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COMPARATIVE BIOCHEMISTRY OF FLAVONOIDS—III.

A CORRELATION BETWEEN CHEMISTRY AND PLANT GEOGRAPHY IN THE GENUS *EUCRYPHIA**

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Abstract—A correlation between flavonoid chemistry and plant geography has been discovered among *Eucryphia* species. The rare quercetin 5-monomethyl- and 3,5-dimethyl ethers, azaleatin and caryatin, have been identified in leaf of the two South American species *E. cordifolia* and *E. glutinosa* and in two of their hybrids. Caryatin occurs in the free state but azaleatin is present as the 3-galactoside, as a 3-arabinosylgalactoside and as a 3-diglucoside; the 3-galactoside, 3-rhamnoside and a 3-diglycoside of quercetin are also present. Neither of the above methyl ethers occurs in leaf of the other three known *Eucryphia* species, all of which are of Australian origin. These three species have a much simpler flavonoid pattern: *E. milliganii* has dihydroquercetin glycoside, *E. moorei* a quercetin 3-triglycoside and *E. lucida* kaempferol 3,7-dimethyl ether as the respective major leaf constituents. This survey represents a complete coverage of the Eucryphiaceae as at present constituted and the bearing of these results on the taxonomic position of the family are discussed.

INTRODUCTION

THE Eucryphiaceae, which consists of a single genus of five species of tree, is an archichlamydous family, the taxonomic position of which is a matter of some dispute. At one time or another, it has been associated variously with at least thirteen other families. It is placed in the latest revision[1] of Engler's Syllabus close to the Dilleniaceae (order Guttiferales) but Bausch earlier[2] considered, from studying its wood anatomy, that it was closely related to the Cunoniaceae (order Rosales).

It is thus a matter of some interest to see if the chemistry of the Eucryphiaceae has any bearing on its taxonomy. An encouragement to examine the chemical constituents came during a leaf survey of dicotyledonous plants,[3] when a flavonoid of unusual yellow fluorescence in u.v. light was detected in *Eucryphia glutinosa*. The substance was subsequently identified as the rare 5-methyl ether of quercetin (azaleatin, (I))[4] and the phenolics of all five species and two hybrids have now been examined and are reported here.

RESULTS

Flavonoid Identifications

Two-dimensional chromatography of leaf extracts of *Eucryphia* species showed (Fig. 1) that some fourteen flavonoids were variously present. The major constituents (Table 1)

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* Part II: J. B. HARBORNE, *Phytochem.* **5**, 589 (1966).

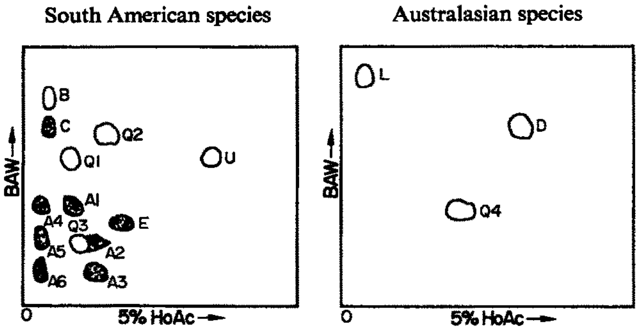
[1] A. ENGLER'S *Syllabus der Pflanzenfamilien*, 12 edn. (Edited by H. MELCHIOR), Vol. 2. Springer-Verlag, Basle (1964).

[2] J. BAUSCH, *Kew Bull.* 317 (1938).

[3] E. C. BATE-SMITH, *J. Linn. Soc.* (*Botany*) **58**, 95 (1962).

[4] E. C. BATE-SMITH, J. B. HARBORNE and S. M. DAVENPORT, *Nature* **212**, 1065 (1966).

were identified as far as was possible by spectral and chromatographic studies of the substances and their products of acid hydrolysis. Identifications in some cases are tentative because the compounds occurred only as trace constituents or else were difficult to isolate in a pure form. Some were unusually acid-labile; the azaleatin and quercetin diglycosides in particular were often completely hydrolysed simply by chromatography on paper in a solvent mixture containing acetic acid. Others were contaminated, even after several different chromatographic developments, with leucoanthocyanidin materials which occur in abundance in all five species investigated.


South American species

Australasian species

FIG. 1. COMPOSITE TWO-DIMENSIONAL CHROMATOGRAMS OF FLAVONOIDS IN LEAF EXTRACTS OF EUCRYPHIA SPECIES.

Flavonoid*	Identification	Distribution in *Eucryphia* *cordifolia*	*glutinosa*†	*moorei*	*milliganii*	*lucida*
C	Caryatin	+	+	−	−	−
A 1	Az 3-galactoside	−	+	−	−	−
A 2	Az 3-diglucoside	+	+	−	−	−
A 3	Az 3-arabinosylgalactoside	+	−	−	−	−
A 4–6	Azaleatin (Az)	+	+	−	−	−
E	Unidentified flavanone (?)	+	+	−	−	−
Q 1	Quercetin 3-galactoside	+	+	−	+	−
Q 2	Quercetin 3-rhamnoside	+	+	−	−	−
Q 3	Quercetin 3-diglycoside	+	+	−	−	−
Q 4	Quercetin 3-triglycoside	−	−	+	−	−
D	Dihydroquercetin 3-glycoside(s)	−	−	−	+	−
L	Kaempferol 3,7-dimethyl ether	−	−	−	−	+
B	Cordifolia constituent	+	−	−	−	−
U	Unidentified flavan	+	+	−	−	−

* Colours in u.v. light without and with NH_3: C, blue (green); A 1–3, blue fluorescence (yellow); A 4–6, E, yellow fluorescence (yellow fl.); Q 1–4, dark ochre (dull green); L, dark ochre (dull yellow); B, colourless (green); U, absorbing (absorbing).

† The hybrid *E.* × *nymansensis* is similar to *glutinosa* and, in addition, has component A 3.

Derivatives of azaleatin (I), A 1–A 3, were readily recognized by their intense yellow fluorescence in u.v. light (with or without ammonia) and the aglycone, present on chromatographs (spots A 4–A 6) as a breakdown product of the glycosides and also obtained by hydrolysis in alcoholic acid of A 1, A 2 and A 3, was identified by direct comparison with authentic material earlier isolated from *Plumbago capensis* (Plumbaginaceae). Azaleatin, previously known only as the 3-rhamnoside azalein in *Rhododendron*[5, 6] and *Plumbago*,[5] was identified in *Eucryphia* as the 3-galactoside. That A 1 (Fig. 1) is the simple galactoside is based upon its spectral properties and its R_f value in relation to azalein and the 3-galactoside and 3-rhamnoside of quercetin; it was also identical in R_f to a sample of azaleatin 3-galactoside isolated from *Ceratostigma plumbaginoides* leaf.[7] Two other azaleatin glycosides A 2 and A 3 were recognized by R_f values, spectral shifts and hydrolysis products as 3-diglycosides.

TABLE 1. SPECTRAL AND CHROMATOGRAPHIC PROPERTIES OF *Eucryphia* FLAVONOIDS

	λ_{max} in EtOH (nm)				
Component	Alone	+NaOAc	+NaOAC–H_3BO_3	+NaOH	+$AlCl_3$
A 1, A 2 and A 3	249, 345	250, 350 272	257, 372	268, 399	269, 350 413
C	253, 347	253, 347	258, 366	267, 392	253, 347
Q 1, Q 3 and Q 4	257, 364	266, 375	269, 385	277, 415	268, 390
Q 2	257, 353	268, 363	263, 374	274, 405	272, 344, 404
D	225, 292 330 (infl.)	—	—	245, 322	309, 363
E	282, 330 (infl.)	—	—	—	—
L	269, 346	269, 346	269, 346	277, 393	279, 356 394 (infl.)

	R_f value (×100) in			Products of acid hydrolysis
Component	BAW	5% HOAc	PhoH	
A 1	51	15	73	Azaleatin and galactose
A 2	27	34	19	Azaleatin and glucose
A 3	32	24	18	Azaleatin, arabinose and galactose
C	73	03	89	Unchanged on acid treatment
Q 1	56	16	39	Quercetin and galactose
Q 2	65	31	50	Quercetin and rhamnose
Q 3	36	31	11	Quercetin, galactose and glucose*
Q 4	30	49	06	Quercetin, rhamnose and glucose
D	63	54	—	Dihydroquercetin, rhamnose, xylose and arabinose*
E	29	47	—	Glucose and aglycone with yellow–green fluorescence in u.v. light, λ_{max} 273, 279, R_f 0·69 in BAW, 0·61 in Forestal
L	94	04	97	Unchanged on acid treatment

* Sugar analyses on these pigments are possibly unreliable, because of contamination with leucoanthocyanidin material.

A 2 gave only glucose on hydrolysis and appears to be a 3-diglucoside; A 3 gives arabinose and galactose and may be a 3-arabinosylgalactoside, a sugar combination reported recently in quercetin glycosides of *Quercus incana* leaf[8] and of *Rumex maritimus* fruit.[9] Both are very

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[5] J. B. HARBORNE, *Arch. Biochem. Biophys.* **96**, 171 (1962).

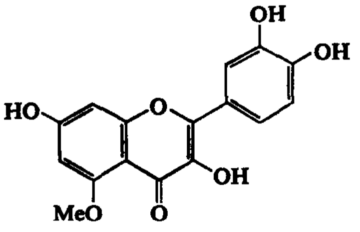
[6] E. WADA, *J. Am. Chem. Soc.* **78**, 4725 (1956).

[7] J. B. HARBORNE, *Phytochem.* **6**, 1415 (1967).

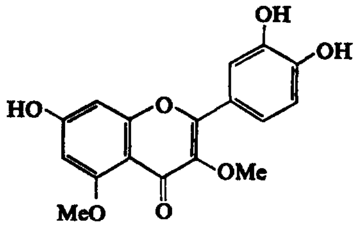
[8] V. K. KALRA, A. S. KUKLA and T. R. SESHADRI, *Current Sci.* (*India*) **35**, 204 (1966).

[9] A. K. BAGRI, B. V. KURMEZ and V. I. LITVINENKO, *Chem. Abstr.* **65**, 15489 (1966).

acid-labile, as is Q 3, a quercetin diglycoside for which no reliable sugar analysis could be obtained, and it is possible that the disaccharides in all three pigments have β,1 → 2 links, since flavonol sophorosides are known to be especially readily hydrolysed by acid. Q 1 and Q 2 were simply identified (Table 1) as the 3-galactoside and 3-rhamnoside of quercetin.



(I) Azaleatin



(II) Caryatin

The most unusual constituent present in *Eucryphia* is the compound C which has a blue colour in u.v. light, changing to green with ammonia, properties which are usually associated with caffeic acid esters. However, its spectral properties (λ_{max} 253 and 347 nm) show it to be a quercetin derivative related to azalein (λ_{max} 249 and 345 nm) and also indicate that it must be the 3,5-dimethyl ether. Thus it gives (Table 1) a positive sodium acetate shift (free 7-hydroxyl), a positive boric acid shift (a free 3',4'-dihydroxyl grouping), a stable alkaline spectrum (3-hydroxyl blocked) but no aluminium chloride shift (3- and 5-hydroxyls blocked). Structure (II) for C was confirmed by demethylation with pyridinium chloride when it gave a mixture of 3- and 5-methyl ethers and free quercetin. This structure was further confirmed by mass spectral analysis. Identification of quercetin 3,5-dimethyl ether in *Eucryphia* is of particular phytochemical interest, since the compound has only been isolated (as caryatin) from one other plant source, the heartwood of *Carya pecan* (Juglandaceae).[10]

The flavonoids severally present in the Australasian *Eucryphia* species (Fig. 1) all lack distinctive colours in u.v. light. Because of lack of material, none has been completely identified but enough has been done to show that the structures differ from any of the flavonoids in South American species. Thus, compound D present only in *E. milliganii* gives dihydroquercetin on acid hydrolysis. Although clearly present in glycosidic combination, D could not be equated with any one glycoside. Even after chromatographic purification, it gave three sugars on hydrolysis (arabinose, xylose and rhamnose), in spite of the fact that the spectral data and R_f indicate that it should be a 3-monoside. The results suggest that D, if it was obtained pure, may be an inseparable mixture of the 3-arabinoside, 3-xyloside and 3-rhamnoside of dihydroquercetin but further work is needed to substantiate this.

Again, compound Q 4 in *E. moorei*, although readily recognized as a quercetin 3-glycoside, could not be equated with any known substance. From the fact that it gives rhamnose and glucose on hydrolysis and from its R_f values in relation to the common 3-rhamnosylglucoside (rutin), it appears to be a 3-triglycoside. However, it differs in R_f from both of the known 3-rhamnodiglucosides of quercetin, which occur in *Camellia sinensis* leaf and *Solanum tuberosum* petal respectively.[11]

Finally, compound L in *E. lucida* has the chromatographic behaviour and colour reactions of a flavonol 3-methyl ether and indeed yields kaempferol on demethylation. It, however,

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[10] T. SASAKI, *Yakugaku Zasshi* **84**, 195 (1964).

[11] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 69. Academic Press, New York (1967).

differs from the kaempferol 3-methyl ether, isolated recently from *Begonia*[12] and its R_fs and its spectral properties, particularly the lack of a sodium acetate shift, indicate that it is the 3,7-dimethyl ether. Since this dimethyl ether has only been isolated once previously, from an unnamed *Beyeria* species,[13] no direct comparison with natural material has been possible. However, the 3,7-dimethyl ether was prepared by selective methylation of kaempferol and the material so obtained (see Experimental) was identical in R_f, colour reactions and spectrum with the *lucida* constituent.

Flavonoid Distribution

The distribution of leaf flavonoids within the species of *Eucryphia* (Fig. 1) show that there is a clear division between the two South American species *E. glutinosa* (Chile) and *E. cordifolia* (Chile) and the three Australian species, *E. moorei* (New South Wales and Victoria), *E. milliganii* (Tasmania) and *E. lucida* (Tasmania). Azaleatin and caryatin, which characterize the first two species, are presumably controlled biosynthetically by dominant genes, since they appear not only in the hybrid *E. cordifolia* × *E. glutinosa* (*E.* × *nymansensis*) but also in *E. glutinosa* × *E. lucida* (*E.* × *intermedia*). *E. glutinosa* and *E. cordifolia* differ mainly in that the former has azaleatin 3-galactoside in the leaf whereas the latter has the arabinosylgalactoside instead; both glycosides are present in equal amounts in the hybrid *E.* × *nymansensis*. *E. cordifolia* also has an unidentified constituent B not present in *glutinosa*.

The three Australian species, besides lacking azaleatin and caryatin, generally contain few flavonoids in the leaves. Each has a single major constituent which appears to be species-specific. Thus, *E. milliganii* has dihydroquercetin, *E. lucida* kaempferol 3,7-dimethyl ether and *E. moorei* an unidentified quercetin triglycoside.

The present work has been restricted to leaf flavonoids, mainly because of the limited availability of plant material, but there are indications that the leaf is, in fact, the richest source of flavonoids. Thus, examination of the various organs of *E.* × *nymansensis* showed that the bark, heartwood and petals were similar in phenolic patterns to the leaf, except that constituents Q 1, A 1 and A 3 were absent (A 1 was also missing in the petals).

DISCUSSION

Few correlations between plant geography and flavonoid chemistry have been noted at the present time so that the discovery of one in *Eucryphia* is of general interest. Three comparable examples may be mentioned. Thus, Williams[14] found the flavones luteolin and apigenin in leaves of Eastern Asian *Pyrus* species, whereas the flavonols kaempferol and quercetin occurred in Western Asian and European species. Similarly, Bate-Smith[15] discovered that there were differences in flavonoid pattern that distinguished Eastern from Western Mediterranean *Crocus* species. Again, Eugster (cf. Ref. 15) records that anthocyanins occur as leaf pigments only in Asiatic *Coleus* species, being replaced by red quinones in East African species. In *Eucryphia*, equally marked chemical differences distinguish species geographically isolated from each other.

The fact that the two rare quercetin methyl ethers in *Eucryphia* only occur in two of the five species limits the value of this data in determining the position of this anomalous plant group. However, it is worth pointing out that all four families now known to contain

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[12] J. B. HARBORNE and E. HALL, *Phytochem.* **3**, 453 (1964).

[13] P. R. JEFFERIES and T. G. PAYNE, *Australian J. Chem.* **18**, 1441 (1965).

[14] A. H. WILLIAMS, *Chem. Ind.* (*London*) 1318 (1964).

[15] E. C. BATE-SMITH and T. SWAIN, *Lloydia* **28**, 267 (1965).

azaleatin (Table 2) are predominantly woody and, in addition, have a common flavonol glycosidic pattern and are abundant in leucoanthocyanidins. In fact, all four families, although placed far apart in most systems, show closer affinities with the Rosalean families (e.g. Leguminosae, Rosaceae) than their present positions suggest. These results therefore tend to support Bausch in placing the Eucryphiaceae near the Cunoniaceae, although there is no evidence as yet of azaleatin or caryatin in the latter family. Further chemical confirmation is clearly necessary here and a study of terpenoid and other constituents might well yield more decisive data. That *Eucryphia* contains other interesting secondary compounds follows from the use of the vernacular "stinkwood" for *E. moorei* and from the observation made during the present study that the leaves of all five species give off an obnoxious sulphurous odour when heated in aqueous acid.

Table 2. Natural distribution of azaleatin (quercetin 5-*O*-methyl ether) and its glycosides

Plant family, genus and species	Organ	Glycosidic form	Co-occurring quercetin glycosides
Eucryphiaceae			
*Eucryphia glutinosa**	Leaf	3-Galactoside	3-Galactoside
*E. cordifolia**		3-Arabinosylgalactoside	
		3-Diglucoside	3-Rhamnoside
Juglandaceae			
*Carya pecan**	Heartwood	Free state	—
Ericaceae			
Rhododendron, 43 spp.	Petal	3-Rhamnoside	3-Rhamnoside 3-Galactoside 3-Arabinoside
Erica vagans	Leaf	Not known	
Plumbaginaceae			
Plumbago spp.	Petal	3-Rhamnoside	3-Rhamnoside
Ceratostigma spp.	Leaf	3-Galactoside	3-Galactoside

* These taxa also contain quercetin 3,5-dimethyl ether (caryatin).

EXPERIMENTAL

Plant Materials

Fresh leaves of the following taxa were kindly provided by staff of the Royal Botanic Gardens, Kew: *Eucryphia lucida* (Labill.) Baill., *E. moorei* F. von Mueller, *E. milliganii* Hook f., *E.* × *intermedia* Bausch (*E. glutinosa* × *E. lucida*), *E.* × *nymansensis* Bausch (*E. cordifolia* × *E. glutinosa*), *E. glutinosa* (Poepp. et Endl.) Baill. and *E. cordifolia* Cav. Fresh material of the latter three taxa was also obtained from plants growing at Ness Gardens (University of Liverpool).

Methods

Spectra were measured on a Unicam S.P. 800 Automatic Spectrophotometer. Chromatography was carried out on Whatman No. 1 paper at 18–20°. Solvents used were: BAW, butanol–acetic acid–water (4:1:5, top layer); 5% HOAc, 5% aqueous acetic acid; PhOH, water-saturated phenol; and BEW, butanol–ethanol–water (4:1:2·2). Preliminary separations of leaf extracts were carried out in BAW and components were purified by chromatography in 5% or 15% HOAc and then BEW. On purifying the major flavonoid of *E. lucida* by chromatography two minor components with very similar properties were found to be present but they did not occur in sufficient quantity for further study.

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[16] J. B. Harborne, *Nature* **208**, 1051 (1965).

Caryatin

This was obtained as pale yellow needles from aqueous ethanol. Molecular weight, by mass spectra, was 330 (required value for $C_{17}H_{14}O_7$, 330). On demethylation with pyridinium chloride at 120° for 1 hr, it gave a mixture of quercetin, quercetin 3-methyl ether and quercetin 5-methyl ether. On reductive cleavage with sodium amalgam, caryatin yielded phloroglucinol monomethyl ether, *p*-hydroxyphenylpropionic acid and *p*-hydroxyphenylpropanol.

Dihydroquercetin

This was identified, in hydrolysates of *E. milliganii* leaf extracts, by chromatographic and spectral comparison with authentic material. It was readily distinguished from the closely related 5,7,3′,4′-tetrahydroxyflavanone (eriodictyol) by its higher R_f in 5% HOAc (0·37 as compared with eriodictyol, 0·14) and by the magnitude of the alkaline shift in the u.v. spectrum ($\Delta\lambda^{\text{alk.}}$ 29 nm; eriodictyol, $\Delta\lambda^{\text{alk.}}$ 40 nm).

Methylation of Kaempferol

Methylation of kaempferol with dimethyl sulphate in aq. alc. Na_2CO_3 at room temperature for 15 min[17] yields a mixture of six methyl ethers which can be separated by chromatography on Whatman No. 3 paper in 50% HOAc and then BAW. Two of the components have dark-brown colours on paper in u.v. light, changing to dull yellow with ammonia, colour reactions which indicate they are methylated in the 3- but not the 5-position. The one of lower R_f in 50% HOAc (0·79, kaempferol has R_f 0·45 in the same system) is the 3-methyl ether.[12] The one of higher R_f (0·82) appears to be the 3,7-dimethyl ether. Thus, it has $\lambda_{\max}^{95\%\ \text{EtOH}}$ at 269 and 348 nm, $\Delta\lambda^{\text{NaOAc}}$ 0 nm, $\Delta\lambda^{\text{NaOEt}}$ +43 nm and $\Delta\lambda^{\text{AlCl}_3}$ +46 nm and the following R_fs: 0·89 in Forestal, 0·93 in BAW, 0·96 in PhOH, 0·22 in 15% HOAc, 0·39 in 30% HOAc and 0·58 in toluene–ethyl formate–HCO_2H (5:2:3, with silica gel support). The 3,7-dimethyl ether did not separate chromatographically from the *E. lucida* constituent L in any of the above systems. Furthermore, it is distinct from the 7,4′-dimethyl ether, which was prepared by methylation of kaempferol 3-rhamnoside under the same mild conditions as above, followed by acid hydrolysis of the product. The 7,4′-dimethyl ether has $\lambda_{\max}^{\text{EtOH}}$ 268, 322 (inflection), 364 nm, $\Delta\lambda^{\text{NaOEt}}$ +46 nm, $\Delta\lambda^{\text{AlCl}_3}$ +55 nm and $\Delta\lambda^{\text{NaOAc}}$ 0 nm. It is bright yellow on paper in u.v. light and, while having similar R_fs to the 3,7-dimethyl ether in most solvents, it is clearly distinguished from it on Whatman No. 1 paper in 50% HOAc (R_f 0·63, 3,7-dimethyl ether 0·82).

Acknowledgements—The authors are grateful to Dr. F. M. Dean for arranging the mass spectral analysis of caryatin. S. M. D. and J. B. H. are grateful to the Science Research Council for financial support.

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[17] T. H. Simpson and J. L. Beton, *J. Chem. Soc.* 4065 (1954).