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A Preliminary Chemical Investigation into the Heritability of the Metabolic Pathway for the Cardiac Glycoside Cymarin within the Family Apocynaceae

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ABSTRACT

Cardiac glycosides are secondary metabolites of plant tissues having a powerful action on the mammalian heart muscle. Although cardiac glycosides have been identified in a number of unrelated plant families, little is known of the heritability of the biosynthetic pathway for the cardiac glycoside cymarin within the family Apocynaceae. Two species of Apocynaceae, *Apocynum cannabinum* and *Vinca minor* Linn., were harvested and prepared for chemical analysis on an alumina column, using a benzene-chloroform solvent system, for the presence of cymarin. Modifications of the extraction procedure for safety and convenience precluded the isolation of cymarin from *A. cannabinum* and necessitated the postponement of the analysis of *Vinca minor* extracts for the presence of cymarin.

INTRODUCTION

Secondary metabolites of plant tissues include saponins, tannins, and the alkaloids, which include the cardiac glycoside cymarin. The cardiac glycosides constitute a group of steroids having a powerful action on the mammalian heart muscle. The molecular site of action is a membrane-bound ATP-ase that regulates cation transport across the cardiac muscle cell membrane. Hydrolysis of plant glycosides yields one or more sugars and a steroidal aglycone containing an unsaturated lactone ring attached to C-17 (Robinson, 1983).

Cardiac glycosides have been found in several quite unrelated plant families such as Apocynaceae (Harris, et al, 1964; Lee, et al, 1972), Moraceae (Adams & Wilkinson, 1960), Liliaceae, and Ranunculaceae (Robinson, 1983). The cardiac glycosides of *Apocynum cannabinum* include cymarin, K-strophanthin, appocannoside, cynocannoside, and the genin strophanthidin (Adams & Wilkinson, 1960).

Potential medicinal uses of cardiac glycosides range from their use as microbicides to the treatment and control of diseases of the cardiovascular system (Lewis,1977). The efficacy of the cardiac glycoside cymarin in the treatment of carcinomas of the nasopharynx was reported by Kupchan, et al (1964). Native Americans used extracts of root material from *A. cannabinum* as a tonic, a purgative, a febrifuge, and as a treatment of dropsy, ague, and syphilis (Kindscher, 1992).

As a folk remedy *A. cannabinum* was used extensively, and was described as an active hydrogogue cathartic, a diuretic, and as a remedy for dropsy (Lloyd, 1921).

The root extracts of *A. cannabinum* have been analyzed and found to contain a principle cardioactive compound, cymarin. While cymarin is found throughout the plant, it is most concentrated in the root tissue, with harvesting recommended in late autumn. Another species of Apocynum, *A. androsaemifolium* was also listed in the National Formulary (1916-1960) as a diuretic, cathartic, and expectorant (Kindscher, 1992).

Efforts to determine the presence of cymarin in the tissues of other members of the family Apocynaceae appear to be limited (Harris, et al, 1964). Alkaloids from *Vinca rosea* Linn. were analyzed by Cone, et al. as early as 1963, but never concomitantly with alkaloids from *A. cannabinum* . The objective of this investigation was the extraction of the cardiac glycoside cymarin from whole plant extracts of *A. cannabinum* and *Vinca minor* , a related species from the family Apocynaceae (Great Plains Flora Assn., 1991). Using the technique of Adams and Wilkinson with modifications found in Research Methods in Plant Science (Klein, 1970), extracts from *A. cannabinum* were analyzed using liquid column chromatography (Snyder & Kirkland, 1979). Having failed to isolate the cardiac glycoside cymarin from extracts of *A. cannabinum* , the preliminary chemical investigation of *Vinca minor* tissues was postponed

MATERIALS AND METHODS

Preparation of Plant Samples

A catalog of locations of Apocynum cannabinum in McPherson County was obtained from the R.L. MacGregor Herbarium, University of Kansas, Lawrence, Kansas. In combination with A Manual of the Flowering Plants of Kansas (Barkley, 1968) and Roadside Wildflowers of the Southern Great Plains (Freeman & Schofield, 1991), Flora of the Great Plains (Great Plains Flora Assn, 1991) was consulted for identification of *A. cannabinum* . *Vinca minor* was harvested from cultivated plots in downtown McPherson, courtesy of the City of McPherson.

Plants were cut into small pieces of leaf, stem, and root, and dried in marked containers at 105C for 48 hours. Extraction of Cymarin can be carried out on fresh or dried plant tissue, but drying is preferred in the event of a long delay between plant acquisition and extraction. Dried plant material was ground in the Wiley mill using a 1mm screen pore size, and transferred to marked storage bags.

Extraction of the Cardiac Glycoside

A 50 ml burett was prepared for liquid column chromatography by placing a glass wool plug at the

base for support of the adsorbent. A small amount of benzene:chloroform solvent (1:2 v/v) was added to the column and the glass wool plug was compressed with a glass rod until no more air escaped (Klein, 1970). Neutral alumina (Activity grade 1) was combined with enough benzene:chloroform solvent to form a slurry which was added to the column in small increments and allowed to settle under gravity until a level of 32 ml was reached. The solvent was drained off until the level of the adsorbent was reached. The column was capped until the sample was prepared.

Twenty grams of ground *A. cannabinum* tissue (whole plant) was combined with 1/10 volume of chilled acid-washed sand and 25 ml chilled Ligroin (Aldrich Chemical) in a chilled mortar and macerated for two minutes with a pestle. The viscuous mixture was transferred to a glass funnel lined with Whatman's #2 filter paper. The extract was collected in a flask, and included an additional 15 ml of Ligroin, with a combined volume of 40 ml.

Twenty ml of plant extract was combined with enough alumina to make a friable mixture. The sample was transferred to the prepared column and separation began using benzene:chloroform solvent system (Adams & Wilkinson, 1960) with a flow rate of 4 ml/min. Fractions were somewhat arbitrarily collected in that only the approximate volume of the individual samples was monitored, until 25 tubes were collected, ensuring that the column had been fully developed.

Identification of Cymarin

A sheet of Whatman's # 1 Chromatographic paper was used to outline a grid of 1 cm x 1 cm squares to which alliquots of the individual samples were added and numbered from 1 thru 25 . the grid was allowed to air-dry and the process repeated three times. After drying completely, the grid was sprayed with a dinitrobenzene solution (Raymond's reagent) and observed for the expected color reaction, a short-lived but distinct purple color indicating the presence of the cardiac glycoside cymarin (Harris, et al, 1964).

RESULTS

Extracts of the tissues of *A. cannabinum* failed to yield evidence of the presence of the cardiac glycoside cymarin, specifically, the expected color reaction between dinitrobenzene and the α,β-unsaturated lactone ring of the steroidal aglycone.

DISCUSSION

There exist several explanations for the failure of the extraction and separation procedure to yield evidence of cymarin. The most significant source of error is the exclusion of the treatment of the whole plant extract with methanolic ether as described in the work of Adams & Wilkinson (1960), upon which the procedure for the separation in this investigation was based. While a large number of terpenoids and steroids are decidedly non-polar, the cardiac glycosides are an exception, and are commonly extracted in ether--with high molecular weight alcohols--follwed by treatment with hot alcohol (Robinson, 1983). The decision to eliminate the use of diethyl ether in the experimental procedure was based on the fact that diethyl ether is known to form explosive peroxides, and seemed too great a hazard for the undergraduate laboratory operated without close faculty supervision.

The procedure used by Adams and Wilkinson (1960) required the use of neutral alumina deactivated with 10% acetic acid. This investigation utilized Alumina with Activity grade of 1 instead of deactivated alumina due to the difficulty in filling the small i.d. column with the deactivated adsorbent. However, according to Robinson (1983) in most cases highly active alumina is undesirable because it may cause degradative reactions. If degradative reactions occurred in this procedure due to the presence of highly active alumina then it is likely that those reactions contributed to the absence of cymarin from the fractions that came off the column.

Another possible source of error involves the degradation of the dinitrobenzene used to test for the presence of the unsaturated lactone ring of cymarin. The use of dinitrobenzene for the estimation of digitoxin in digitalis was investigated by J. M. Rowson in 1955, and the results of his investigation include the stability of dinitrobenzoic acid reagent. Rowson reports that dinitrobenzene solution is stable for periods up to 7 days if stored in the dark. The dintrobenzene solution used in this investigation to detect the presence of cymarin was stored for 45 days prior to use. The ability of the solution to detect the presence of purified Cymarin(Sigma Chemical) was not checked.

The question remains of the heritability of the metabolic pathway for the cardiac glycoside cymarin within the family Apocynaceae. There is evidence of similarity in the biosynthesis of cardenolides and steroidal saponins, in particular, the starting point, which can begin with cholesterol, and the formation of a 3-keto intermediate (Robinson, 1983). Robinson notes that cholesterol, while not a common constituent of plants, may be an important precursor for many plant steroids, including water-soluble glycosides and acylated glycosides, along with the more common C-24 ethyl compounds, derived by successive additions of carbon atoms from the methyl group of methionine.

The relationships between the various genera of a plant family are based on assumptions gathered through observation of morphological characteristics. The morphological characters for systematic classification of plants within the family Apocynaceae, like the enzymatic processes which give rise to a plants various metabolic activities, are dictated at the level of the genome.

It follows quite naturally that relatedness of physical characteristics on the morphological level may suggest similarities at the level of the genome, including those genes coding for enzymes involved in the synthesis of cardiac glycosides like cymarin within the family

Apocynaceae.

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