FLAVONOID ISOLATION, IDENTIFICATION AND CHEMOTAXONOMY OF *TRICHIPTERIS SP.* (CYATHEACEAE)

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Jack Farah Mustaklem

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A B S T R A C T

As part of a larger chemotaxonomic project to evaluate the relationship between the Cyatheaceae and Dicksoniaceae and the separation of Trichipteris sp. and Cyathea sp., seven species of Jamaican and Costa Rican Cyatheaceae were studied for their flavonoid content.

The flavonoids were extracted from the dried samples using a 50% ethanol solvent. A preliminary investigation of the extract components was carried out using two-dimensional paper chromatography followed by ultra-violet spectroscopy. Components having flavonoid spectra were isolated and purified using three solvent systems on chromatography paper.

The identification of the flavonoids was then attempted by comparing the ultraviolet spectra and R_f values with the literature with the aid of a punch-card system developed for the retrieval of information on flavonoids. The flavonoids were acid-hydrolyzed and identification of the sugars and the aglycones was attempted using paper and thin-layer chromatography.

Isovitexin and kaempferol-3-glucoside were found in certain ferns. Other flavonoids have been isolated but not identified.

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I N T R O D U C T I O N

The purpose of this research was the isolation and identification of flavonoids in seven Trichipteris sp. (Cyatheaceae) as part of two larger but related chemotaxonomic projects, namely to compare the flavonoid content of Trichipteris with Cyathea and that of the Cyatheaceae with the Dicksoniaceae.

From purely classical taxonomic studies, the Cyatheaceae and the Dicksoniaceae have been considered separately and classified as two different tree fern families.[1] Upon closer morphological study, Holttum decided in 1961 that they should have a common classification.[1-4] This concept was supported in 1970 by Nayar who asserted that they are derived from the same Cyatheaceae origin.[5] Consensus is still not reached yet since Tryon, for example, was able to differentiate the two families using hairs and scales.[6]

On the question of comparing Cyathea with Trichipteris, some taxonomists[7-9] refer to Tryon's separation in 1970 of Trichipteris from Cyathea.[10] In 1972, Tryon[7] found new species of American Cyatheaceae and classified some of them under the two separate genera Trichipteris and Cyathea. Lucansky and White[8] in 1974 compared the nodal and vascular anatomy of Cyatheaceae and found basic agreement with Tryon's phyletic scheme, although Trichipteris and Cyathea were found to be very similar. In their study of the tree fern indusia

in 1975, Tryon and Feldman[9] state that all species of Trichipteris are exindusiate, while there are sphaeropteroid, cyatheoid and hemitelioid indusia among the species Cyathea. Although they go along with Tryon's classification system which separates Trichipteris from Cyathea, they feel that classification based on the type of indusia is inadequate. Tryon[11], himself, recently revised the genus Cyathea and found it morphologically related to Trichipteris.

It seems obvious that more taxonomic studies have to be performed before a conclusion can be made as to whether the Dicksoniaceae can be classified with the Cyatheaceae or to whether the separation of Trichipteris from Cyathea is justified. Perhaps the present chemotaxonomic study can make a very small contribution towards that conclusion.

B A C K G R O U N D

CHEMICAL TAXONOMY

The occurence and comparative biochemistry of certain substances, especially the secondary substances which developed late in the evolution of plants, could form theoretically the basis for a self-sufficient taxonomic and phylogenetic classification system. Data on the distribution of these substances in most plants is scarce except for substances of medical or economic value. It is not possible, at this stage, to develop a phylogenetic classification system by exclusively using the distribution of biochemical substances in plants. Chemical plant taxonomy can only be used in conjunction with the other taxonomic methods available to try to solve specific problems such as the one that forms the basis of this study.

It has been argued that because the distribution of a substance does not always have a relation with plant relationships, chemotaxonomy is not very useful. Studies on biochemical genetics have proven that it could be useful. It must be remembered that sometimes morphological similarity does not neccessarily imply phylogenetic relationship. Alston and Turner,[12] although they do not advocate abandonment of morphological studies, presume that only quantitative modification occurs in the chemical characteristics of plants, whereas their morphological characteristics can

change qualitatively by different modifiers. This assumption, they conclude, renders the use of chemical characters more advantageous.

Although the usefulness of chemistry in plant classification has long been realized by many scientists, Alston and Turner[12] explain the slow advancement in this area by i) the competition provided by genetics and enzymology, both of which attracted researchers who may have otherwise been interested in biochemical systematics, ii) the pharmacological approach to surveys of natural products and iii) the inadequacy of the techniques to produce the necessary chemical information. Recently, chromatography has allowed for the isolation of small quantities of plant constituents, the techniques for their identification have improved and their chemical surveys are more easily performed. Biochemical systematics, including chemotaxonomy, have thus progressed and many classes of compounds have been studied in this respect.

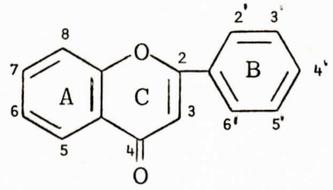
Alston and Turner's Biochemical Systematics[12] includes chapters on such compounds as amino acids, fatty acids, carbohydrates, alkaloids, cyanogenetics, phenolics, quinones, terpenoids and others. Swain's Chemical Plant Taxonomy [13] includes chapters by various authors dealing with the use of specific kinds of compounds such as alkanes, alkynes, fatty acids, aliphatic polyols and cyclitols, glycosides, alkaloids, anthocyanins and sulfur compounds. Harborne outlines more recent developments in biochemical systematics in one of the

chapters in Progress in Phytochemistry.[14] The first volume of Recent Advances in Phytochemistry[15] includes a chapter by Erdtman discussing the chemical principles of chemosystematics and papers that emphasize chemotaxonomy using nitrogen and sulfur compounds, acetate and mevalonate compounds as well as flavonoids.

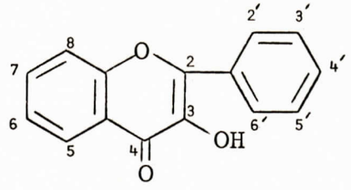
CHEMICAL TAXONOMY OF THE FLAVONOIDS

Phenolic substances in general and flavonoids in particular form a major class of compounds as far as their usefulness and use in chemical plant taxonomy and biochemical systematics. The phenolics occupy the longest chapter in Alston and Turner's Biochemical Systematics.[12] Five of the chapters in Swain's ChemicalPlant Taxonomy[13] deal directly or indirectly with flavonoids as taxonomic markers. Books dealing exclusively with the flavonoids such as Geissman's The Chemistry of Flavonoid Compounds[16] and Harborne's Comparative Biochemistry of the Flavonoids[17] include at least one chapter each on the chemotaxonomical use of these substances. Other studies and reviews[14-5,18-23] also show that the use of flavonoids has a large appeal and value in chemical plant taxonomy.

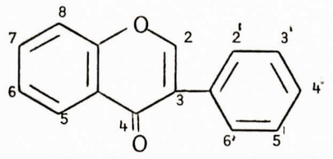
For use in chemotaxonomy, a class of compounds has to have the following characteristics. First, it is neccessary that the compounds be present in most, if not all, the plants incorporated in the study. Flavonoids are known to be present in all the angiosperms, gymnosperms and pteridophytes that have been investigated for their presence.[24] Secondly the compounds have to exhibit structural variability. Flavonoids exist in a number of classes the skeletons for some of which appear with their numbering schemes in figure I. A great number of variations on the basic skeletons is possible considering the number of possible methyl, hydroxyl,



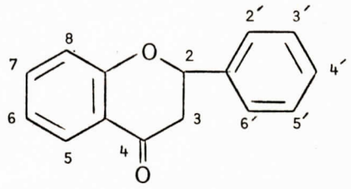
Flavones



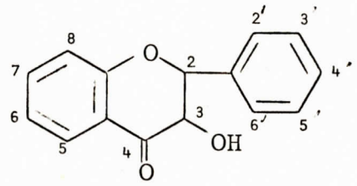
Flavonols



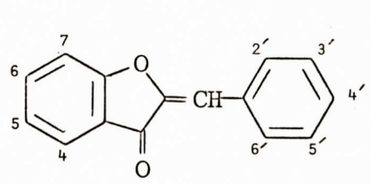
Isoflavones



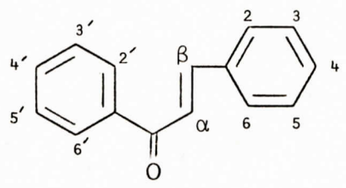
Flavanones



Dihydroflavonols



Aurones



Chalcones

Figure I. Skeletons and Numbering Schemes for the Main Classes of Flavonoids

glycosyl and other groups on each of the skeletons. The number of flavonoids isolated and characterized reached 600 in 1972.[25] Thirdly, a class of compounds has to be stable to be useful in plant chemotaxonomy. The flavonoids are generally stable in plants. Finally, it is very important that the compounds can be easily isolated and identified. The flavonoids can be isolated and identified easily and rapidly making them very suitable for chemotaxonomic investigations.

One of the best examples of the use of favonoids in chemotaxonomy is the study of Alston et al on the Baptista species in which they found a number of species-specific flavonoids.[26 - 29] Their study suggests " a generally high degree of enzyme specificity controlling the production of flavonoid types." They "conclude that truly hybrid compounds may be produced in interspecific hybrids, and that considerable enzyme specificity governing even these secondary compounds may be expected."[29]

Other chemotaxonomic findings involving the flavonoids include those of Baker and Ollis who found that the biflavonyls are almost restricted to the Gymnospermae and absent from the Pinaceae.[30-1] ; the presence of 3-deoxyanthocyanins in most of the species in the sub-family Gesneriaceae and their absence from the sub-family Cyrtandroideae[31] ; the absence of anthocyanins from most of the families in the order Centrospermeae [31-2] ; and others.

In 1973 Harborne et al identified a number of species-specific derivatives of kaempferol in the ferns of the Appalachian Asplenium complex.[33] The above are just a few selected examples of the flavonoids in chemotaxonomy.

FLAVONOIDS IN FERNS

Flavonoids are known to be present in all the angiosperms, gymnosperms and pteridophytes that have been investigated[24]. Their general distribution in higher plants and their comparative biochemistry are the subjects of Harborne's Comparative Biochemistry of the Flavonoids.[17] Studies on the chemical constituents of ferns are scarce and non-systematic, usually concentrating on certain compounds from specific ferns. A lot of work has been done on the Dryopteris species and its chemical constituents, especially the phloroglucinols[34-5].

A few examples of the presence and chemotaxonomy of flavonoids in certain plants have already been cited. Of particular interest to this study are the flavonoids present in ferns. The kaempferol derivatives found by Harborne in the ferns of the Appalachian Asplenium complex have already been mentioned[33].

Early investigations by Bate-Smith showed the presence of leuco-anthocyanins in ferns[36]. Harada and Saiki found such flavonoids as apigenin, luteolin, kaempferol, quercetin, farrerol and cyrtominetin to be widely distributed in ferns and they reported the results for 290 kinds of ferns.[34,37-8] Using paper chromatography, Lee examined the distribution of flavonoids in 173 species of Formosan ferns obtaining positive results with Marsiliaceae and the genera Cheilanthes and Thelypteris but not with Asplenium[39]. Voirin studied the

flavonoids present in 48 species of Filicinae fromCentral Africa, Spain and Southeastern France. He determined the leuco-anthocyanins and flavonoids qualitatively and quantitatively[23]. In a general phytochemical survey conducted on 100 West Indian fern species, Lynch found leuco-anthocyanins in 17 species[40].

More specific studies on flavonoids in ferns are reviewed by Berti and Bottari[35] who reported a number of flavonoids, many of which are glucosides, found mainly by Japanese investigators. Kobayashi isolated a flavonoid which was probably kaempferitrin (kaempferol 3,7-dirhamnoside) from Onychium japonicum and he found kaempferol 3-rhamnoglucoside in Dryopteris oligophlebia[41]. Kishomoto isolated and identified quercitrin (quercetin 3-rhamnoside) and afzelin (kaempferol 3-rhamnoside) from Dicranopteris dichotoma[42], From Cyrtomium falactum and other Cyrtomium species, Kishomoto isolated and identified isoquercitrin, astragalin, cyrtopterin, cyrtomin, cyrtominetin and farrerol.[43-6] Ueno isolated two kinds of flavone glycosides, phegopolin (genkwanin 4'-glucoside) and phegokaempferin (kaempferol 3-glucorabinoside), from Phegopteris polypodioides and no other plant[47]. Astragalin (kaempferol 3-glucoside), isoquercitrin (quercetin 3-glucoside) and rutin (quercetin 3-rhamnoglucoside) were isolated by Nakabayashi from Pteridium aquilinum[48]. Berti and Bottari[35] report additional flavonoids found in ferns including anthocyanins,

leucoanthocyanidins, chalcones, dihydrochalcones as well as xanthones, which are not flavonoids,

Bohn and Tryon surveyed some tree ferns for cinnamic acid and benzoic acid derivatives[49] and Lynch found leuco-anthocyanins in certain tree ferns[40].

In the Cyatheaceae, Ueno[50] found vitexin in Cyathea fauriei. Soeder[51] found vitexin in Cyathea tueckheimii and C. onusta and isovitexin in C. divergens. Hiraoka[52] found kaempferol 3-sophoroside and kaempferol 7-rhamnoglucoside in Cyathea fauriei, C. metensiana and C. leichardtiana and kaempferol 3-galactoside and kaempferol 3-rhamnoglucoside in Cyathea podophylla and C. hancockii. In all the five species Hiraoka also found vitexin, orientin, kaempferol 3-glucoside, kaempferol 3-rhamnoside and kaempferol 3-arabinoside.

CHROMATOGRAPHY OF FLAVONOIDS

Harborne[53] has reviewed the different chromatographic methods and electrophoresis and assessed their usefulness in the separation and identification of phenolic compounds. Thin layer chromatography is widely used for rapid identification and for separations that are not easily performed on paper. Stahl[54] describes the possibilities for the separation by TLC of plant phenolic derivatives and provides information on some useful separation systems. Gas liquid chromatography is useful for the separation of the smaller, more volatile phenols[53]. Electrophoresis generally has no advantage over paper chromatography, although it has been used in a few limited cases when other methods failed[53]. Conventional column chromatography on polyamide and Sephadex is frequently used for the large scale partial purification of phenolic substances from plant extracts, for the separation of different classes of phenolics and for separating individual components in milligram quantities[53,55].

Paper chromatography, first used by Bate-Smith[56] to separate phenolic pigments in 1948, continues to be the most utilized method for the separation of theses substances, which include the flavonoids. These compounds are suitable for separation by paper chromatography because they have the appropriate range of solubility and usually exhibit a pattern in their separation on paper chromatograms related to their chemical structures. The R_f values of the flavonoids are

determined mostly by the number of hydroxyl, methyl and glycosyl groups. Other factors, such as the planarity of the molecules, also affect the R_f values[53].

Although an infinite number of solvent systems can be used for separating the flavonoids and other phenolics, there are a few standard systems commonly used as an aid in their characterization by comparing R_f values with the literature. Harborne[53] lists the most common ones. Soeder uses three standard solvent system in a data retrieval system. They are TBA, HOAc and BAW which will be described in the experimental section.[57]

In addition to their R_f values, the appearance of flavonoids on the paper chromatograms aids in their identification. Except for a relatively few exceptions, they can be detected without using chromatographic sprays and reagents[53]. Usually all that is needed is as ultraviolet lamp and ammonia vapor.

There are some complications which occasionally arise of which the chemical taxonomist has to be aware.[53] Isomers can separate in certain solvents and can be thought to be quite different compounds. Alcohols can be esterified. Esters can be hydrolyzed. Two flavonoids can form a complex. Other complications can occur but the advantages of paper chromatography in the chemical taxonomy of flavonoids still outweigh its disadvantages.

E X P E R I M E N T A L

1) COLLECTION AND IDENTIFICATION OF SAMPLES

Four Costa Rican tree ferns were collected in June 1972 and June 1973 by R.W. Soeder, Appalachian State University, Boone, North Carolina and identified by R.M. Tryon, Gray Herbarium, Harvard University, Cambridge, Massachusetts. A fifth Costa Rican tree fern was collected by B. McAlpin, San Vito, Costa Rica in the Spring of 1974 and identified by R.M. Tryon. Two Jamaican tree ferns were collected by R.W. Soeder in December 1973 and identified by R.G. Proctor, Institute of Jamaica, Kingston, Jamaica. All the ferns were cut fresh and dried before extraction.

Table I lists the seven species investigated, their voucher numbers, the date and place of collection as well as the names of the persons collecting and identifying each fern sample.

2) REMOVAL OF NON-POLAR MATERIALS

Between 10 and 50 grams of green or brownish dry fronds were minced in a blender and extracted twice by shaking, at least overnight, on a platform shaker with 100-200 ml hexane to remove the non-polar constituents. The hexane

TABLE I

Voucher No.*	Species	Collection date	Location	Collected by	Identified by
72-26	Trichipteris stipularis (Christ) Tryon	June 1972	Osa Peninsula, near Rincon, Costa Rica El. 50 m	Soeder	Tryon
72-27	T. trichiata (Maxon) Tryon	June 1972	Same as 72-26	Soeder	Tryon
72-56	T. schiediana (Presl) Tryon	June 1973	Calle de Los Angeles, beyond Angele Del Norte, Costa Rica	Soeder	Tryon
73-71	T. chnoodes (Christ) Tryon	June 1973	Same as 72-26	Soeder	Tryon
73-96	T. armata (Sw.) Tryon	Dec. 1973	Hardwar Gap Road, Jamaica El. 3850 ft	Soeder	Proctor
73-100	T. aspera (L.) Tryon	Dec. 1973	Hardwar Gap Road, North of Hardwar Gap, Jamaica El. 3600 ft	Soeder	Proctor
74-6	T. conjugata (Hook.) Tryon	Spring 1974	Las Cruses Tropical Botanic Gardens #73-422, San Vito de Java, Costa Rica	McAlpin	Tryon

* Vouchers are on deposit in the Phytochemical Herbarium, Appalachian State University

solution was then filtered by suction and the extracted fronds were then allowed to dry in a hood.

3) EXTRACTION OF POLAR MATERIALS

The polar materials, including the flavonoids, were then extracted three times from the dry fronds with 100-200 ml of a 50% ethanol solution by shaking on a platform shaker, at least overnight, and filtering each ethanol extract. The 50% ethanol extracts were then combined and their volume was reduced by evaporation. The reduced volume was usually 15-75 ml. The evaporation was done using a rotatory evaporator, under reduced pressure and at a temperature between 50°C and 55°C to prevent the decomposition which might take place at higher temperatures. The color of the extracts was either brown or dark green.

4) ANALYSIS OF THE 50% ETHANOL EXTRACT COMPONENTS

The analysis of the 50% ethanol extracts for their flavonoid components was performed by using a procedure similar to that described by Mabry in The Systematic Identification of Flavonoids. [58]

A. Two-dimensional paper chromatography

Part of the concentrated solution was spotted on six 46cm x 57cm Whatman No. 3 paper sheets, 10cm away from two adjacent sides. An area 1/2 inch to 3/4 inch in diameter

was spotted with the extract and dried using a heat gun until the paper was almost saturated in that spot.

The six chromatograms were folded in the short dimension and hung in a chromatocab saturated with the vapors of TBA (t-butyl alcohol, glacial acetic acid, water - 3:1:1) for about an hour. After the paper chromatograms have equilibrated with the TBA vapors, freshly prepared TBA was added in the trays at the top of the chromatograms, since all chromatograms were to be descending. The chromatograms were allowed to develop in the long direction of the paper. When the TBA solvent front reached to within a few centimeters of the lower edge, the chromatograms were removed, hung on a drying rack and dried in a hood.

The dry chromatograms were then folded along the longer side and allowed to develop, after equilibration, in the short direction using 15% acetic acid (HOAc) as the solvent. When the solvent front reached to within a few centimeters from the lower edge they were removed and dried. Development of the chromatograms with TBA lasted about 27 hours, while with HOAc it lasted about six hours.

The spots on the two-dimensional chromatograms were outlined with a pencil as they were being observed with and without ammonia vapors, under ordinary light and under ultraviolet light. Information about the appearance of each spot under the different conditions were recorded on the chromatography paper itself.

Each spot was assigned a number and the same number was given to the corresponding spots on the six chromatograms. The R_f values of each spot were determined for TBA and HOAc. A list was made for each chromatogram containing the spot numbers, their appearance (color) under the different conditions and their R_f values in TBA and HOAc.

B. Preliminary spectral analysis of the extracts

The spots were then cut off the chromatograms and spots with the same number were placed in a 125ml erlenmeyer flask. In each erlenmeyer flask 20-100ml methanol were added, depending on the size of the spot. The spot constituents were extracted by shaking on a platform shaker, usually overnight.

The methanol extracts were then suction filtered through a Buchner funnel and placed in 50-250ml round-bottom flasks. They were then evaporated to dryness by rotatory evaporation at 50-55°C under reduced pressure. After evaporating each spot extract to dryness, 1-2ml of spectral grade methanol were used to dissolve the material remaining in the flask. The resulting solutions were placed in appropriately labelled 3-5ml vials.

A preliminary spectral analysis was carried out on all the spot extracts by scanning the wavelength of the UV-visible spectrometer from 200nm to 500nm. The spectrophotometer used was a Beckman DB-G Grating Spectrophotometer with a Beckman Hydrogen Lamp Power Supply. All spectra were

recorded on a Model SRLG Sargent-Welch Recorder.

Many spot extracts had to be diluted to be within the appropriate absorbance range of the spectrophotometer. All the spot extracts were scanned and only those with characteristic flavonoid spectra were further analyzed. Typical flavonoid spectra[58,61] include two characteristic bands, Band I usually occuring above 300nm and Band II between 220nm and 290nm. For flavones and flavonols Band I has a single, well-defined peak at 320-350nm for the former and at 340-380nm for the latter. Band II has a single, well-defined peak between 240nm and 270nm for both the flavones and the flavonols. Three or four maxima are seen in the ultraviolet-visible spectra of aurones, the principal band being in the 350-430nm region. Chalcones have a strong absorption in Band I between 300nm and 400nm and a weaker absorption in Band II between 220nm and 270nm, Band I having an intense peak (Band Ia) at 340-390nm and a small peak or inflection (Band Ib) at 300-320nm. For isoflavones Band I is very weak or absent and Band II is very intense at 250-270nm and there may be a small peak or inflection at 300-330nm. Similarly for flavanones there is strong absorption in Band II in the 270-290nm region and a weak inflection at about 320-330nm.

In this study all spectra with no peaks above 300nm were considered belonging to non-flavonoid components. Solutions whose spectra portrayed flavonoid characteristics were further analyzed using a procedure outlined by M.L.

Soeder based on Mabry's procedure for determining the ultraviolet absorption spectra of flavonoids[59]. The outline of the procedure, also used for the analysis of the purified flavonoids, is reproduced below.

PROCEDURE:

Cut out a spot on the chromatogram containing a flavonoid. Cut a matching spot from a blank area of the chromatogram. Elute each for 30 min.-1 hr. with 50 ml. spectral grade MeOH. Filter each and evaporate each to dryness. Dissolve each residue in 10 ml. spectral grade MeOH. The blank will be used in the UV reference cell without added reagents for the initial screaning of possible flavonoids. Run the following spectra on the flavonoid:

1. MeOH spectrum of flavonoid - Determine wavelength accurately for each maximum in this and all other spectra.

2. NaOMe - Add 3 drops NaOMe stock solution to flavonoid solution used for spectrum no. 1. Measure spectrum immediately and again after 5 minutes to check for flavonoid decomposition. Discard solution.

3. $AlCl_3$ spectrum - To a fresh sample of flavonoid solution, add 6 drops of $AlCl_3$ stock solution. Run spectrum.

4. $AlCl_3$/HCl spectrum - Add 3 drops HCl stock solution to the flavonoid-$AlCl_3$ solution used for spectrum no. 3. Run spectrum immediately. Discard solution.

5. NaOAc spectrum - To a fresh sample of flavonoid solution and excess powdered NaOAc with gentle shaking until 1-2 mm NaOAc remain in the bottom of UV cell. Run spectrum within 2 min. and again after 5-10 min. to check for decomposition.

6. a. NaOAc/H_3BO_3 spectrum - Add excess boric acid to the solution used for spectrum no. 5. Run spectrum after solids have settled. Discard.

6. b. In cases where decomposition of the flavonoid was observed during the recording of the NaOMe spectrum, the following procedure is used to determine the NaOAc/H_3BO_3 spectrum: Add 5 drops of spectral grade MeOH saturated with boric acid to 2-3 ml. fresh flavonoid solution. Quickly add excess NaOAc with gentle shaking and run the spectrum.

5) ANALYSIS OF PURIFIED FLAVONOIDS

Without attempting to identify the flavonoids from the preliminary spectra of the impure extracts, it was felt neccessary to i) secure larger amounts of the isolated flavonoids in order to be able to carry out the aglycone and sugar analyses after hydrolyzing the glycosides; ii) purify the flavonoids for a more accurate spectral analysis.

A. Isolation and purification of flavonoids

The partially purified flavonoids obtained by two-dimensional paper chromatography had to be purified using

a third solvent system. Instead of obtaining the partially purified flavonoids by two-dimensional paper chromatography as in the preliminary analysis, and , in order to isolate more easily larger amounts of flavonoids, one dimensional chromatography in three solvent systems was carried out.

First, the original 50% ethanol extract was applied as a band on each of six 46 cm x 57 cm Whatman No. 3 paper sheets, ten centimeters away from the top short edge using a heat gun until the paper was almost saturated along that band. The six chromatograms were developed using TBA as the solvent system. After the chromatograms were removed and dried, they were viewed, as described before, with and without ammonia, with ordinary light and under ultraviolet light. Information about the bands or 'stripes' formed was recorded after they were outlined with a pencil. The information included the appearance of the different bands under the different conditions and their R_f values in TBA.

It was neccessary to cut, extract and further purify bands whose appearance and R_f values in TBA were close to the spots whose spectra portrayed flavonoid characteristics in the preliminary investigation. All the bands on the TBA chromatograms were UV analyzed for their flavonoid content after extraction with methanol and concentration. This was done to try to find flavonoids that may have not been detected in the preliminary analysis because of their lower concentration, since smaller amounts of fern extracts were used for the two-dimensional chromatography.

Each band extract which had flavonoid characteristics was then applied as a band on another 46 cm x 57 cm Whatman No. 3 paper sheet and developed using HOAc. Here again, not only bands that had similar appearance and R_f values to the flavonoids found in the preliminary investigations, but all the bands on the HOAc chromatograms were extracted with methanol, concentrated and analyzed spectroscopically.

All band extracts that had UV flavonoid characteristics were further purified by being applied as a band on a 23 cm x 57 cm Whatman No. 1 paper and the chromatograms were developed using a third solvent system, BAW. The BAW solvent system was the top layer obtained after mixing 4 volumes n-butyl alcohol, one volume glacial acetic acid and 5 volumes water.

After the bands of the BAW chromatograms were extracted with methanol and analyzed, those that contained the flavonoids were saved for i) accurate R_f determinations in three solvent systems, ii) accurate spectral analysis and iii) the aglycone and sugar analyses after the glycoside hydrolysis.

B. Accurate R_f measurements

A small portion of the methanol extracts of the purified flavonoids was used for the final determination of the R_f values to be used for the identification of the flavonoids. Each of three Whatman No. 1 chromatography sheets, 23 cm x 57 cm, was spotted with three small spots,

about 1/2 cm in diameter, 10 cm away from the top edge and about 6 cm apart. The chromatograms were developed using the freshly made solvent systems described above, namely TBA, HOAc and BAW. The flavonoid spots were viewed under ultraviolet light after drying and their R_f values were determined accurately by averaging the distances travelled by the three spots divided by the distance travelled by the solvent.

C. UV analysis of purified flavonoids

Another portion of the purified flavonoid solution was used for the careful UV analysis needed for identification. The procedure for this analysis has already been described on page 21. Peak maxima were determined carefully from the recorded spectra and, whenever possible, from the spectrophotometer itself.

D. Aglycone and sugar analysis

The remainder of the pure flavonoid solution was hydrolyzed with 6% hydrochloric acid solution after evaporating most of the methanol. The hydrolysis was performed in a watch glass over a steam bath for 1-2 hours.

The hydrolysis products were then partitioned between ether and water, the aglycones being in the former and the sugars in the latter. After the two layers were separated, the aqueous solution was concentrated by the evaporation of most of the water and the ether was evaporated to dryness and its aglycone constituents were dissolved in a minimum amount of methanol.

Identification of the sugars and the aglycones was attempted by paper and thin layer chromatography. The aqueous sugar solution was chromatographed, on Whatman No. 1 paper or on a cellulose TLC plate, in a solvent made by mixing 12 parts ethyl acetate, 5 parts pyridine and 4 parts water, together with some common sugar standards such as glucose, galactose, mannose, arabinose, xylose, ribose and rhamnose. After development, the paper or thin layer chromatogram was dried and sprayed with a solution made by dissolving 1 gm p-anisidine and 0.1 gm sodium hydrosulfite in 10 ml of methanol and diluting to 100 ml with n-butanol. This procedure, adopted from Mabry[60], provided good separation of the sugars and was satisfactory for the identification of the unknown sugars when they were present in sufficient amounts.

Thin layer chromatography was used for the identification of the aglycones. The most common solvent systems used were chloroform, acetic acid, water (50:45:5) on a cellulose plate and toluene, chloroform, acetone (40:25:35) on a silica gel plate. These and other solvent systems and plates suitable for the separation and identification of flavonoids and other plant phenol derivatives are described in a section of Stahl's Thin Layer Chromatography.[54] The unknown aglycones were compared with such standard aglycones as are commonly found in ferns including kaempferol, apigenin and quercetin. The spots were viewed using ultraviolet light.

6) IDENTIFICATION OF FLAVONOIDS

The R_f values, UV-visible spectra and sugar and aglycone analysis results were all used in the attempt to identify the isolated flavonoids. Comparing the data obtained directly with the literature would have been a very difficult task. The task was made much easier by the use of two main aids.

Mabry[58] gives the chromatographic data as well as the UV spectral data for 175 flavonoids. The data obtained for the isolated flavonoids was compared with Mabry's tabulated data and with the UV spectral curves given for each flavonoid. Mabry's book is very useful, but it reports only 175 out of the more than 600 flavonoids that have been isolated and fully classified.

Soeder[57] has developed a data retrieval system for the flavonoids using punch cards. The punch cards, 19 x 8.5 cm, have a row of holes on all four sides of each card. Coded information, represented by one hole or more, is punched by having the part of the card between the hole and the edge of the card notched away. There is written information, including references, on both sides of the card. Coded and written information includes R_f values for the three standard solvents, color appearance under different conditions, UV maxima and the glycoside sugars. In this study, a sorting needle was used when the cards were searched for certain information, the wanted cards falling off the needle while the cards remaining on it were considered inappropriate

for that bit of information. This was very helpful in the attempt to identify the isolated flavonoids using the R_f, UV and sugar data obtained from the analyses. Figure II shows a typical punch card.

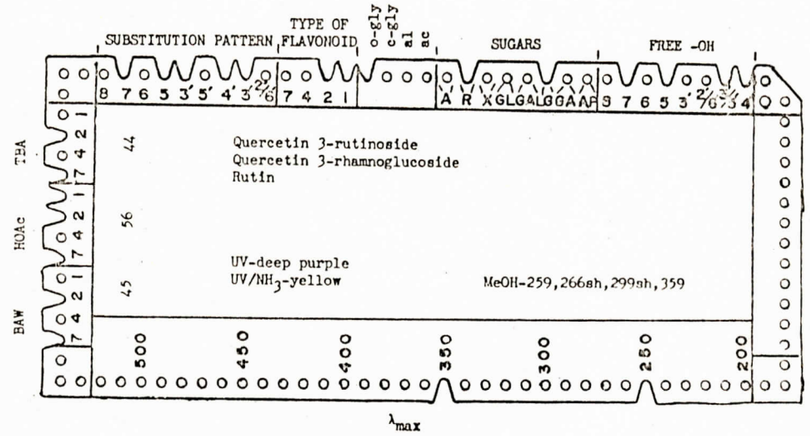


Figure II. Typical Flavonoid Punch Card

R E S U L T S

Seven tropical Cyatheaceae tree ferns of the genus Trichipteris were studied for their flavonoid constituents. Table I lists the species, their voucher numbers, their date and place of collection as well as the names of the people responsible for their collection and identification.

The experimental procedures described above allowed for the isolation of a few flavonoids from the seven tree ferns and the identification of two.

It is needless to say that probably not all the flavonoids present in the species studied have been detected. Most of the undetected flavonoids, undoubtedly present in the ferns, probably exist in low concentrations. Detection of these flavonoids would require larger samples and more time for the investigation of their presence.

The techniques used can probably be improved, especially by using high performance liquid chromatography. This technique can isolate larger amounts of flavonoids faster. The flavonoids thus isolated will be enough in amount and in purity to allow for the identification of old and new compunds.

The flavonoids isolated in this study are probably the ones that exist in higher concentrations.

72-26 TRICHIPTERIS STIPULARIS

Two flavonoids were isolated from 50 gms of Trichipteris stipularis. One was identified as kaempferol 3-glucoside (astragalin) and the other is another kaempferol glycoside.

1) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	dull yellow-green
R_f values:	(TBA)	0.67
	(HOAc)	0.44
	(BAW)	0.75

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	267, 302sh, 350
NaOMe	272, 325, 395
$AlCl_3$	272, 302, 350, 390-95
$AlCl_3$/HCl	275, 302, 345-50, 390
NaOAc	273, 302, 345-50
NaOAc/H_3BO_3	267, 350

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: glucose (barely seen on TLC plate)

IDENTIFICATION

Kaempferol 3-glucoside (astragalin). All the data support this structure although the glucose was not very clear.

2) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	deep purple-green
R_f values:	(TBA)	0.74
	(HOAc)	0.48
	(BAW)	0.86

SPECTRAL DATA (λ_{max}, nm)

MeOH 265, 300sh, 345

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: unknown

IDENTIFICATION

The only spectral data available is in methanol. From the above data it appears that this flavonoid is a a kaempferol glycoside; probably the same one found in T. conjugata.

72-27 TRICHIPTERIS TRICHIATA

One flavonoid was isolated from 22 gms of dried Trichipteris trichiata. This flavonoid was identified as kaempferol 3-glucoside.

CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	dull yellow-green
R_f values:	(TBA)	0.67
	(HOAc)	0.45
	(BAW)	0.76

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	265, 305sh, 345
NaOMe	270, 325, 395
$AlCl_3$	272, 302, 345, 380-90
$AlCl_3/HCl$	270, 302, 345, 380-90
NaOAc	270, 307, 385
$NaOAc/H_3BO_3$	265, (320sh), 350

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: glucose

IDENTIFICATION

Kaempferol 3-glucoside (astragalin).

73-56 TRICHIPTERIS SCHIEDIANA

Two flavonoids were isolated from 16 gms dry Trichipteris schiediana. One was identified as kaempferol 3-glucoside and the other as isovitexin.

1) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	dull yellow-green
R_f values:	(TBA)	0.69
	(HOAc)	0.42
	(BAW)	0.74

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	265, 300sh, 345-50
NaOMe	272, 323, 395
$AlCl_3$	270, 302, 345-50, 390-95
$AlCl_3/HCl$	268, 300, 345, 390-95
NaOAc	272, 305, 385
$NaOAc/H_3BO_3$	265, 350

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: glucose (very light yellow-brown spot)

IDENTIFICATION

Kaempferol 3-glucoside (astragalin).

2) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	dark purple
	(NH_3)	yellow
	(UV/NH_3)	yellow-green
R_f values:	(TBA)	0.56
	(HOAc)	0.55
	(BAW)	0.60

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	268, 300sh, 340
NaOMe	277, 325-30, 400
$AlCl_3$	274, 305, 350, 385sh
$AlCl_3/HCl$	260sh, 277, 302, 345, 380sh
NaOAc	275, 303, 385-90
$NaOAc/H_3BO_3$	268, 300sh, 350

HYDROLYSIS PRODUCTS

Aglycone: apigenin (and kaempferol?)

Sugar: glucose

IDENTIFICATION

Isovitexin (saponaretin).

73-71 TRICHIPTERIS CHNOODES

Isovitexin and a few unidentified flavonoids were isolated from 10 gms of dry Trichipteris chnoodes.

1) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	dark purple
	(NH_3)	yellow
	(UV/NH_3)	yellow-green
R_f values:	(TBA)	0.56
	(HOAc)	0.53
	(BAW)	0.59

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	268, 340
NaOMe	277, 325-30, 400
$AlCl_3$	275, 305, 350, 385sh
$AlCl_3$/HCl	275, 302, 345, 382sh
NaOAc	275, (300sh), 385-90
NaOAc/H_3BO_3	265, 300sh, 350

HYDROLYSIS PRODUCTS

Aglycone: apigenin (and kaempferol?)

Sugar: glucose

IDENTIFICATION

Isovitexin (saponaretin).

2) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	dark purple
	(NH_3)	yellow
	(UV/NH_3)	yellow-green
R_f values:	(TBA)	0.55
	(HOAc)	0.54
	(BAW)	0.61

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	268, 300sh, 340
NaOMe	277, 325-30, 400
$AlCl_3$	274, 305, 350, 385sh
$AlCl_3$/HCl	280, 302, 345, 385sh
NaOAc	275, 305sh, 385
NaOAc/H_3BO_3	265, 300sh, 350

HYDROLYSIS PRODUCTS

Aglycone: kaempferol - no apigenin observed

Sugar: glucose

IDENTIFICATION

Spot appearance, R_f values and UV spectra indicate that the substance is isovitexin (another portion of which has already been identified in this species) but the aglycone is exclusively kaempferol as seen on the TLC plate.

3) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	blue
	(NH_3)	yellow
	(UV/NH_3)	blue
R_f values:	(TBA)	0.59
	(HOAc)	0.70 & 0.77 (two spots)
	(BAW)	0.67

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	260sh, 305
NaOMe	295sh, 310sh, 325sh, 350
$AlCl_3$	300, 350-60
$AlCl_3/HCl$	305, 350sh
NaOAc	310, 350sh
$NaOAc/H_3BO_3$	260sh, 305

HYDROLYSIS PRODUCTS

Aglycone: small light yellow spot corresponding to kaempferol barely visible.

Sugar: unknown

IDENTIFICATION

This flavonoid, possibly a kaempferol glycoside, could not be identified. This compound separated on the final chromatogram developed by 15% HOAc.

4) CHROMATOGRAPHIC DATA

Spot appearance:	(ordinary light)	light yellow
	(UV)	light yellow
	(NH_3)	yellow
	(UV/NH_3)	fluorescent green
R_f values:	(TBA)	0.51
	(HOAc)	0.75
	(BAW)	0.56

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	245sh, 300sh, 325
NaOMe	250sh, 300sh, 325sh, 350sh, 370
$AlCl_3$	255sh, 330sh, 355
$AlCl_3/HCl$	245sh, 320
NaOAc	260sh, 320sh, 345sh, 375
$NaOAc/H_3BO_3$	250sh, 305sh, 325sh, 340, 350sh

HYDROLYSIS PRODUCTS

Aglycone: very light yellow spot with same R_f as kaempferol, surrounded by fluorescent blue-green

Sugar: unknown

IDENTIFICATION

This flavonoid has not been identified.

5) CHROMATOGRAPHIC DATA

Spot appearance:	(ord. light)	faint yellow
	(UV)	blue
	(NH_3)	yellow
	(UV/NH_3)	blue
R_f values:	(TBA)	0.48 & 0.56 (two spots)
	(HOAc)	0.51
	(BAW)	0.59

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	315, 350sh
NaOMe	245sh, 295sh, 325sh, 360
$AlCl_3$	310, 350sh
$AlCl_3$/HCl	310, 350sh
NaOAc	275sh, 310, 325sh, 365
NaOAc/H_3BO_3	315, 350sh

HYDROLYSIS PRODUCTS

Aglycone: light yellow spot corresponding to kaempferol, surrounded by fluorescent blue

Sugar: unknown

IDENTIFICATION

Possibly a kaempferol derivative

73-96 TRICHIPTERIS ARMATA

Two flavonoids were isolated from 32 gms of dry Trichipteris armata. One was identified as kaempferol 3-glucoside and the other as another kaempferol glycoside.

1) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	dull yellow-green
R_f values:	(TBA)	0.73
	(HOAc)	0.45
	(BAW)	0.72

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	266, 300sh, 350
NaOMe	274, 323, 400
$AlCl_3$	273, 302, 345, 390-95
$AlCl_3$/HCl	275, 302, 348, 390
NaOAc	273, 307, 390
NaOAc/H_3BO_3	266, 350

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: glucose

IDENTIFICATION

Kaempferol 3-glucoside (astragalin).

2) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	green-purple
R_f values:	(TBA)	0.81
	(HOAc)	0.48
	(BAW)	0.82

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	267, 295sh, 342
NaOMe	272, 320, 390
$AlCl_3$	274, 304sh, 345, 390-95
$AlCl_3$/HCl	274, 304sh, 342, 385sh
NaOAc	266, 305sh, 385
NaOAc/H_3BO_3	266, 343

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugars: three sugar spots could vaguely be seen on TLC corresponding to rhamnose, xylose and arabinose. Not definite.

IDENTIFICATION

Spectra almost indistinquishable from astragalin. Apparently there are two or three sugars on kaempferol.

IN addition to the three paper chromatography solvents, a few TLC systems failed to separate the glycoside. Apparently, this flavonoid is a kaempferol di- or tri-glycoside.

73-100 TRICHIPTERIS ASPERA

A very small amount of one flavonoid was isolated from 11 gms of dry Trichipteris aspera.

CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	faint yellow
	(UV/NH_3)	purple
R_f values:	(TBA)	0.84
	(HOAc)	0.46
	(BAW)	0.87

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	267sh, 295sh, 345sh
NaOMe	273sh, 295sh, 385
$AlCl_3$	273sh, 300sh, 350
$AlCl_3/HCl$	273sh, 300sh, 350
NaOAc	270sh, 300sh, 345
$NaOAc/H_3BO_3$	270sh, 300sh, 345

HYDROLYSIS PRODUCTS

Aglycone: not observed- very low concentration

Sugar: not observed- very low concentration.

IDENTIFICATION

Low concentration did not allow observation of hydrolysis products and accurate spectra. Spectra have trend of kaempferol glycosides. R_f values and appearance similar to kaempferol di- or tri-glycoside found in T. armata. They are possibly the same compound.

74-6 TRICHIPTERIS CONJUGATA

Two flavonoids were isolated from 12 gms of dry Trichipteris conjugata. One of the flavonoids was kaempferol 3-glucoside, the other another kaempferol derivative.

1) CHROMATOGRAPHY DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	dull yellow-green
R_f values:	(TBA)	0.69
	(HOAc)	0.43
	(BAW)	0.74

UV SPECTRAL DATA (λ_{Max}, nm)

MeOH	267, 300sh, 345-50
NaOMe	272, 322sh, 395
$AlCl_3$	272, 302, 345-50, 390sh
$AlCl_3/HCl$	270sh, 302, 345-50, 390sh
NaOAc	273, 305, 385
$NaOAc/H_3BO_3$	266sh, 345

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: undetected

IDENTIFICATION

Kaempferol 3- glucoside (astragalin). This can be concluded from the above data although the sugar could not be detected.

2) CHROMATOGRAPHIC DATA

Spot appearance:	(UV)	purple
	(NH_3)	yellow
	(UV/NH_3)	dull yellow-green
R_f values:	(TBA)	0.74
	(HOAc)	0.47
	(BAW)	0.82

UV SPECTRAL DATA (λ_{max}, nm)

MeOH	262, 302sh, 340-45
NaOMe	270, 385
$AlCl_3$	272, 302, 340-45, 385-90sh
$AlCl_3/HCl$	270, 302, 340-45, 385-90sh
NaOAc	270, 386
$NaOAc/H_3BO_3$	262, 340-45

HYDROLYSIS PRODUCTS

Aglycone: kaempferol

Sugar: unknown

IDENTIFICATION

From the above data it seems that this flavonoid is a kaempferol glycoside, possibly the same as the one found in T. stipularis which has similar R_f values and appearance, as well as a similar methanol spectrum.

SUMMARY OF RESULTS

Kaempferol 3-glucoside was found in five Trichipteris species out of the seven investigated. Four were Costa Rican and one was Jamaican. This is a very common constituents of the Cyatheaceae. It has been found in all five of the Cyathea species investigated by Hiraoka et al, for example.[52]

Isovitexin was found in two of the Costa Rican ferns studied. It was reported for the first time in ferns by Soeder and Babb when they found it in Cyathea divergens.[51]

After the hydrolysis of isovitexin, both apigenin and kaempferol, which have different R_f values and colors, were observed on the TLC plates. In addition, after hydrolyzing a different flavonoid portion, also believed to be isovitexin, only kaempferol was visible.

An unidentified kaempferol derivative was found in T. stipularis and T. conjugata, both Costa Rican ferns.

Another kaempferol derivative, which is probably a di- or a tri-glycoside, was found in T. armata and T. aspera, both Jamaican ferns.

In two, separate, cases purified unidentified kaempferol derivatives separated when their R_f values were to be determined. One of the compounds separated when its chromatogram was developed in TBA; the other in HOAc. Separation of isomers in certain solvents is known to occur as previously mentioned in the section about the chromatography of the flavonoids.

The number of flavonoids found in each fern ranged from one to five, without any correlation to the amount of dry fern used.

Table II summarizes some of the results obtained in this investigation.

TABLE II

Voucher No.	Species	(gms) wt. used	Kaempferol 3-glucoside	Isovitexin	No. isolated not identified
72-26	T. stipularis	50	+	-	1
72-27	T. trichiata	22	+	-	-
73-56	T. schiediana	16	+	+	-
73-71	T. chnoodes	10	-	+	4
73-96	T. armata (J)*	32	+	-	1
73-100	T. aspera (J)*	11	-	-	1
74-6	T. conjugata	12	+	-	1

(J)* These ferns were collected in Jamaica; the rest in Costa Rica.

C O N C L U S I O N

Kaempferol 3-glucoside, being a common constituent of ferns, cannot be used as a taxonomic marker. The significance of the fact that isovitexin was found only in two Costa Rican ferns is uncertain, especially since the number of Jamaican ferns investigated was limited.

Similarly uncertain is the significance of the fact that an unidentified kaempferol derivative was found in two of the Costa Rican ferns and in neither of the Jamaican ferns.

Perhaps more significant is the apparent presence of the unidentified di- or tri-glycoside derivative of kaempferol in both of the Jamaican ferns and in none of the Costa Rican ferns.

Although vitexin, found by Soeder and Babb[51] in two Cyathea species, was not found in any of the Trichipteris species, isovitexin, found in one Cyathea species, was found in two Trichipteris species.

This thesis will neither provide a complete answer about the distribution of flavonoids in the Trichipteris species, nor will it answer the questions as to whether the Cyatheaceae and the Dicksoniaceae should have a common classification and whether Trichipteris sp. and Cyathea sp. should be combined or separated. At best, it may offer a very minor contribution towards those questions.

REFERENCES

1. Holttum, R.E. and U. Sen, Phytomorphology, 11, 406 (1961)
2. Holttum, R.E., Amer. Fern Journal, 54, 1 (1964)
3. Roy, S.K. and R.E. Holttum, Amer. Fern Jour.,55,35(1965)
4. Roy, S.K. and R.E. Holttum, Amer. Fern Jour.,55,160(1965)
5. Nayar, S., Taxon, 19, 229 (1970)
6. Tryon, R., Amer. J. Bot., 53, 638 (1966)
7. Tryon, R., Rhododora, 74(800), 441 (1972)
8. Lucansky, T.W. and R.A. White, Amer. J. Bot.,61,818(1974)
9. Tryon, A.S. and L.J. Feldman, Can. J. Bot., 53,2260(1975)
10. Tryon, R., Contrib. Gray Herb. Harv. Univ.,200,1 (1970)
11. Tryon, R., Contrib. Gray Herb. Harv. Univ., 206,19 (1976)
12. Alston, R.E. and B.L. Turner, Biochemical Systematics, Prentice-Hall, N.J. (1963) p.45
13. Swain, T. ed., Chemical Plant Taxonomy, Academic Press, London (1963)
14. Harborne, J.B., Progress in Phytochemistry, ed. by L. Reinhold and Y. Liwschitz, Vol. 1, p. 545, Interscience Publishers, N.Y (1968)
15. Mabry, T.J, R.E. Alston and V.C. Runeckles, eds., Recent Advances in Phytochemistry, Vol. 1 , Appleton-Century-Crofts, N.Y (1968)
16. Geissman, T.A., ed., The Chemistry of Flavonoid Compounds, McMillan, N.Y. (1962)
17. Harborne, J.B., Comparative Biochemistry of the Flavonoids, Academic Press, London (1967)
18. Pakosi, M., Acta. Univ. Debrecen., 7, 159 (1961) Chemical Abstracts, 57, 10125a (1962)

19. Haensel, R., Planta Medica, 10, 361 (1962)
20. Feenstra, W.J., Planta Medica, 10, 412 (1962)
21. Bate-Smith, E.C. and T. Swain, Lloydia, 28(4),313 (1965)
22. Leifertova-Hajkova, L., Cesk. Farm., 14(8), 427 (1965) Chemical Abstracts, 64, 11549b (1965)
23. Voirin, M.B., C.R. Acad. Sc., Paris, Ser. D., 264,665(1967)
24. Harborne, J.B., Comparative Biochemistry of the Flavonoids, Ch. 4
25. Harborne, J.B., Recent Advances in Phytochemistry, ed. by V. Runeckles and J. Watkin, Vol. 4, Appleton-Century-Crofts, N.Y (1972)
26. Alston, R.E. and B.L. Turner, Nature, 184, 285 (1959)
27. Turner, B.L. and R.E. Alston, Amer. J. Bot., 46, 678(1959)
28. Alston, R.E. and B.L. Turner, Biochemical Systematics, pp. 35, 43, 45, 49, 74, 111, 113, 166, 175, 316
29. Alston, R.E., H. Rosler, K. Naifeh and T.J. Mabry, Proc. Nat. Acad. Science USA, 54, 1485 (1965)
30. Alston, R.E. and B.L., Biochemical Systematics, p. 219
31. Harborne, J.B., Comparative Biochemistry of the Flavonoids, pp. 306-10
32. Mabry, T.J., Comparative Phytochemistry, ed. by T. Swain, pp. 231-44 Academic Press, London (1966)
33. Harborne, J.B., Williams, C.A. and D.M. Smith, Biochemical Systematics, 1, 51 (1973)
34. Harada, T, Y. Saiki and U. Amano, Shizuoka Yakka Daigaku Kaigaku 5-Shunen Kinen Rombushu, 1958, 76 Chemical Abstracts, 53, 1473c (1959)
35. Berti, G. and F. Bottari, Progress in Phytochemistry, ed. by L. Reinhold and Y. Liwschitz, Vol. 1, p.589
36. Bate-Smith, E.C. and N.H. Lerner, Biochem. J., 58,126(1954)
37. Harada, T. and Y. Saiki, J. Pharm. Soc. Japan, 75, 267(1955) Chemical Abstracts, 49, 9884g (1955)

38. Harada, T. and Y. Saiki, Pharm. Bull. Japan, 3,469(1955)
Chemical Abstracts, 50, 14878e (1956)

39. Lee, K.H., F.C. Huang and T.H. Yang, J. Taiwan Pharm Assoc, 13, 25 (1961)
Chemical Abstracts, 58, 1721c (1961)

40. Lynch, B., A. Fay and C. Seaforth, Lloydia, 33,284(1970)

41. Kobayashi, K., J. Pharm. Soc. Japan, 64, 35 (1944)
G. Berti and F. Bottari, op. cit., p.636

42. Kishomoto, Y., J. Pharm. Soc. Japan, 75, 1437 (1955)
Chemical Abstracts, 50, 10091c (1956)

43. Kishimoto, Y., J. Pharm Soc. Japan, 76, 246 (1956)
Chemical Abstracts, 50, 13894b (1956)

44. Kishomoto, Y., J. Pharm. Soc. Japan, 76, 250 (1956)
Chemical Abstracts, 50, 13894b (1956)

45. Kishomoto, Y., Pharm Bull (tokyo), 4, 24 (1956)
Chemical Abstracts, 51, 2761h (1957)

46. Arthur, H.R. and Y. Kishomoto, Chem Ind (london) 1956,738

47. Ueno, A., J. Pharm Soc Japan, 82, 1479 (1962)
Chemical Abstracts, 59, 7361 (1962)

48. Nakabayashi, T., Vitamins (Kyoto), 8, 410 (1955)
Chemical Abstracts, 49, 14826i (1955)

49. Bohm, B.A., R.M. Tryon, Can. J. Bot., 45, 585 (1967)

50. Ueno, A., K.H. Oguri, Y. Saiki and T. Harada, J. Pharm. Soc. Japan, 83, 420 (1963)
Chemical Abstracts, 59, 3716h (1963)

51. Soeder, R.W., and M.S. Babb, Phytochemistry, 11,3079(1972)

52. Hiraoka, A. and M. Hasegawa, Bot. Mag. Tokyo, 88,127(1975)

53. Harborne, J.B., Chromatography, ed. by E. Heftman p.759
Van Nostrad Reinhold, N.Y. (1975)

54. Stahl, E., ed., Thin-Layer Chromatography, Springer-Verlag N.Y. (1969) p. 686

55. Woof, J.B and J.S. Pierce, J. Chromatogr., 28, 94 (1967)

56. Bate-Smith, E.C., Nature, 161, 835 (1948)

57. Soeder, M.L. and R.W. Soeder, Planta Medica, 30,189(1976)

58. Mabry, T.J., K.R. Markham and M.B. Thomas, The Systematic Identification of the Flavonoids, Springer, New York (1970)

59. Ibid, Ch. 4

60. Ibid, Ch. 3

61. Jurd, L., The Chemistry of Flavonoid Compounds, ed. by T.A. Geissman, Ch. 4 , McMillan, N.Y. (1967)