SECTIONS

A PHYTOCHEMICAL STUDY OF KALMIA ANGUSTIFOLIA L.*

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Kalmia angustifolia L. is a small, hardy shrub growing in the Atlantic Coast States from Labrador to Ontario and southward through Pennsylvania, Virginia and the Carolinas to Alabama (1). It is known in various regions by the synonyms *Wichy, Sheep Laurel, Wicky, Lambkill, Dwarf Laurel* and *Sheepkill.* It contains a poisonous principle which is common to the species of Kalmia and which occurs in many Ericaceæ. This poison acts upon both man and animal, with the exception of the deer and the grouse. The flesh of the deer or grouse which has eaten the leaves is, however, poisonous to man. Holmes (2) reports the Cree Indians of the Hudson Bay area used *K. angustifolia* as a tonic, and the leaves have also been used as an arterial sedative and for the itch (3).

In 1886, Deibert (4) identified arbutin in the leaves of *K. angustifolia.* In the benzin extract, he reported *a wax, a resin* and *a fixed oil.* Stabler (5) and Bullock (6) and Matusow (7) reported studies of the constituents of *Kalmia latifolia* L., a similar shrub which grows somewhat larger than *K. angustifolia.* A survey of the literature reveals three characteristic compounds frequently present in members of the Ericaceæ. These are *arbutin, andromedotoxin* and *ursolic acid.* Kennedy (8) reported arbutin in *K. latifolia,* and Deibert (4) and Tunnman (9) reported arbutin in the leaves of *K. angustifolia.* Deibert (4) and Plugge (10) have reported andromedotoxin present in *K. angustifolia.*

The constituents present in the two species of Kalmia which have been reported in the literature are shown in Table I.

TABLE I.—CONSTITUENTS OF KALMIA AS REPORTED IN THE LITERATURE.

Constituent.	K. latifolia.	K. angustifolia.
Wax	Present (8)	Present (4)
Fixed oil	.....	Present (4)
Volatile oil	Present (5)	.....
	Absent (6)	.....
Andromedotoxin (phlorhizin)	Present (7, 10)	Present (4, 10)
Arbutin	Present (8)	Present (4, 9)
Tannin	Present (7, 11)	Present (4)
Phlobaphene	Present (7)	.....
Resin	Present (7)	Present (4)
Mucilage	Present (7)	Present (4)

Since *Kalmia angustifolia* grows widely in eastern North Carolina, and was therefore readily available, it was chosen for study in a series of North Carolina

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plants having possible medicinal use. The leaves were gathered in Duplin County, near Rose Hill, North Carolina, and at three different times, August 1936, February 1937 and July 1937. The plants used in this study were identified by Dr. H. R. Totten of the Department of Botany of the University of North Carolina.

EXPERIMENTAL.

Ash Determinations.—The total ash and acid insoluble ash determinations were run by the U. S. P. XI methods and the results are shown in Table II. A qualitative analysis of the ash disclosed the presence of potassium, sodium, calcium, iron and magnesium as metals, and sulfates, chlorides and carbonates as acid radicals.

Moisture Determination.—The distillation with xylene method (12) was used to determine the moisture content of the plant. The results are shown in Table II. The ash determinations were then recalculated upon the basis of the moisture-free drug, and these results are shown in Table II.

TABLE II.—CONSTANTS OF KALMIA ANGUSTIFOLIA L.

I. Gathered August 1936 and February 1937.
II. Gathered July 1937.

Calculated on the Basis of Air-Dried Drug.

Total Ash.		Acid Insoluble Asb.	
I	2.80% av.	I	0.407% av.
II	2.99% av.	II	0.634% av.

Moisture.

I	7.30% av.
II	7.29% av.

Calculated on the Basis of Moisture-Free Drug.

Total Ash.		Acid Insoluble Ash.	
I	3.02% av.	I	0.439% av.
II	3.22% av.	II	0.683% av.

The Process of Stas-Otto.—Twenty grams of the air-dried drug were subjected to the process of Stas-Otto (13) by boiling with 100 cc. of alcohol made faintly acid with tartaric acid for half an hour under a reflux. After cooling and filtering, the alcoholic filtrate was evaporated to dryness, and the residue was taken up in water, and the aqueous solution extracted with ether. The aqueous solution was then treated with the following alkaloidal reagents and no precipitate was formed in any case: phosphotungstic acid, mercuric chloride, picric acid, iodine-potassium odide, Froehde's reagent, Mayer's reagent or tannic acid. A precipitate was formed with phosphomolybdic acid which is characteristic in the test for arbutin with ammonium hydroxide and phosphomolybdic acid. This failure to obtain positive tests was taken to indicate the absence of alkaloids.

The ether solution was then evaporated to dryness, and the residue was taken up in water. When treated with a few drops of 20% alpha-naphthol in alcoholic solution and sulfuric acid, a violet ring was produced at the junction of the solution and the acid, which indicates the presence of carbohydrates or glucosides.

The General Method.—Three portions of 400 Gm., 700 Gm. and 1200 Gm. of the air-dried drug were extracted separately in the continuous extraction apparatus of Wester (14) with petroleum ether, ether, chloroform and alcohol until extraction was complete. The amounts obtained in these extracts are shown in Table III. Each extract was then investigated according to the procedure outlined below.

TABLE III.—EXTRACTS OF KALMIA ANGUSTIFOLIA OBTAINED BY CONTINUOUS EXTRACTION.

Petroleum ether	8.41% av.
Ether	2.59% av.
Chloroform	0.69% av.
Alcohol	28.75% av.

Petroleum Ether Extract.—The petroleum ether extract was saponified with alcoholic potassium hydroxide on a water-bath under a reflux. The saponification mixture was then poured into water and allowed to stand over night, and the mixture was extracted with ether. The ether layer (A) was then dried over anhydrous sodium sulfate.

The aqueous solution (B) was then acidified, and the precipitate filtered off. The filtrate was then extracted with ether and the ether layer was added to the above precipitate (C) and the whole evaporated to dryness. This residue was taken up in alcoholic potassium hydroxide and precipitated with lead acetate (15). The lead salts formed were extracted with ether, and the ether soluble lead salts (D) on evaporation proved to be in too small a quantity to be worked with. The ether insoluble lead salts (E) were suspended in water and decomposed with hydrochloric acid and extracted with ether. The ether solution so obtained yielded only crystals of potassium chloride.

The aqueous filtrate from the extraction of the saponifiable matter was allowed to evaporate to dryness, and the residue extracted with a mixture of three volumes of alcohol and one volume of ether. On evaporation, the residue was tested for glycerin with potassium acid sulfate, but the tests were negative.

The ether soluble, non-saponifiable fraction (A) was evaporated to dryness. Thirty-five grams of the petroleum ether extract yielded 25 Gm. of this residue. The residue was treated with hot alcohol, a residue (G) of 10 Gm. being insoluble. The hot alcohol solution (H) was then allowed to cool.

The insoluble portion (G) was treated with boiling acetic anhydride, two layers forming. The insoluble portion was separated from the solution while hot, and this portion cooled to a brown wax-like solid, melting at 48–50° C. From the melting point and the behavior toward solvents this substance was concluded to be a hydrocarbon (15). The acetic anhydride layer deposited a precipitate on cooling, and on the addition of water, precipitation was complete. On recrystallization from alcohol, it darkened and decomposed.

On cooling, the hot alcohol solution (H) formed 0.2 Gm. of a yellowish precipitate (K) which on recrystallization from alcohol and acetone gave a solid melting at 73° C. The acetate of this substance melted at 62° C. The substance did not give a positive Liebermann-Burchard test for sterols, so it was concluded to be an alcohol. Ceryl alcohol melts at 79° C. and its acetate melts at 65° C. While wet, this substance held a large volume of the solvent used to crystallize it from, but on drying, it decreased considerably in volume. This behavior is characteristic of carnaübyl alcohol, which melts at 68° C. (15).

The cold alcohol solution (L) was evaporated to dryness and the residue was taken up in benzene. On concentration, this solution yielded a white precipitate which on recrystallization from alcohol melted at 134° C., and gave a positive Liebermann-Burchard test for sterols. The acetate melted at 121° C. Sitosterol and its acetate melt at 136° C. and 125° C. respectively. The substance was concluded to be sitosterol (15).

On evaporation, the benzene solution yielded a brown, sticky mass, which when steam distilled gave no evidence of a volatile component.

Ether Extract.—A greenish white precipitate was noticed in the solvent reservoir during the extraction of the drug with ether. This precipitate was removed from the extract before concentration, and was recrystallized from alcohol as the potassium salt. On purification, this substance melted at 275° C. and gave a positive Liebermann-Salkowski test (16). This behavior on extraction, the melting point and the Liebermann-Salkowski test indicated ursolic acid. The substance was converted into the acetyl derivative which melted at 196° C., agreeing with that of the diacetyl derivative of ursolic acid (201° C.).

The ether was then removed from the remainder of the ether extract and the residue taken up in 10% potassium hydroxide solution. The mixture was filtered, and the aqueous solution (C) was precipitated with acid, and the mixture again filtered. This aqueous solution was precipitated with lead acetate, and since no further precipitate was given with lead subacetate, the mixture was filtered and the filtrate freed from lead with hydrogen sulfide. The aqueous solution was then extracted with chloroform. On evaporation of the solvent, a resinous substance resulted which could not be obtained in a crystalline form, but which gave a yellow color with sulfuric acid, turning red on heating; a blue color with hydrochloric acid; and a red

color with phosphoric acid. These color reactions are characteristic of andromedotoxin (phlorhizin).

The aqueous solution (D) was then evaporated to dryness under reduced pressure, and the residue was extracted with alcohol. On evaporation, a resinous substance was found which could not be obtained in a crystalline form, but which gave a blue color with ferric chloride and a blue color with ammonia and phosphomolybdic acid. These tests are characteristic of arbutin (10). Further attempts to obtain the glucosides in a crystalline form met with the same results.

Chloroform Extract.—The chloroform extract after the removal of the solvent was heated with water on a bath of water. The aqueous solution so obtained was evaporated to dryness, but again no crystalline substance could be obtained. The resinous substance so obtained gave the above tests for andromedotoxin.

Alcohol Extract.—Fifty grams of the alcohol extract after the removal of the solvent were taken up in 50 cc. of alcohol and precipitated with 100 cc. of ether. The precipitate (A) was removed and taken up in water. A residue (D) was left behind which was taken up in potassium hydroxide solution and precipitated with acid. 2.5 Gram. of phlobaphene were thus obtained. On heating in alcoholic solution with acid, the phlobaphene reduced Fehling's solution.

The aqueous solution (C) of the ether precipitate was then precipitated with lead acetate and lead subacetate and the lead precipitates were filtered off. These precipitates were then suspended in water, and decomposed with acid The mixture was then extracted with ethyl acetate, and the solvent removed. After several such treatments, an amorphous red tannin was obtained, which when dissolved in water, gave a greenish black color with ferric chloride and a flesh-colored precipitate with bromine water. These tests are characteristic of catechol tannins (17).

Treatment of the aqueous solution from the extraction of the tannins, and the ether soluble portion (B) of the alcohol extract by the method outlined above for the isolation of arbutin, yielded the same resinous substance as before, which gave the characteristic tests for arbutin.

Quantitative Determination of Tannins.—The total tannins present in the leaves of *K. angustifolia* were determined by the gravimetric method of Schröder (18), in which a water soluble percolate of the leaves is treated with hide powder for the complete absorption of the tannins. The leaves of *K. angustifolia* were found to contain 6.51% tannin.

Quantitative Determination of Glucosides.—One hundred fifty grams of the air-dried leaves were extracted with 200 cc. of boiling water and the decoction on cooling was precipitated with lead acetate and lead subacetate. The precipitates were filtered off and the solution freed from lead with hydrogen sulfide, and the aqueous solution so obtained evaporated to a volume of 50 cc. under reduced pressure. This solution was extracted with chloroform and 0.1 Gm. of phlorhizin obtained, amounting to 0.066% of the total weight of the air-dried drug.

The concentrated solution was then allowed to evaporate to dryness in the air, and the residue taken up in alcohol and boiled with charcoal. On evaporation, this solution yielded 1.4 Gm. of a resinous substance which gave positive tests for arbutin. This amounted to 0.93% of the total weight of the air-dried drug.

SUMMARY.

The total ash, acid insoluble ash, moisture and the inorganic constituents of the drug have been determined. A hydrocarbon, an alcohol and sitosterol were found in the petroleum ether extract. Ursolic acid and traces of andromedotoxin (phlorhizin) and arbutin were identified in the ether extract. Phlorhizin was identified in the chloroform extract. In the alcohol extract, catechol tannins, phlobaphene and arbutin were identified. The total tannins were determined to be 6.51%, the total content of andromedotoxin (phlorhizin) was found to be 0.066% and the total content of arbutin was found to be 0.93%.

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BIOASSAY OF PHENOLPHTHALEIN USING THE RHESUS MONKEY.*

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Being interested in laxatives in general, and especially phenolphthalein, we were very desirous of having some satisfactory method for evaluation of laxative action. A review of the literature showed that no satisfactory chemical assay was available and that all animals tried in connection with bioassays were not satisfactory, with one exception. The work of Fleig (1), back in 1908, suggested that it would be interesting to try experimental work on the monkey and twenty-six years later the work of Williams, Abramowitz and Killian (2) which, to our knowledge, is the first real work utilizing the monkey, showed definite promise of the development of a satisfactory bioassay. However, this work was not carried on to the point of establishing a definite assay method.

In view of the monkey showing more possibility than any other animal, we decided to further study the monkey in the hope that we might be able to develop a satisfactory bioassay method for laxatives. Our work over the past two years has enabled us to develop a satisfactory method for determining the relative potency of various samples of phenolphthalein. While this work has been conducted particularly in connection with phenolphthalein, we have found in our experiments that the rhesus monkey responds to Epsom Salts, Milk of Magnesia, Cascara, Castor Oil and Aloes. The method which has been evolved and which we are using at the present time follows.

We are using animals not commonly used in drug assays and believe it desirable to go into more detail concerning this method than would normally be considered necessary.

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