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Research Articles

Phytochemical Investigation of *Carya illinoensis*

By LEON O. WILKEN, JR.†, and FRANK P. COSGROVE

The leaves and petioles of *Carya illinoensis* and a lyophilized aqueous extract of this plant were investigated. Examination of the petroleum ether, ether, and chloroform extracts revealed the presence of unidentified phytosterols and a squalene-like substance in the unsaponified portions, and the presence of capric, lauric, myristic, palmitic, stearic, arachidic, oleic, linoleic, and linolenic acids in the saponified portions. Nonhydrolyzable tannins containing a phloroglucinol and a catechol nucleus were found in the ethanol and methanol extracts. Investigation of the plant extracts revealed the presence of carbohydrates and the absence of discernible amounts of glycosides and alkaloids. A crystalline neutral substance obtained from a neutral lead acetate treated aqueous extract was identified as the *m*-inositol. A crystalline acidic substance isolated from an aqueous extract of the crude drug was identified as 3,4-dihydroxybenzoic acid. Further pharmacologic studies of various extracts are presently in progress.

THIS PHYTOCHEMICAL STUDY was undertaken primarily on the basis of our preliminary screening tests which indicated that certain extracts of the leaves and petioles of *Carya illinoensis* (Wangh) K. Koch (Fam. *Juglandaceae*), common name—pecan, possessed the property of temporarily lowering the blood pressure of test animals on intravenous administration. A search of the available literature revealed little (1, 2) or no scientific information concerning the evaluation of this plant for medicinal properties. The fact that this species has been assigned eight official names (3) throughout its relatively short history may account for the lack of investigation in the *Carya* genus. In 1951, the plant was reclassified under the international code as *Carya illinoensis*.

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† Present address: School of Pharmacy, Auburn University, Auburn, Ala.
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EXPERIMENTAL

Source and Preparation of Material.—The plant parts used in this investigation consisted of the air dried leaves and petioles collected in late summer from pecan trees growing near Covington, La. This material, sampled and authenticated,[1] was reduced to a moderately coarse powder with the aid of a W. J. Fitzpatrick, model D, comminuting machine.

Moisture Content and Ash Determination.—The moisture content of the powdered plant material, as determined by the toluene method (4) of the U.S.P.

[1] The authors are deeply indebted to Dr. B. L. Turner, Professor of Botany and Director of the Herbarium, The University of Texas, and Dr. M. Johnston, Lecturer in Botany, The University of Texas, for authenticating the plant.

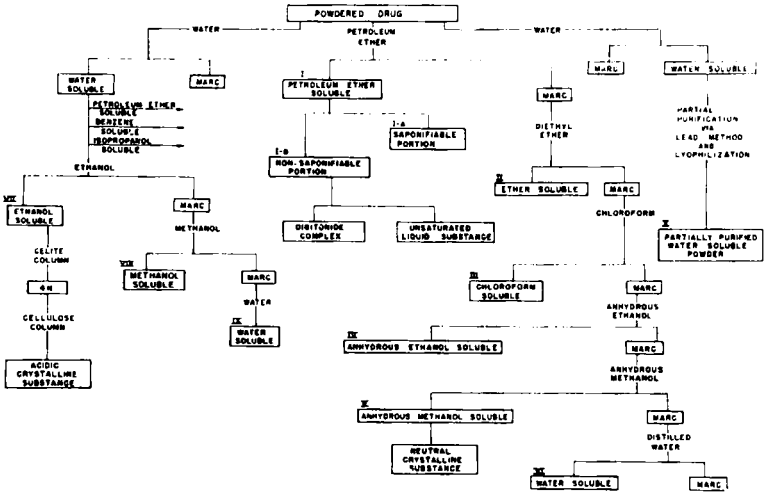

Fig. 1.—A schematic diagram of the preparation of fractions for phytochemical studies.

XVI, was shown to be 9.42%. The determinations for ash content as recommended by the U.S.P. XVI (5) afforded the following results: total ash, 7.03%; acid-insoluble ash, 1.40%; and acid-soluble ash, 5.63%. Further examination of the acid-soluble ash revealed the presence of K, 0.71%; Na, 0.048%; Ca, 0.97%; Mg, 0.34%; and Al, 0.92%. These mineral content values are generally in accordance with the values reported by Robinson and Edgington (6).

Preparation of Extracts for Phytochemical Studies.—Suitable samples of the powdered drug were extracted successively with a series of selective solvents in a soxhlet apparatus according to a modified Rosenthaler procedure (7). The solvents utilized and the per cent of extractives obtained with each solvent were: petroleum ether (b.p. range 30–60°), 2.07%; anhydrous diethyl ether, 2.51%; chloroform U.S.P., 0.79%; anhydrous ethanol, 6.97%; anhydrous methanol, 5.75%; and distilled water (percolation), 7.10%.

A schematic diagram of the preparation of the fractions for the phytochemical studies is shown in Fig. 1.

Fatty Acids.—Accurately weighed samples of the saponifiable portions of the petroleum ether fraction (Fraction I-A in Fig. 1), the diethyl ether fraction (Fraction II-A), and the chloroform fraction (Fraction III-A) were individually esterified according to the method of Fischer, as described by Fieser (8), in order to provide the more volatile methyl esters of the fatty acids. Analyses of these mixtures in an Aerograph gas chromatograph, model A-90-P, equipped with a Brown 1 mv. automatic recorder and comparisons of the resulting chromatograms with a standard chromatogram produced under similar conditions showed the presence of the methyl esters of capric, lauric, myristic, palmitic, stearic,

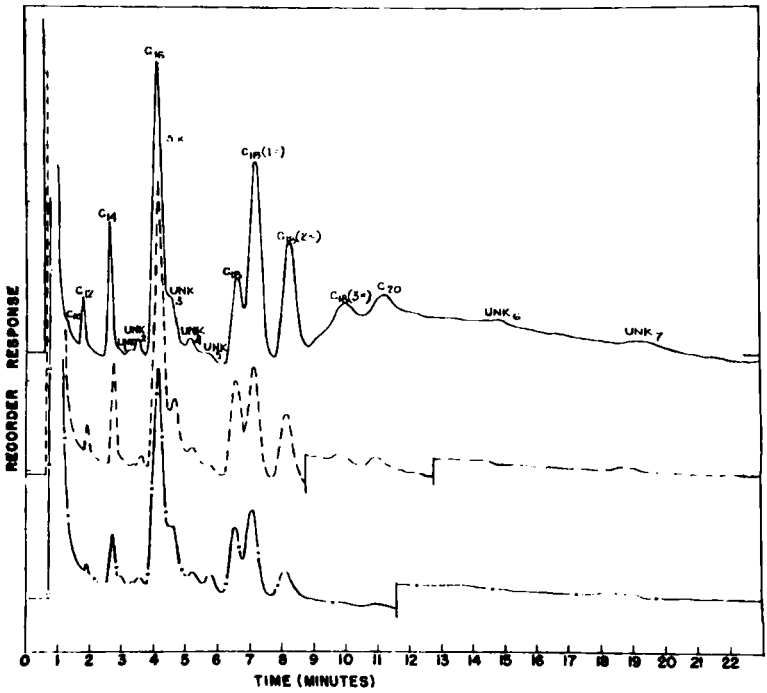

Fig. 2.—Gas chromatograms of the methyl esters of fatty acids found in the petroleum ether extract (——), the diethyl ether extract (- - - - -), and the chloroform extract (—·—·—). Conditions: sample 6.0 μl.; column packing, 20% diethyleneglycol succinate on firebrick; column 62 × 0.25 in.; helium, flow rate, 30 ml./min.; temp., column 223°, collector 183°, detector 240°, injector 260°; current 200 ma.; sensitivity 2.

arachidic, oleic, linoleic, and linolenic acids. The sample for the standard chromatogram contained pure methyl esters of all these named acids. Although seven additional peaks were observed, they were not studied further. The chromatograms obtained are shown in Fig. 2.

Phytosterols.—The unsaponifiable fractions of the petroleum ether extract, (Fraction I-B in Fig. 1), the diethyl ether extract (Fraction II-B), and the chloroform extract (Fraction III-B) were examined for the presence of phytosterols by the digitonin reaction described by Fieser and Fieser (9). Although the quantities of the insoluble digitonides formed in the petroleum ether and the chloroform extracts were too slight for further study, the diethyl ether extract afforded a white digitonide which melted between 205–208°. The presence of phytosterols in all three extracts was verified by the positive results obtained with the reactions of Hesse, Moleschott, and Hirschsohn (10) as well as the Liebermann-Burchard reaction (11).

Squalene-like Substance.—A suitable portion of the unsaponifiable fraction of the petroleum ether extract was extracted with boiling anhydrous methanol. When the alcohol soluble portion was decanted and placed in a freezer at a temperature of −18° overnight, a white flocculant precipitate formed which upon drying resulted in a viscous liquid having solubility and unsaturation characteristics similar to squalene. The isolation, purification, and characterization of this substance will be reported in a separate paper at a later date.

Glycosides.—Since a preliminary qualitative examination of the anhydrous ethanol, anhydrous methanol, and distilled water extracts, (Fractions IV, V, and VI, respectively, in Fig. 1) with the aid of Molisch reagent yielded positive results for carbohydrates and/or glycosides, the conventional method of Stas-Otto (12) was utilized in an attempt to verify these findings.

Powdered plant material (200 Gm.) was refluxed for 12 hours with 600 ml. of a 2% ethanolic solution of tartaric acid and then filtered. Evaporation of the filtrate under reduced pressure, using a Rinco rotatory evaporator with steam as the source of heat, afforded a residue which was extracted with hot distilled water. This aqueous extract was cooled, then extracted in a separator with diethyl ether.

The residue from this ethereal extract was dissolved in ethanol and the resulting solution tested for glycosides and/or carbohydrates with Molisch reagent. The negative results both before and after boiling this alcoholic solution with diluted hydrochloric acid indicated that glycosides in significant amounts were absent.

Alkaloids.—The purification procedures of the proximate assay method of the "National Formulary XI" (13) were applied to the diethyl ether extract and the chloroform extract (Fractions II and III, respectively, in Fig. 1) in order to analyze qualitatively for alkaloids. The resulting extracts gave negative tests with mercuric iodide T.S., gold chloride T.S., mercuric-potassium iodide T.S., and iodine-potassium iodide solution.

The negative tests obtained with these same reagents and the aqueous portion of the Stas-Otto extraction indicate the absence of any significant or discernible amounts of alkaloids in the plant material.

Tannins.—Since the conventional method of Feist and Bestehorn (14) for the extraction of tannins compares favorably with the method of obtaining the Rosenthaler extractives, the ethanol and methanol extracts (Fractions IV and V, respectively, in Fig. 1) from the Rosenthaler process were examined for the presence of tannins. Aliquots of 50% ethanolic solutions of these dried extracts gave positive results with the general tannin test reagents. A green solution was produced by the addition of ferric chloride T.S., and precipitates were obtained upon the addition of bromine T.S., quinine hydrobromide solution (1%), lead acetate T.S., gelatin T.S., and potassium dichromate T.S. to samples of each of the aliquots. These positive reactions are those characteristic of a tannin (15). The presence of a condensed (nonhydrolyzable) tannin was indicated by the formation of green with ferric chloride T.S. and by the development of a brown precipitate with bromine T.S. (16). The presence of a catechol nucleus (two adjacent phenolic groups) was indicated by the green obtained with ferric chloride T.S. and by precipitation with lead acetate T.S. (17). The presence of a phloroglucinol group was revealed by the production of a red coloration upon the addition of vanillin-HCl solution and confirmed by a positive Coward-Harris test (18). The absence of a gallic acid nucleus was indicated not only by the negative results with ammonium molybdate but also by the absence of a blue with ferric chloride T.S.

Isolation, Purification, Classification, and Chemical Characterization of a Neutral Crystalline Substance.—A small portion of the anhydrous methanol extract (Fraction V in Fig. 1) from the Rosenthaler procedure was redissolved in methanol and partly purified by treatment with a slight excess of a 20% solution of neutral lead acetate in methanol. The precipitate which formed was removed by filtration, the excess lead in the filtrate precipitated with hydrogen sulfide, and the mixture filtered. When the resulting filtrate, having been reduced in volume on a steam bath, was allowed to stand over calcium chloride for several days, a small quantity of colorless platelet crystals formed. These were shown to be only slightly soluble in methanol but very soluble in water.

To obtain an ample supply of this crystalline substance, 7.460 Kg. of the powdered plant material was extracted with seven portions ($1^1/_2$ gal. each) of distilled water. The 12 gal. (approx.) of aqueous extract collected was reduced in volume to 2 gallons using steam as a source of heat.

The resulting concentrate, partly purified with lead acetate in the manner previously described for the anhydrous methanol extract, was reduced to a

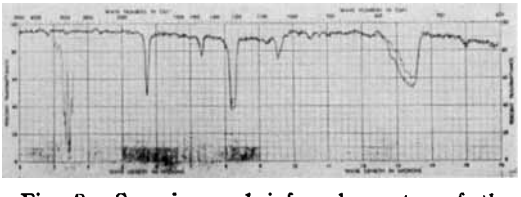

Fig. 3.—Superimposed infrared spectra of the hexa-acetate derivatives of the unknown (dark line) and a known myoinositol (light line) in chloroform.

fine powdery consistency with the aid of a F. S. Stokes lyophilizer, model 200 4LX3. This lyophilized extract was dissolved in a minimum of hot water, methanol added to cloudiness, and placed over phosphorous pentoxide for several days. A large crop of crystals resembling those observed with the previous methanol extract was collected.

Purification.—After six recrystallizations from methanol and water, the colorless needles which were dried *in vacuo* at 80° with potassium hydroxide in an Abderhalden pistol for 12 hours melted sharply at 224°. A yield of 0.08% was obtained by this procedure.

Classification and Chemical Characterization.—The reactions of the crystalline material to some of the chemical classification tests are summarized as follows: sodium fusion test (19) for nitrogen, sulfur, and halides, negative; bromine T.S., negative; potassium permanganate T.S., negative; ferric chloride T.S., negative; ceric nitrate T.S., positive; fuschin-aldehyde test, negative; and Molisch test for carbohydrates, negative. The collected data indicated the presence of a polyhydroxy alcohol, a conclusion which was substantiated by a mixed melting point of the unknown and a known C.P. grade inositol.

Esterification.—A sample of the unknown and the known C.P. inositol were separately acetylated according to the method of the "National Formulary XI" (20). The purified hexa-acetate derivatives melted at 216° both individually and as an intimate mixture.

Infrared Analysis.—To confirm the identity of the inositol, comparative studies of the infrared spectra of the unknown, the known myoinositol, and their hexa-acetate derivatives were made. The nearly identical spectra of the known and unknown samples offer confirmatory evidence of the identity of the two samples. The spectra of the hexa-acetate derivatives are superimposed and shown in Fig. 3.

Chromatographic Analysis.—In an attempt to identify which of the isomeric forms of hexahydroxycyclohexane had been isolated, the method of Angyal, *et al.* (21), utilizing paper chromatography was effectively employed. The unknown and a sample of myoinositol of known purity were spotted alone and together as a mixture with the control, dextrose.

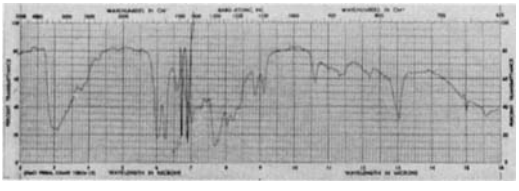

Fig. 4.—Infrared spectrum of the unknown acidic crystalline material in KBr pellet.

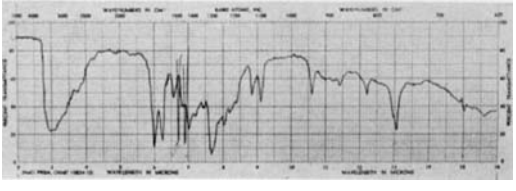

Fig. 5.—Infrared spectrum of a sample of 3,4-dihydroxybenzoic acid in KBr pellet.

The unknown migrated to the same degree as the known sample for four different solvent systems. These results indicate that the unknown is 1,2,3,-5/4,6-hexahydroxycyclohexane or, more commonly, myoinositol. A comparison of an elemental analysis of the unknown with the calculated values for inositol were obtained.

Anal.—Calcd.: C, 40.00; H, 6.71. Found: C, 40.81; H, 6.75.

Isolation of an Acidic Crystalline Substance.—Since preliminary studies of an aqueous extract of the crude material showed that solvent partition techniques were inadequate for the separation of constituents, the combined procedures of selective solvent extraction and column chromatography were employed for this purpose.

Successive extractions of a sample of the lyophilized aqueous extract in a soxhlet extractor with petroleum ether, benzene, isopropanol, ethanol, and methanol yielded significant quantities of extractives only in the ethanol and methanol fractions.

Further separation of the ethanol fraction (Fraction VII in Fig. 1) was accomplished through the use of a Celite 545[2] chromatographic column with elution effected by 1000-ml. portions of isopropanol, isopropanol-ethanol (1:1), ethanol, ethanol-methanol (1:1), and methanol applied in the order of increasing polarity. Since the third, fourth, and fifth fractions (500 ml. each) afforded significant quantities of similar residues when reduced in volume to a viscous syrup, they were combined and labeled 4N.

Separation of this fraction, 4N, was accomplished with the aid of a powdered cellulose chromatographic column utilizing as eluating liquids mixtures (500 ml. each) of solvents—skellysolve C, diethyl ether, ethanol, methanol, and water—applied in the order of increasing polarity. The 18th, 19th, and 20th fractions (100 ml. each) when dried afforded crops of similar brownish crystals and therefore were combined.

Purification.—After five recrystallizations from methanol and water, a crop of grayish-yellow crystals were collected which, after drying for 12 hours *in vacuo* at 80° with potassium hydroxide in an Abderhalden pistol, melted sharply at 199–200°.

Classification and Chemical Characterization.—Some of the qualitative classification tests applied to the crystals are summarized as follows: negative tests with bromine T.S., vanillin-HCl solution; positive tests with potassium permanganate T.S., ferric chloride T.S., lead subacetate T.S., and lead acetate T.S. Blue litmus was reddened and a sodium fusion test (22) for nitrogen, sulfur, and halides proved negative.

The accumulated data indicated the presence of both a phenolic and a carboxylic acid group. Determinations of the molecular weight and neutralization equivalent of the unknown material showed a formula weight of 154 Gm. and a neutralization equivalent of 154. An elemental analysis yielded: C, 54.58%, H, 4.07%.

The collected data compare favorably with the corresponding theoretical values for 3,4-dihydroxybenzoic acid, which are C, 54.55%; H, 3.92%; F.W., 154 Gm.; neutralization equivalent, 154.

A mixed melting point determination of the

[2] The Celite 545 used in this investigation was generously supplied by Johns-Manville, Houston, Tex.

unknown and a 3,4-dihydroxybenzoic acid of known purity showed no lowering of the melting point.

Preparation of the 3,4-Diacetyl Derivative.—The diacetyl derivative of 3,4-dihydroxybenzoic acid was prepared according to the method of Chattaway (23). The crystals obtained melted at 157–158.5°. The reported melting range is 157–159° (24). A mixed melting point determination of this derivative with one similarly prepared from a known 3,4-dihydroxybenzoic acid showed no lowering of the melting point.

Infrared Spectra as Proof of Identity.—The infrared spectra of both the isolated compound and a sample of 3,4-dihydroxybenzoic acid of known purity both in KBr pellets, were determined in an infrared spectrophotometer, model 4-55, Baird Associates, Inc. The tracings of the unknown and the known 3,4-dihydroxybenzoic acids are shown in Figs. 4 and 5, respectively. A comparison of the two spectra clearly shows the high degree of similarity existing between the spectrum of the known and that of the unknown. This identity of spectra, in addition to the other accumulated data, affords conclusive evidence for the identification of the unknown as 3,4-dihydroxybenzoic acid.

SUMMARY

The gas chromatographic analysis of the methylated saponified material of the petroleum ether, the diethyl ether, and the chloroform fractions indicated the presence of capric, lauric, myristic, palmitic, stearic, arachidic, oleic, linoleic, and linolenic acids in these fractions.

The qualitative tests for the presence of phytosterols in unsaponifiable material were positive.

On the basis of preliminary chemical studies, the liquid isolated from the unsaponifiable material of the petroleum ether fraction was partially characterized as an unsaturated squalene-like substance.

The qualitative tests performed for the presence of glycosides and alkaloids were negative.

A series of reactions with chemical reagents were positive for the presence of condensed tannins containing a phloroglucinol nucleus and a catechol nucleus.

From the results of the chemical, infrared, and paper chromatographic studies, the neutral crystalline substance was identified as 1,2,3,5/4,6-hexahydroxycyclohexane (myoinositol).

On the basis of chemical, physical, and infrared studies, the crystalline acidic substance was identified as 3,4-dihydroxybenzoic acid (protocatechuic acid).

Pharmacologic and chemical studies of certain extracts of the leaves and petioles of *Carya illinoensis* are in progress in an attempt to isolate the hypotensive activity and possibly identify the agent responsible.

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