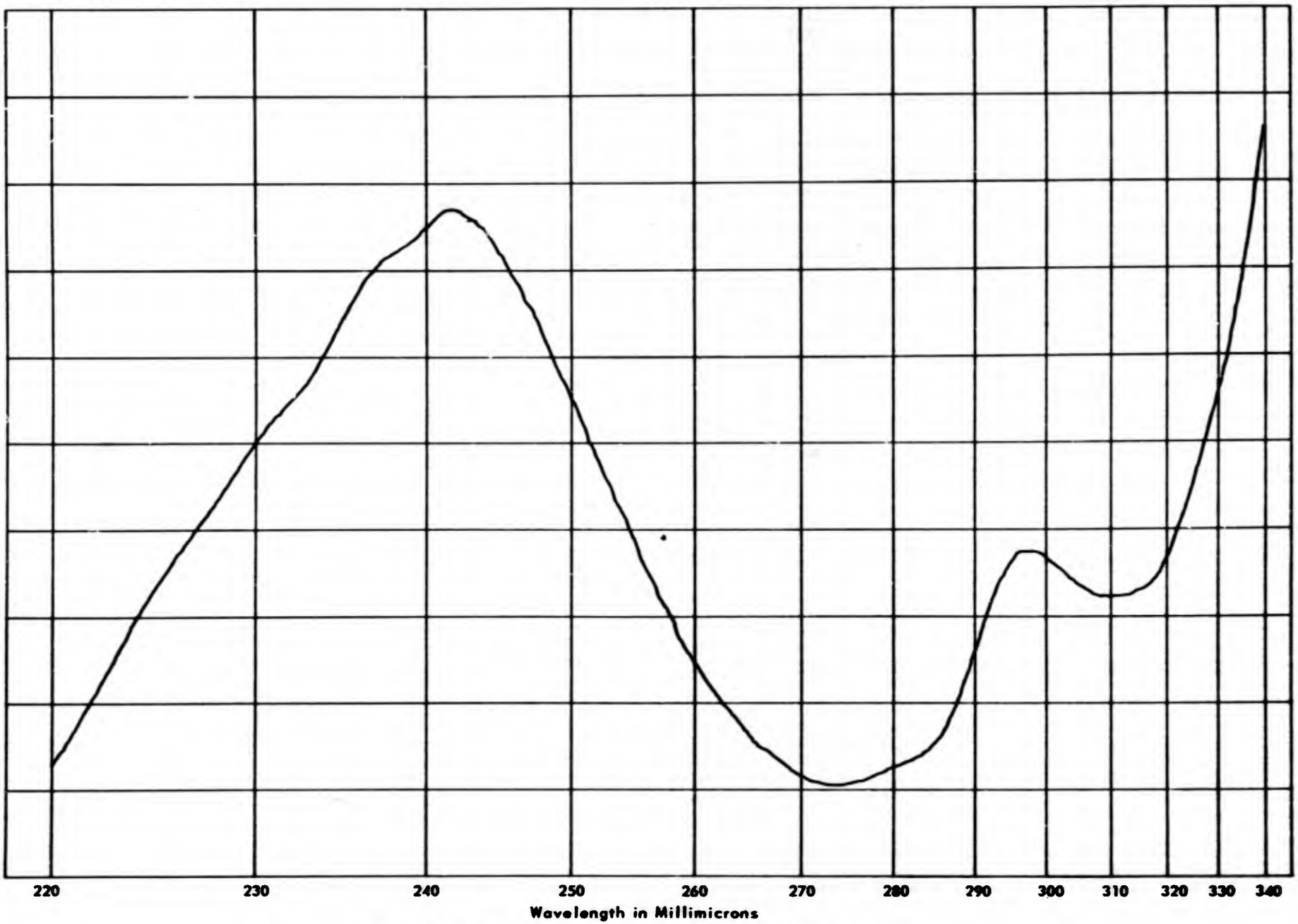


Figure 2. Ultraviolet spectrum of phenolic fraction.

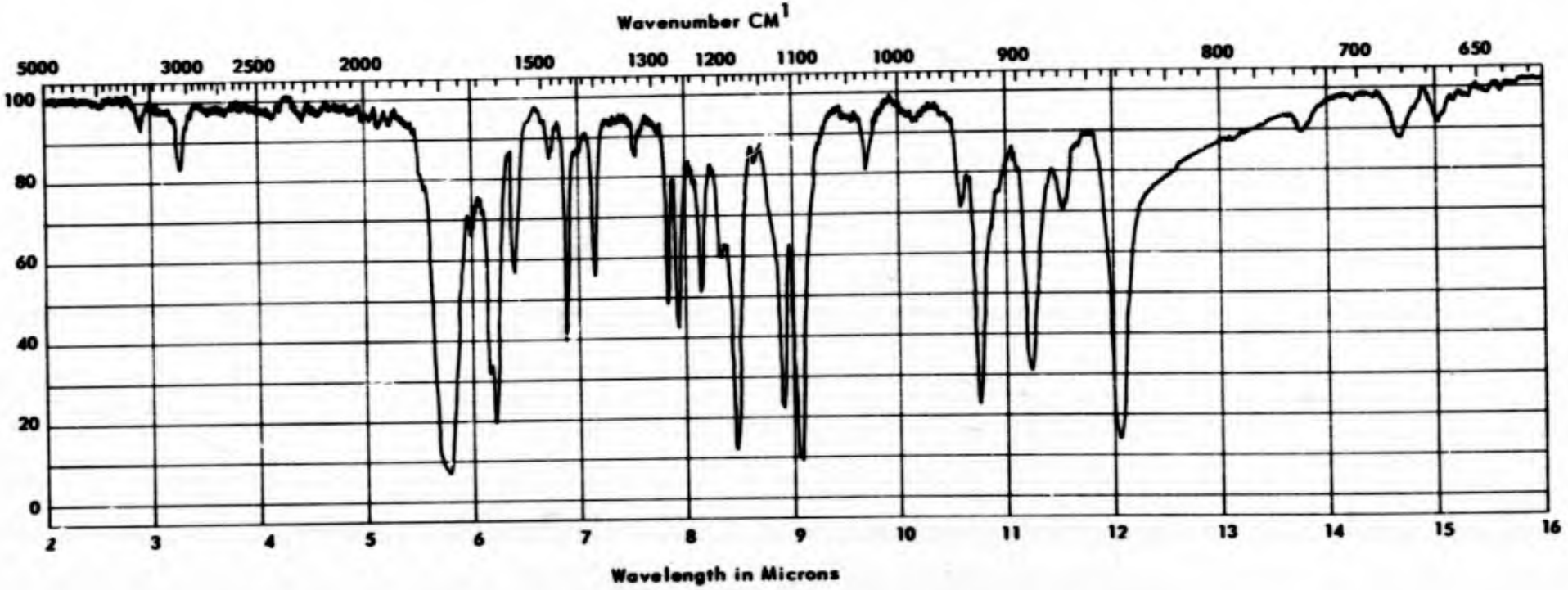


Figure 3. Infrared spectrum of phenolic fraction.

The Rast molecular weight was determined to limit the structural possibilities presented by the infrared data. A mixture of 5.6 mg of phenolic fraction in 53.4 mg of camphor depressed the melting point of camphor 27.8 degrees, from 174.8°C to 147.0°C. The molecular weight of the phenolic compound, as determined by the following equation, was 150.

$$M = \frac{39.7\,(w)\,(1{,}000)}{\Delta(W)}$$

where w = weight of compound

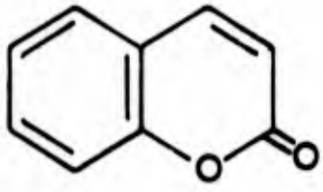
W = weight of camphor

Δ = depression of melting point

On the basis of the infrared spectrum, the phenolic compound contains:

1. A phenyl group, $-C_6H_5$, weight 77.
2. An α,β-unsaturated keto group, $-C(=O)-CH=CH-$, C_3H_2O, weight 54.
3. A C-O-C ether linkage, O, weight 16.

The weights of these groups total 147. Almost the only compound containing these groups within the 150 molecular weight range is coumarin:



The melting point of the mixture of coumarin and the phenolic compound showed no depression (Table III), and their separate infrared spectra were identical. The major phenolic fraction is therefore coumarin.

Table III. Melting Points of Coumarin and Isolated Phenol

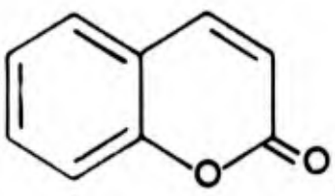
Test Specimens	Melting Point (°C)
Isolated phenol	68.4 to 69.8
Coumarin*	68.4 to 69.8
Mixture of both	68.4 to 70.3

*Purchased from Eastman Chemical Co.

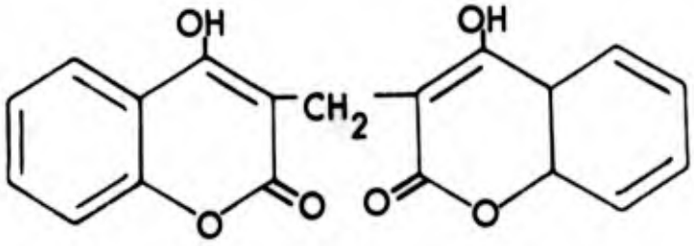
DISCUSSION

Gliricidia sepium is a member of the family Leguminosae. Since several genera within this family are known to contain coumarin, its presence in the genus Gliricidia is not surprising. The relationship between the presence of coumarin and its rodenticidal properties, however, is not readily discernible, because none of the other genera have gained a reputation as a rodenticide.

Coumarin is a comparatively nontoxic substance. It can, however, be converted by bacterial action into a powerful anticoagulant. Campbell and Link[5] isolated this anticoagulant and named it dicoumerol.

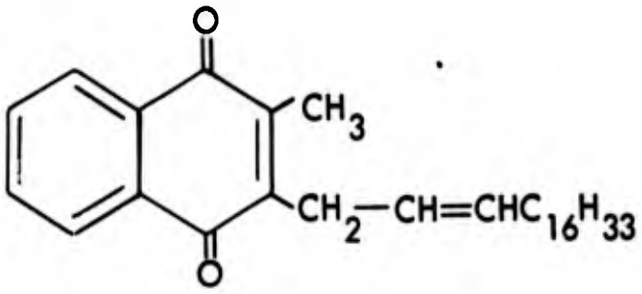


Coumarin



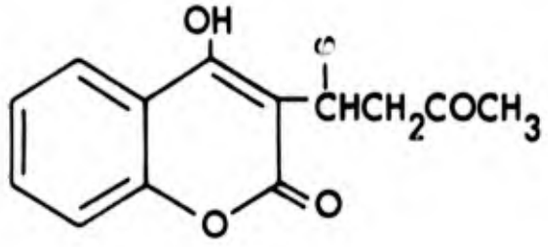
Dicoumerol

Dicoumerol is a structural analog of vitamin K, and its anticoagulant activity is counteracted by massive doses of this vitamin.



Vitamin K_1

In 1948 O'Connor[6] showed in field tests that dicoumerol is an effective rodenticide. It is not a rapidly acting substance, but repeated insult by sublethal doses induces fatal hemorrhages within a few days. Rats fed on baits containing dicoumerol feed freely and do not develop the bait shyness that is so common with other rodenticides. A number of coumarin derivatives have been synthesized that are effective in smaller dosages than is dicoumerol. One of the earliest and still one of the most common derivatives in field use is Warfarin, 3-(α-acetonylbenzyl) -4-hydroxycoumarin:



Warfarin

Since coumarin was the first pure compound to be isolated from Gliricidia sepium, and since coumarin, by its subsequent conversion to dicoumerol, develops rodenticidal properties, it was necessary to decide whether the rodenticidal properties of this plant could be explained by the presence of coumarin or whether another toxic principle should be sought. To help make this decision it was necessary to determine the manner in which the natives of Central America use this plant as a rodenticide and the mechanism by which this plant exerts its toxicity.

In southern Mexico the bark or leaves of Gliricidia sepium is ground and mixed with damp corn flour or spread on bananas.[2] In Panama the leaves are ground or mashed and then mixed with grain too.* At this point, however, there are two versions of the proper procedure. One method requires that the bait be cooked or steeped and dried before use, and the other that the uncooked mixture be used. At either locality it is worthy of note that the ground leaves are mixed with grain and allowed to ferment under the conditions of high humidity and temperature that exist in these tropical or subtropical areas.

The results of the chemical analysis for dicoumerol are self-evident: incubation under tropical or subtropical conditions increases the dicoumerol content of the leaves (Appendix A). After 24 hours of incubation, the dicoumerol content was increased from nontoxic to minimal toxic levels. This confirms the theory that toxic concentrations of dicoumerol can be built up in the baits.

Finally, it is necessary to consider the clinical observations. It was observed of Gliricidia sepium that "when rats eat it, their hair stands straight up and they bloat up and die in 4 or 5 days."[2] This is the type of clinical picture one would expect from a hemorrhagic poison. Unfortunately, no autopsy had ever been performed on the animals that were killed by the baits.

Preliminary tests on the bioassay of leaf extracts by the Bureau of Sport Fisheries and Wildlife, Fish and Wildlife Service, U. S. Department of Interior, showed these extracts to have no rodenticidal properties. The hemorrhagic mechanism, however, was confirmed by a controlled experiment conducted by Dr. E. L. Alpen at the U. S. Naval Radiological Defense Laboratory (Appendix B). Animals fed a normal diet of unincubated Gliricidia sepium leaves in amounts of 1.5 grams three times a day

* Private communication from Mr. Charles W. Hummer, Jr.

for 6 days showed no pathological changes. Those fed on incubated leaves in amounts of 0.5 gram three times a day for 6 days presented equivocal findings with respect to a blood coagulating defect. Those fed on incubated leaves in amounts of 1.5 grams three times a day for 6 days showed clear signs of hemorrhage in the gut, lung, and spleen.

CONCLUSION AND RECOMMENDATION

The evidence points to the conclusion that the rodenticidal properties of Gliricidia sepium are a result of the conversion of one of its normal nontoxic constituents, coumarin, into the anticoagulant dicoumerol. The conditions under which the plant is normally used favor this conversion, and the examination of experimental animals fed on incubated leaves shows the hemorrhagic reaction consistent with an anticoagulant toxic mechanism.

Gliricidia sepium exerts its rodenticidal properties by the bacterial conversion of its coumarin contents into the hemorrhagic poison dicoumerol. There are more efficient hemorrhagic poisons available. Further study of Gliricidia sepium is therefore not recommended.

ACKNOWLEDGMENT

The author would like to express his appreciation to Mr. Charles R. Southwell and Mr. Charles W. Hummer, Jr., both formerly with the Canal Zone Corrosion Laboratory of the U. S. Naval Research Laboratory, for procuring properly identified specimens of Gliricidia sepium and for ascertaining the procedures by which this plant is used by the natives of Panama as a rodenticide; to Dr. Quentin Jones, New Crops Research Branch, Crops Research Division, Agricultural Research Service, U. S. Department of Agriculture, for initial quantities of plant material; to Dr. Edward L. Alpen, Head, Biological and Medical Sciences Division, U. S. Naval Radiological Defense Laboratory, for conducting the rodenticidal tests; to Dr. William H. Robinson and Dr. D. Glen Crabtree, Chemical, Physiological and Pesticide — Wildlife Studies Section, Bureau of Sport Fisheries and Wildlife, Fish and Wildlife Service, U. S. Department of Interior, for preliminary rodenticidal tests on leaf extracts.

Appendix A

DETERMINATION OF DICOUMERAL

Dicoumeral was determined in ground leaves by the method of Roseman and Green,[4] which depends on solvent extraction and spectrophotometric absorption at 313 mμ. The determinations were made on both incubated and unincubated moistened leaves. Corn meal has frequently been used to accelerate bacterial action on a substrate by furnishing a carbohydrate source. The incubation was also conducted with ground leaves containing an equal weight of corn meal. All determinations were repeated three times, and the results are given in Table IV.

Table IV. Dicoumeral Content of Ground Leaves and Corn Meal

Substrate (5 gm)	Incubation (days)	Dicoumeral* (μgm/gm leaves)
Leaves	0	176
Leaves	1	338
Corn meal	1	15
Leaves and corn meal	1	270
Leaves and corn meal	2	240

* Average of three determinations.

Appendix B

LETTER FROM DR. E. L. ALPEN ON HEMORRHAGIC EFFECTS OF *Gliricidia sepium*

U. S. NAVAL RADIOLOGICAL DEFENSE LABORATORY
SAN FRANCISCO, CALIFORNIA 94135

In Reply Refer To:
920-137
ELA:eb
9 Jun 1965

Dr. Harry Hochman
Senior Research Chemist
Applied Science Department
U. S. Naval Civil Engineering Laboratory
Port Hueneme, California 93041

Dear Doctor Hochman:

I have completed my examination of the microscopic tissue preparations on the Gliricidia sepium rats and this is a summary of the data:

1. Four groups of 10 rats each were used, males weighing 140-160 g at the start of the experiment.

Group A - Maintained on normal diet.

Group B - Fed 0.5 g of ground leaves which had been incubated 24 hours in the moist state at 37°C. Feeding was by stomach tube, three times a day, total dose 1.5 g/day for 6 days.

Group C - Same as B except fed 1.5 g X 3 per day, total daily dose 4.5 g, for 6 days.

Group D - Fed 1.5 g X 3 per day for 6 days of unaltered Gliricidia sepium.

2. No deaths occurred in Groups A or D. In Group B, 7 deaths occurred in the period 7-20 days with a mean survival of 14 days. The survivors of this group were sacrificed on day 20 and pathology done on them at the same time as on the decedents. In Group C all animals died, with survival times of 6-14 days, mean survival time 8 days.

3. Pathological examination of tissues were conducted on all animals of Groups B and C and on 4 animals from Groups A and D. The tissues examined were: spleen; lymph node; bone marrow; liver; small intestine; and stomach. As I told you during the last phone call, the findings in the rats receiving 0.5 g X 3 were equivocal with respect to a blood coagulation defect. There were signs of intra-organic hemorrhage in the spleen, liver and gut wall, but no evidence of frank hemorrhage into hollow organs. There was also some depression of the bone marrow consistent with a cytotoxic effect of the drug.

At the higher dosage level, clear signs of hemorrhage into gut, lung and spleen were seen. I cannot tell whether the bleeding seen is the result of an anti Vitamin K effect or due to a suppression of platelet count. One peripheral blood smear which I performed showed adequate platelets.

4. I'm sorry for the further delays, but this work has had to be done on a "time available" basis.

Sincerely,

EDWARD L. ALPEN
Head, Biological and
Medical Sciences Division

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A study was made of the mechanism by which Gliricidia sepium (Yaite) exerts its rodenticidal properties. Extraction of the leaves of this plant, followed by physical and chemical fractionation, revealed the presence of coumarin as a constituent of the phenolic fraction. Consideration of the conditions under which these leaves are used as rodenticides, the known bacterial conversion of coumarin into the hemorrhagic agent dicoumerol, and pathological evidence in rats fed on incubated leaves point to coumarin as the basis for the rodenticidal properties of this plant.

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