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COMPARATIVE BIOCHEMISTRY OF THE FLAVONOIDS—IV.

CORRELATIONS BETWEEN CHEMISTRY, POLLEN MORPHOLOGY AND SYSTEMATICS IN THE FAMILY PLUMBAGINACEAE*

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Abstract—Fifty-five species, representing ten of the eleven genera of the family Plumbaginaceae have been surveyed for flavonoids and other phenolic constituents in root, leaf and flower. The results reveal a close correlation between chemistry, pollen morphology and taxonomy. The most useful chemical "marker" is 5-hydroxy-2-methyl-naphthoquinone (plumbagin) which occurs in roots of all ten taxa examined of the tribe Plumbagineae (plants with monomorphic pollen) and which is uniformly absent (0 out of 44 species) from plants of the tribe Staticeae (with dimorphic pollen). There are also characteristic differences between the tribes in their flavonoid pigments. Thus, all but two of the *Plumbago* and *Ceratostigma* species studied contain one or other of five rare *O*-methylated flavonoids, namely azaleatin, 5-*O*-methylmyricetin, capensinidin, pulchellidin and europinidin. The two latter pigments are new anthocyanidins, provisionally identified as 5-*O*-monomethyl and 5,3′-di-*O*-methyldelphinidin. In addition, *Plumbago europea* leaf contains a new flavonol 7-*O*-methylmyricetin (europetin), a substance which forms a link between the Plumbaginaceae and a neighbouring family, the Primulaceae. Of the seven *Plumbago* species examined, *P. rosea* is the most distinctive, for besides lacking 5-*O*-methylated flavonols, it contains in its flowers a mono- and a digalloyl-glucose. The anthocyanidins in the Plumbagineae occur as 3-rhamnosides, 3-galactosides or 3-glucosides. By contrast, in the tribe Staticeae, the main anthocyanins are malvidin 3,5-diglucoside (*Armeria* spp.) and petunidin 3-rhamnoside-5-glucoside (most *Limonium* spp.). Members of the Staticeae characteristically have large amounts of myricetin glycosides in the leaves. Examination of the most primitive member of the Staticeae, *Aegialitis annulata*, which is anomalous in having monomorphic (instead of dimorphic) pollen, showed it to be unusual chemically. The main leaf flavonoids were 3-*O*-methyl ethers of quercetin and myricetin; the latter is a new pigment, annulatin. The absence of plumbagin and 5-*O*-methylated flavonols from this plant indicates that it has been correctly placed, in spite of its pollen morphology, in the Staticeae.

INTRODUCTION

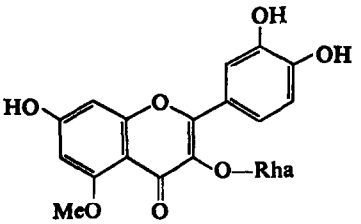
ALTHOUGH the chemotaxonomy of higher plants has been widely studied in recent years,[1,2] there are relatively few groups in which a clear relationship between chemistry and taxonomy has been established. In the case of the phenolic constituents, which are amongst the most favoured of taxonomic marker substances,[3] few intensive surveys have been carried out at the generic or family level.[4–8] In order to explore further the relationship between phenolic

* Part III. E. C. BATE-SMITH, S. M. DAVENPORT and J. B. HARBORNE, *Phytochem.* **6**, 1407 (1967).

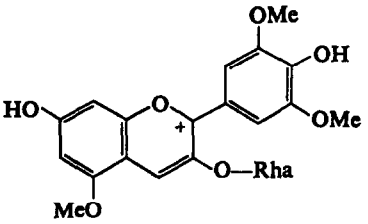
[1] T. SWAIN (Editor), *Chemical Plant Taxonomy*. Academic Press, New York (1962).
[2] T. SWAIN (Editor), *Comparative Phytochemistry*. Academic Press, New York (1966).
[3] E. C. BATE-SMITH, *J. Linnean Soc. London (Botany)* **58**, 95 (1962).
[4] W. E. HILLIS, *Phytochem.* **5**, 1075 (1966) and subsequent papers.
[5] H. ERDTMAN, In *Chemical Plant Taxonomy* (Edited by T. SWAIN), p. 89. Academic Press, New York (1962).
[6] A. H. WILLIAMS, In *Comparative Phytochemistry* (Edited by T. SWAIN), p. 297. Academic Press, New York (1966).
[7] R. E. ALSTON, In *Comparative Phytochemistry* (Edited by T. SWAIN), p. 33. Academic Press, New York (1966).
[8] J. B. HARBORNE, *Phytochem.* **5**, 589 (1966).

chemistry and plant systematics, an intensive study of the flavonoids and related phenols in the family Plumbaginaceae has been undertaken.

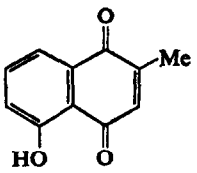
The Plumbaginaceae was chosen primarily because earlier studies[9] of the flavonoid pigments of flowers of *Plumbago capensis* and *P. rosea* revealed that the family is one exhibiting considerable biosynthetic versatility. The rare flavonol, azalein (I), and a novel anthocyanin, capensinin (II), were among the pigments identified. More conventional flavonoids—myricetin, quercetin, leucodelphinidin and leucocyanidin—are also known to occur in the leaves of members of the family.[3] In addition, another unusual phenolic, plumbagin (III), has been known for some time to be present in the roots of four *Plumbago* species (*P. europea*, *P. pulchella*, *P. rosea* and *P. zeylanica*).[10]



Azalein (I)



Capensinin (II)



Plumbagin (III)

The Plumbaginaceae is a conveniently sized family for chemical study since it contains only some 320 species, classified into eleven genera and two tribes. Furthermore, many species are of ornamental value so that living plant material is reasonably accessible. Yet another reason for studying this family is the fact that the pollen morphology, breeding behaviour and cytology of members of the family have been well surveyed by Baker[11,12] and it was of interest to relate chemistry to the general biology of the group.

RESULTS

Identification of Flavonoids and Other Phenolics in the Plumbaginaceae

Commonly occurring flavonoids (e.g. quercetin 3-rhamnoside, myricetin 3-rhamnoside, malvidin 3,5-diglucoside, etc.) found in the Plumbaginaceae during the course of the present survey were identified by spectroscopic and chromatographic comparisons with authentic samples (see Experimental). Three rare flavonol methyl ethers present in the plants (5-*O*-methylquercetin, 5-*O*-methylmyricetin and 3-*O*-methylquercetin) were similarly identified. Four new methylated flavonoids were discovered during the survey and their identification is described below. They are europetin (7-*O*-methylmyricetin), annulatin (3-*O*-methylmyricetin), pulchellidin (5-*O*-methyldelphinidin?) and europinidin (5,3′-di-*O*-methyldelphinidin?).

Europetin, a new flavonol present as the 3-rhamnoside in the leaves of *Plumbago europea*, was identified as the 7-*O*-methyl ether (IV) of myricetin on the following evidence. (1) Its R_f values and spectral properties (Table 1) fit those of a myricetin monomethyl ether; the R_f in Forestal, for example, is very similar to quercetin (same number of hydroxyls as (IV))

[9] J. B. HARBORNE, *Arch. Biochem. Biophys.* **96**, 171 (1962).
[10] R. H. THOMSON, *Naturally Occurring Quinones*. Butterworth, London (1957).
[11] H. G. BAKER, *Ann. Botany (London)* **12**, 207 (1948); **17**, 433, 615 (1953).
[12] H. G. BAKER, *Evolution* **20**, 349 (1966).

TABLE 1. PROPERTIES OF THE *O*-METHYLATED FLAVONOIDS OF THE PLUMBAGINACEAE

Flavonol*	R_f value (×100) in BAW	Forestal	PhOH	λ_{max}† (in nm) in 95% EtOH	$\Delta\lambda_{band\ I}^{NaOAc}$	$\Delta\lambda_{band\ II}^{NaOEt}$
Quercetin	77	38	33	255, 374	+11	Decomposes
3-Methylquercetin	93	84	71	257, 362	+3	+52
Azaleatin (5-methylquercetin)	48	49	50	254, 369	+20	Pigment decomposes in alkali
Myricetin	43	28	13	256, 378		
Annulatin (3-methylmyricetin)	69	62	49	257, 362	+6	
5-Methylmyricetin	26	33	21	252, 371	—	
Europetin (7-methylmyricetin)	51	49	55	255, 379	0	

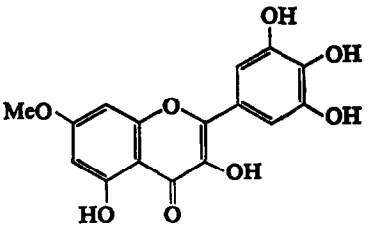
Anthocyanidins‡	R_f value (×100) in Forestal	Formic	BAW	λ_{max} (nm) in MeOH—HCl	$\frac{E_{440}}{E_{max}}$ as %	$\Delta\lambda_{AlCl_3}^{EtOH-}$
Delphinidin	32	13	42	277, 546	16	+23
Pulchellidin (5-methyldelphinidin?)	50	24	48	278, 543	9	Positive shift
7-Methyldelphinidin (prepared from europetin)	44	20	56	274, 542	20	Positive shift
Petunidin (3′-methyldelphinidin)	46	20	52	276, 543	17	+14
Europinidin (5,3′-dimethyldelphinidin?)	64	30	—	271, 541	12	+38
Malvidin (3′,5′-dimethyldelphinidin)	60	27	58	275, 542	19	0
Capensinidin (5,3′,5′-trimethyldelphinidin?)	88	—	79	273, 538	12	0

* Colours on paper in u.v. light: quercetin, myricetin, europetin are bright yellow; 3-methyl ethers are dull brown; 5-methyl ethers are intense yellow fluorescent.

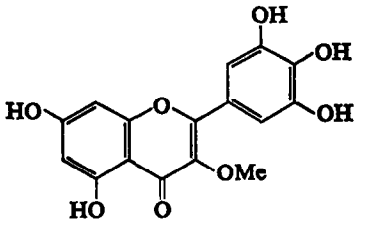
† All the flavonols give bathochromic spectral shifts of 20–60 nm in the presence of sodium acetate–boric acid (free B-ring catechol group) and of $AlCl_3$ (free 3- and/or 5-hydroxyl group).

‡ Colours on paper in visible or u.v. light: all purple.

but the R_f is expectedly lower than quercetin in butanol–acetic acid–water and higher in phenol. (2) The molecular weight, determined by mass spectral methods, is 332, which is that required by a myricetin monomethyl ether. (3) It is resistant to demethylation in exactly the same way as rhamnetin (7-*O*-methylquercetin) is but it does yield myricetin on prolonged treatment. Characteristically, it is the 7-methoxy group in flavonols which is most resistant to demethylation.[13] (4) On reductive cleavage or alkaline fusion, it yields the



Europetin (IV)



Annulatin (V)

[13] T. H. SIMPSON and J. L. BETON, *J. Chem. Soc.* 4065 (1954).

expected A- and B-ring fragments, phloroglucinol monomethyl ether and 3,4,5-trihydroxyphenylpropionic acid. (5) On reductive acetylation, and acid treatment it yields a delphinidin monomethyl ether, differing in properties from the known 3'-monomethyl ether (petunidin) (Table 2) but behaving like the so far undescribed 7-methyl ether. (6) Europetin did not separate chromatographically in five solvent systems from synthetic material, prepared by selective demethylation of myricetin 5,7,3',4',5'-pentamethyl ether.

TABLE 2. PHENOLIC CONSTITUENTS OF MEMBERS OF THE PLUMBAGINEAE

Species	*Root constituents*	*Leaf constituents*	*Petal constituents*
Plumbago capensis Thunb.	Plumbagin	Leucodelphinidin	Capensinidin 3-rhamnoside and azaleatin 3-rhamnoside
P. coerulea H.B. & K.	Plumbagin	Plumbagin and myricetin 3-glucoside	Pulchellidin and delphinidin 3-rhamnosides, kaempferol and quercetin 3-rhamnosides
P. europea L.	Plumbagin	Plumbagin, europetin 3-rhamnoside and 5-*O*-methylmyricetin glycoside	Europinidin 3-glucoside
P. pulchella Boiss.	Plumbagin	Plumbagin, leucodelphinidin, europetin, myricetin and quercetin 3-glucosides	Pulchellidin and delphinidin 3-glucosides, azaleatin and quercetin 3-rhamnosides
P. rosea L.	Plumbagin	Leucodelphinidin	Delphinidin, cyanidin and pelargonidin 3-rhamnosides, kaempferol 3-rhamnoside, galloylglucose, digalloylglucose
P. scandens L.	Plumbagin	Plumbagin	Azaleatin 3-rhamnoside
P. zeylanica L.	Plumbagin	Leucodelphinidin and quercetin 3-rhamnoside	Azaleatin 3-rhamnoside
Plumbagella micrantha Spach	Plumbagin	Plumbagin, cyanidin 3-glucoside* and glycosides of quercetin and kaempferol	Not examined
Ceratostigma plumbaginoides Bunge	Plumbagin	Leucodelphinidin, cyanidin and delphinidin 3-galactosides,* myricetin and quercetin 3-rhamnosides, azaleatin and 5-methylmyricetin 3-galactosides	Europinidin 3-galactoside
C. willmottianum Stapf	Plumbagin	Azaleatin and quercetin 3-galactosides	Europinidin 3-galactoside
Dyerophytum africanum (Lam.) Kuntze	Not examined	Myricetin 3-rhamnoside and glycosides of azaleatin and quercetin	Not examined

* In autumnal leaves only.

Annulation (V), a new flavonol from *Aegialitis annulata* leaf, was identified in a similar manner as the previously undescribed 3-methyl ether of myricetin. It has not yet been compared directly with synthetic material but the fact that it occurs in association with quercetin 3-methyl ether, quercetin and myricetin in *Aegialitis* leaf and that it has precisely the chromatographic behaviour one would predict for it (see Table 1) make any other structural assignment unlikely.

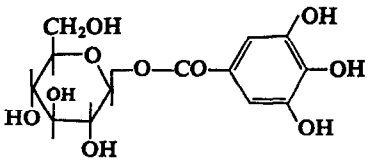
The two new anthocyanidins, pulchellidin and europinidin, occurring in the petals of *P. pulchella* and *P. europea* respectively, have been provisionally identified as 5-monomethyl- and 5,3′-dimethyldelphinidin. In neither case has much material been available for chemical study (the petals of the plants containing them are very tiny and very laborious to collect) and identification has, perforce, had to depend mainly on studying their chromatographic and spectral properties (see Table 1). The first new pigment, pulchellidin, has the R_f and colour properties of a delphinidin monomethyl ether and yields delphinidin on demethylation. It is, however, different from the known 3′-methyl ether (petunidin) and also the 7-methyl ether (prepared from europetin, see above). Since neither methylation in the 3-position nor that in the 4′-position fits the pigment's properties, it must be by elimination the 5-methyl ether. This is confirmed by its low E_{440}/E_{max} ratio. Europinidin, by contrast, has an R_f very close to malvidin (Table 1) and thus appears to be a dimethyldelphinidin. Its spectrum, unlike that of malvidin, gives an aluminium chloride shift, indicating that it has a catechol nucleus in its structure. Like pulchellidin, it has a low E_{440}/E_{max} ratio, indicating that it, too, is 5-substituted. Finally, demethylation of europinidin yields, as expected, an intermediate monomethyl ether (probably petunidin) and delphinidin. Europinidin is therefore presumably the 5,3′-dimethyl ether of delphinidin. The discovery of these two new pigments completes, with capensinidin in *P. capensis*,[9] what appears to be a series of 5-*O*-methylated anthocyanidins based on delphinidin, petunidin and malvidin. The structures advanced for all three pigments are partly based on an assumed biogenetic relationship with the 5-*O*-methylated flavonols (e.g. azaleatin) present in the same plants but clearly other structural assignments are possible. A low E_{440}/E_{max} ratio for example is also shown by 5-deoxyanthocyanidins and the possibility that the three pigments are based on, say, 3,7,8,3′,4′,5′-hexahydroxyflavylium cannot at present be ruled out.

The most important of the simpler phenolics present in the family is the known yellow naphthoquinone, plumbagin. This was detected, in the first instance, on chromatograms of root, leaf or flower hydrolysates as a bright yellow spot (appearing a dull brown unchanged by ammonia vapour in the u.v.) with high R_f values in most solvents. It was then identified by its characteristic spectra (see Experimental) and its mobility in petroleum ether–ethyl acetate (7:3) on thin layers of silica gel. Plumbagin occurs mainly in a bound form, since root tissue containing it is normally colourless; also evidence was obtained for the presence of plumbagin monoglycoside in leaf tissue of *P. europea*. One other simple phenol, glucogallin, was also detected during this work, together with a new digalloylglucose and their identification is described below.

Two unusual phenolic constituents A and B (R_fs 0·31, 0·21 and 0·28, 0·66 in butanol–acetic acid–water and water respectively) were first noted on two-dimensional chromatograms of petal extracts of *P. rosea* as intensely dark absorbing spots under u.v. light, changing to intense bright mauve with ammonia. That they were gallic acid derivatives followed from their u.v. spectra (both had a single maximum at 281 nm) from the intense blue–black colour they gave with ferric chloride and from their yielding only gallic acid and glucose on hydrolysis with acid or esterase. B was hydrolysed rapidly by β-glucosidase to gallic acid and glucose and can only be 1-galloyl-β-D-glucose or glucogallin (VI), a substance isolated earlier from Chinese rhubarb, *Rheum officinale* (Polygonaceae).[14] The second substance A gives, on β-glucosidase treatment, gallic acid and monogalloylglucose different from glucogallin. Thus, A must be a digalloylglucose, in which the glucose moiety is disubstituted (in the 1- and some other position); it cannot be a galloylgalloylglucose. Mass spectral determination

[14] E. FISCHER and M. BERGMANN, *Chem. Ber.* **51**, 1760 (1918).

has confirmed that A is a digalloylglucose. Although penta-, hexa- and hepta- galloylglucoses have been isolated from a number of plants as the hydrolysable tannins in the leaf or bark,[15] no simple digalloylglucose has been found before as a natural substance.



Glucogallin (VI)

Phenolics of the Plumbagineae

The Plumbagineae consists of four genera: *Plumbago* (10–15 spp.), *Plumbagella* (1 species), *Ceratostigma* (10 spp.) and *Dyerophytum* (3 spp.). Living plant material of only ten species representing three of the four genera could be obtained in spite of a consistent search among botanic gardens over a period of 6 years. Herbarium material of one species of the fourth genus *Dyerophytum* was eventually obtained from South Africa: *Dyerophytum africanum* (formerly *Vogelia africana*). The collected results following the isolation and identification of phenolics in these eleven species, are presented in Table 2. These results are best discussed first at the tribal level and then at the species level.

The results of the survey indicate that the tribe has a number of distinctive chemical characters in common, in particular the presence of plumbagin in the root, rare methylated flavonoids in the leaf and/or flower and three simple glycosidic patterns for the flavonoids. Plumbagin, previously found in roots of four *Plumbago* species,[10] has now been found in three further species, in *Plumbagella micrantha* and in two *Ceratostigma* species. It thus represents a consistent tribal character. It is true that sampling is limited to only a third (ten out of about thirty species), but the sample examined is a representative one. Again, 5- and 7-methylated flavonoids replace common flavonols and anthocyanidins in leaf and/or flower of most members of the Plumbagineae examined: these rare flavonoids consistently occur in these taxa as 3-rhamnosides, 3-glucosides or 3-galactosides.

At the species level, these are significant differences in phenolic composition and chemical characters clearly distinguish, for example, all seven *Plumbago* species one from another (Table 3). Of the seven, *P. rosea* is the most distinctive in lacking azalein and related compounds and in having the unique distinction of two galloylglucoses in the flowers. This species is, in fact, very distinct morphologically; it is, for example, the only species with red petal colour, the others having blue or white flowers. *P. europea* and *P. pulchella* are related chemically in being the only two species with 7-*O*-methylated as well as 5-*O*-methylated flavonoids. *P. capensis*, *P. scandens* and *P. zeylanica* might also be grouped together, since they only differ in petal flavonoids in that *P. capensis* has the pigment capensinidin not present in the other two species. There are, however, many differences in minor phenols between these species (see footnote, Table 3) and the white-flowered mutant of *P. capensis*, i.e. the variety *alba*, can readily be distinguished by two-dimensional chromatography of petal extracts, from the two white-flowered species, *P. scandens* and *P. zeylanica*.

Chemical studies here described also have some bearing on the relationship between *Plumbago* and the other genera in the same tribe. *Plumbagella micrantha*, a monotypic

[15] E. HASLAM, *Chemistry of Vegetable Tannins*, p. 105. Academic Press, New York (1966).

species, is sometimes included in the genus *Plumbago*, although it differs in its basic chromosome number ($x=6$, instead of 7) and in being an annual instead of a perennial. Baker,[11] from his studies of pollen morphology, prefers to keep this taxon as a distinct genus and its chemistry, in as much as it lacks any of the 5-*O*-methylated flavonoids of *Plumbago*, supports the separation from *Plumbago*. Similarly, chemical data (Table 2) might be used to support the separation of *Ceratostigma* from *Plumbago*, two taxa that Maury[16] did not consider to be sufficiently different morphologically for generic status. Chemically, there are many

TABLE 3. PHENOLIC AGLYCONES OF THE GENUS *Plumbago*

Substance*	Distribution in *Plumbago* species						
	capensis	*europea*	*pulchella*	*rosea*	*zeylanica*	*scandens*	*coerulea*
Root constituents							
Plumbagin	+	+	+	+	+	+	+
Leaf constituents							
Quercetin	−	−	+	−	+	−	−
Myricetin	−	−	+	−	−	−	−
Myricetin 5-methyl ether	−	+	−	−	−	−	−
Myricetin 7-methyl ether	−	+	+	−	−	−	−
Leucodelphinidin	+	−	+	+	+	−	−
Plumbagin	−	+	+	−	−	+	+
Petal constituents							
Delphinidin	−	−	+	+	−	−	+
Pulchellidin	−	−	+	−	−	−	+
Europinidin	−	+	−	−	−	−	−
Capensinidin	+	−	−	−	−	−	−
Cyanidin	−	−	−	+	−	−	−
Pelargonidin	−	−	−	+	−	−	−
Azaleatin	+	−	+	−	+	+	−
Plumbagin	−	+	−	−	−	−	−
Quercetin	−	−	+	−	−	−	+
Galloylglucoses	−	−	−	+	−	−	−

* Only identified phenols listed, but many unidentified constituents were noted variously in unhydrolysed leaf and petal extracts. Among those present in the leaf are (colours in u.v. without and with NH_3, R_fs in BAW and H_2O): C, colourless/bright blue, 0·78/0·98, λ_{max}^{EtOH} 312 nm, λ_{max}^{NaOEt} 365 nm (*p*-coumaric ester) in *capensis*, *europea*, *rosea*, *zeylanica*; D, bright yellow/quenched, 0·58/0·31 in *pulchella*, *coerulea* and *scandens*; E, colourless/mauve, 0·60/0·31 in *europea*; F, mauve/intense mauve, 0·67/0·33 in *pulchella*, *coerulea*. Petals of four species contain some sixteen phenols of the cinnamic acid type (colours: blue/green, colourless/green blue/light blue or colourless/blue) in trace amounts; there are two in *capensis*, seven in *europea*, two in *rosea* and five in *zeylanica*.

links between *Plumbago* and *Ceratostigma* species (i.e. in the presence in both groups of azaleatin, 5-methylmyricetin and europinidin) but there is a significant difference in anthocyanidin glycosidic pattern, the 3-rhamnosides of *Plumbago* being replaced by 3-galactosides in *Ceratostigma*.

Phenolics of the Staticeae

The tribe Staticeae consists of about seven genera, the most important and largest being *Limonium* Mill. (formerly known as *Statice* L.) with over 150 species. The uncertainty about

[16] P. MAURY, *Ann. Sci. Nat. Botan. 7th Ser.*, **4**, 1 (1886).

the number of genera in the tribe is because certain sections of *Limonium* are in the process of being split off into separate taxa; e.g. the former section Psylliostachys is now recognized as the genus *Psylliostachys* (Jamb. and Spach.) Nevski. Other genera are *Acantholimon* (84 spp.), *Aegialitis* (1 or 2 spp.), *Armeria* (35 spp.), *Goniolimon* (10 spp.) and *Limoniastrum* (6 spp.). Fresh material of forty-four species representing all the above genera except *Limoniastrum* has been examined for phenolics during the survey and the results are shown in Table 4.

TABLE 4. ROOT, LEAF AND PETAL CONSTITUENTS OF THE TRIBE STATICEAE

Plant	Root constituent*	Leaf constituents†
Acantholimon glumaceum Boiss.	Myricetin	Myricetin, quercetin, leucodelphinidin
Aegialitis annulata R. Br.	Ellagic acid, flavonols as leaf	Myricetin, 3-methylmyricetin, quercetin, 3-methylquercetin
Armeria, 17 spp.‡	Kaempferol (4 spp.) Leucodelphinidin (2 spp.)	Myricetin and/or quercetin and/or kaempferol (in all spp. except *A. majellensis*) leucodelphinidin (7 spp.)
Goniolimon tartaricum Boiss.	Leucodelphinidin	Leucodelphinidin, leucocyanidin, myricetin, quercetin
Limonium, 23 spp.§	Leucodelphinidin (6 spp.) Myricetin (1 sp.)	Myricetin and trace of quercetin (13 spp.)
Psylliostachys suvorovii (Regel) Roch K.	Leucodelphinidin	Leucodelphinidin

Plant	Petal constituents	
	Anthocyanins	Flavonols†, flavones
Armeria canescens, *A. cantabrica*, *A. juncea*, *A. labradorica*, *A. maritima* Willd., *A. pseudarmeria*, *A. pubescens*	Malvidin 3,5-diglucoside	Myricetin, quercetin, kaempferol
Limonium binervosum	Petunidin 3-rhamnoside-5-glucoside	Myricetin, quercetin
L. latifolium Moench	Petunidin 3-rhamnoside-5-glucoside and cyanidin 3-glucoside	Not examined
L. sinuatum Mill.	Delphinidin 3,5-diglucoside, delphinidin 3-glucoside	Myricetin, quercetin, kaempferol, luteolin
L. transwallianum *L. vulgare* Mill.	Petunidin 3-rhamnoside-5-glucoside	Not examined
Psylliostachys suvorovii	Cyanidin 3-glucoside	Unidentified flavonols

* Plumbagin uniformly absent from roots of all species.

† Flavonols generally present as 3-rhamnosides, less frequently as 3-galactosides.

‡ *Armeria* species studied were: *A. alpina*, *A. canescens*, *A. cantabrica*, *A. cinerea*, *A. juncea*, *A. labradorica*, *A. leucocephala*, *A. macrophylla*, *A. majellensis* Boiss., *A. maritima* Willd., *A. mauritanica*, *A. plantaginea* Willd., *A. pseudarmeria*, *A. pubescens*, *A. rouyani*, *A. transmontana*, *A. welwitschii*.

§ *Limonium* species studied were: *L. altaica*, *L. articulatum* var. *divergens*, *L. binervosum*, *L. bonduelli*, *L. dictyocladum*, *L. duriusculum*, *L. echioides* Mill., *L. filicaulis*, *L. globularifolium*, *L. gmelinii*, *L. latifolium* Moench., *L. minuatum*, *L. occidentalis*, *L. perezi*, *L. psilocladum* (Boiss.) O. Kuntze, *L. puberulum*, *L. purpurata*, *L. serbica*, *L. sinuatum* Mill., *L. speciosa*, *L. tomentellum*, *L. transwallianum*, *L. vulgare* Mill.

All members of the Staticeae examined lack the naphthoquinone plumbagin as a root (or leaf or flower) constituent. They also differ from members of the Plumbagineae in not having A-ring methylated flavonoids (with one exception, see below). Another distinctive tribal feature is the presence in the leaf of myricetin glycosides, often in high concentration. Anthocyanins based on malvidin, petunidin, delphinidin and cyanidin occur in the flowers (Table 4). Here again, there is a difference from the pigments found in the Plumbagineae in that the glycosides are 3-rhamnoside-5-glucosides or 3,5-diglucosides instead of 3-rhamnosides or 3-glucosides.

Within the tribe, the general pattern of leaf and root constituents is rather similar, with myricetin being abundant in all genera. The 3-rhamnoside of myricetin (myricitrin) has been positively identified in the leaves of *Limonium binervosum*, *L. duriusculum*, *L. latifolium*, *L. occidentalis*, *L. sinuatum* and *L. vulgaris* and probably occurs widely in other Staticeae; thus it has been provisionally identified in several *Armeria* species. Other glycosides are certainly present in addition to the 3-rhamnoside and the 3-galactoside was noted in several species (e.g. *L. latifolium*, *L. serbicum*). In terms of leaf content, the most distinctive species of the Staticeae is the primitive, probably monotypic, Australasian shrub, *Aegialitis annulata* R.Br. This plant contains (as glycosides) the 3-methyl ethers of quercetin and myricetin, substances found nowhere else in the family. The stem of *Aegialitis* has, in addition, ellagic acid, a compound which is generally rare in the Sympetalae and one which has not been detected elsewhere in the Plumbaginaceae. The absence from *Aegialitis* tissue of plumbagin or of 5-*O*-methylated flavonols such as azaleatin indicates that this species has been correctly placed in the Staticeae, in spite of the fact that Baker[11] found it resembled species of the Plumbagineae in its pollen morphology.

Petal constituents in the Staticeae show interesting variations by genera. Thus, all *Armeria* species studied have malvidin 3,5-diglucoside as petal anthocyanin, while four of the five *Limonium* species examined have the rare petunidin 3-rhamnoside-5-glucoside, a pigment only known otherwise in *Lathyrus*[17] and related genera.[18] It is interesting also that the morphologically uniform *Armeria* genus has, apparently, but one pigment pattern, while the much more variable *Limonium* has a range of anthocyanins and other flavonoids. For example, *L. sinuatum* (section Pteroclados), besides having a different anthocyanin from the species in section Limonium (e.g. *L. vulgare*), has the flavone luteolin in the petals. The presence of the rare aurone glycoside, cernuoside, in the yellow corollas of *L. bonduelli* (also section Pteroclados) has already been reported[19] and preliminary studies indicate that a range of other unusual flavonoids are present in the flowers of *Limonium*. *Psylliostachys*, for example (recently split off from *Limonium*), has flavones in the petals which remain so far unidentified (Table 4).

The results with the relatively few *Limonium* species studied indicate that useful chemical differences probably exist at the sectional level in this taxonomically difficult genus. However, attempts to extend the survey of living material have failed, mainly because of inaccessibility of seed and of difficulties in identification and of obtaining sufficient petal material from accessible plants. Attention was therefore turned to herbarium material of species not available in the living state, but the results with *Limonium* were not very encouraging, perhaps because of the antiquity of the specimens available to the author. Of fourteen *Limonium* species only six gave a positive reaction for flavonoids in the leaf. Myricetin was detected

[17] J. B. HARBORNE, *Phytochem.* **2**, 85 (1963).
[18] J. B. HARBORNE, *Comparative Biochemistry of the Flavonoids*, p. 161. Academic Press, New York (1967).
[19] J. B. HARBORNE, *Phytochem.* **5**, 11 (1966).

in five of these and the sixth, *L. ferulaceum* (section Myriolepis), contained azaleatin. Azaleatin was confirmed by co-chromatography and was detected in three specimens of the same species, two dating from 1848 and 1886 respectively. This is the only occurrence so far noted of a typical Plumbagineae constituent in a member of the Staticeae. Results with herbaria specimens of other genera were more consistent; all three *Acantholimon* and all nine *Armeria* species examined were flavonoid positive, myricetin, quercetin, kaempferol and leucodelphinidin being variously present.

DISCUSSION

The present survey of the flavonoids and other phenolics in a representative sample of the Plumbaginaceae has revealed the existence of a close correlation between the chemistry

TABLE 5. CHEMICAL AND BIOLOGICAL DIFFERENCES AT THE TRIBAL LEVEL IN THE FAMILY PLUMBAGINACEAE

	Plumbagineae	Staticeae
No. genera studied	4/4	6/7
No. species studied	11/30	44/286
	Chemical characters	
Quinones in root	Plumbagin uniformly present	Plumbagin uniformly absent
Flavonols in leaf and/or flower	5-*O*-methylation frequent, 7-*O*-methylation in 2 spp.	Myricetin characteristically in leaf, 5-*O*-methylation rare (1 sp.), 3-*O*-methylation in 1 sp.
Anthocyanins in flowers	A-ring methylation common. Glycosidic patterns: 3-glucoside, 3-galactoside or 3-rhamnoside	A-ring methylation absent. Glycosidic patterns: 3-rhamnoside-5-glucoside or 3,5-diglucoside
Leucoanthocyanidins	Rare in root, uncommon in leaf or flower	Frequent in root, leaf and flower
	Biological characters	
Basic chromosome number	$x=6$ or 7	$x=8$ or 9
Pollen type	Uniformly monomorphic	Mainly dimorphic
Key systematic character	Stamens free from the corolla	Stamens attached to the corolla

and the biology of the family. The distribution of phenols (Table 5) is correlated not only with flower anatomy, but also with pollen morphology and with cytology. The best chemical marker distinguishing the two tribes is plumbagin, which has been found uniformly in the Plumbagineae but which is absent from the Staticeae. The only drawbacks with this marker are that it occurs characteristically in an inaccessible plant organ, the root, and that it must be looked for in living plants, since it has not been detected in herbarium material.

Other chemical characters are not as consistent as plumbagin but some could probably be useful in classificatory work. Azaleatin, which occurs in eight of the eleven taxa of the Plumbagineae, is a particularly good marker substance since it is easily detected by its intense fluorescence on paper in u.v. light. Its presence in a single taxonomically "good" species of the Staticeae, *Limonium ferulaceum*, of course, may limit its systematic value. The novel anthocyanidins of the Plumbagineae, europinidin and pulchellidin, could also be looked for

in systematic studies, particularly since they have not as yet been found at all in the Staticeae. There are practical difficulties here in that their R_f values (Table 1) are rather close to those of the commonly occurring pigments petunidin and malvidin. Indeed, in two earlier surveys[20, 21] of anthocyanidins in *Plumbago* and *Ceratostigma* species, they have been mistaken for common anthocyanidins.

The differences in flavonoid complement at the tribal level seem to be quite exceptional and no other obvious chemical differences were observed. A study, for example, of the free amino acids and of the free and bound monosaccharides in the leaves of representative species of both tribes showed that the pattern was uniform throughout the family. Differences between alkaloids and terpenoids at the tribal level were not deliberately sought, since these classes of secondary constituent appear to be absent from the family (see, e.g., Ref. 22). The differences in flavonoid pattern are presumably related to the importance of flavonoids as floral pigments and the fact that the two tribes, because of their geographical separation, have evolved different breeding systems.

Although some of the present chemical data have a bearing on the generic status in the Plumbagineae (see p. 1420), the results do little to help the most difficult taxonomic problem in the family, the grouping of the 150 or so taxa classically assigned to the genus *Limonium*. However, there are indications from the species studied that *Limonium* is heterogeneous in its phenolic chemistry and a more complete chemotaxonomic survey of the genus should be especially profitable.

The Plumbaginaceae usually occupies an isolated position in the Sympetalae as the only family in the order Plumbaginales[23] and the chemical findings are in general agreement with this. Its phenolic pattern does little to indicate an association with the Primulaceae, with which it is occasionally linked.[24] The flavonoid glycosidic patterns are, for example, quite different in the two families, *Primula* leaf and flower being characterized by flavonol 3-gentiotriosides, not by 3-rhamnosides. The only obvious chemical link is the presence of 7-*O*-methylmyricetin (europetin) in *Plumbago* and of hirsutidin (7-*O*-methylmalvidin) in *Primula*. An equally good case could be made for linking the Plumbaginaceae with the Ebenaceae, some plants of which have plumbagin (*Diospyros*) or with the Ericaceae, the only other sympetalous family to have azaleatin.[9]

Azaleatin also occurs in two archichlamydous families, Eucryphiaceae and Juglandaceae,[25] but there is little general chemical support for linking the Plumbaginaceae with archichlamydous families, in particular with those in the order Centrospermae as has often been proposed (cf. Ref. 21). Airy Shaw has recently suggested[26] that the Plumbaginaceae is connected (morphologically?) with the Linaceae through the genus *Anisadenia*. No chemical evidence could be found for this; examination of leaf extracts of the only two known species *Anisadenia saxitalis* and *A. pubescens* Griff. showed that their phenolic pattern was quite different from members of the Plumbaginaceae.

[20] W. J. C. Lawrence, J. R. Price, G. M. Robinson and R. Robinson, *Phil Trans. Roy. Soc., London* **230**, 149 (1939).
[21] E. Beck, H. Merxmuller and H. Wagner, *Planta* **58**, 220 (1962).
[22] R. Hegnauer, *Comparative Phytochemistry* (Edited by T. Swain), p. 211. Academic Press, New York (1966).
[23] A. Engler, *Syllabus der Pflanzenfamilien*, 12th edn. (Edited by H. Melchior), Vol. 2. Borntraeger, Berlin (1964).
[24] J. Hutchinson, *Families of Flowering Plants*, 2nd edn. Macmillan, London (1959).
[25] E. C. Bate-Smith, S. M. Davenport and J. B. Harborne, *Phytochem.* **6**, 1407 (1967).
[26] H. K. Airy Shaw (Editor), *J. C. Willis' Dictionary of Flowering Plants and Ferns*, 7th edn. Cambridge University Press (1966).

EXPERIMENTAL

Plant Material

Plants were obtained from a variety of sources, but the majority were grown from Botanic Garden supplied seed at the John Innes Institute, Hertford. As many species as possible were verified taxonomically and these are shown in Tables 2 and 4 with the taxonomic authority. A plant of *Plumbago rosea* was kindly supplied by the staff of the Royal Botanic Garden, Kew, and fresh material of several *Limonium* and *Armeria* species of known origin came from the Cambridge Botanic Garden. The *Aegialitis annulata* used was dried material collected in Airlie, North Queensland, and identified by Dr. W. T. Jones of the Phytochemical Survey section of the C.S.I.R.O., Australia. Fresh seed of *Aegialitis* was kindly collected by Dr. J. S. Womersley, Dept. of Forests, Division of Botany, Lae, New Guinea, and airmailed to England, but in spite of being sown immediately only a small percentage of the seeds germinated and these all died at the seedling stage. Herbaria material was generously supplied by the British Pharmaceutical Association (*Plumbago* and *Limonium* spp.), the Royal Botanic Garden, Edinburgh (*Anisadenia*), the University of Liverpool (*Limonium*, *Armeria* and *Acantholimon* spp.) and the Botanic Gardens at Kirstenbosch, South Africa (*Dyerophytum africanum*).

Methods

Phenolics were isolated, purified and identified by paper chromatography.[27] Chromatographic solvents used were: BAW, butanol–acetic acid–water (4:1:5); BuHCl, butanol–2 N HCl (1:1); PhOH, water-saturated phenol; Forestal, conc. HCl–acetic acid–water (3:30:10); Formic, conc. HCl–formic acid–water (2:5:3); 1% HCl, water–conc. HCl (97:3); and HOAc–HCl, acetic acid–conc. HCl–water (15:3:82). Spectra were measured on a Unicam SP500 or SP800 Spectrophotometer. All plant material was subjected to a minimal examination for phenolics by means of two-dimensional paper chromatography of alcoholic extracts, using solvents BAW and H_2O, and by means of direct acid hydrolysis and examination for aglycones by one-dimensional chromatography in Forestal, 50% HOAc, BAW and H_2O.

Anthocyanins

Pulchellidin was isolated from petals of *Plumbago pulchella*, where it occurs as the 3-glucoside along with delphinidin 3-glucoside, and from petals of *P. coerulea*, where it occurs as the 3-rhamnoside, accompanied by delphinidin 3-rhamnoside. The two glycosides have the following R_fs in BAW, BuHCl, 1% HCl and HOAc–HCl: 3-glucoside, 0·40, 0·39, 0·12 and 0·32; 3-rhamnoside, 0·50, 0·55, 0·30 and 0·53. On hydrolysis, both yielded pulchellidin, which had the properties shown in Table 1; the aglycone is identical in colour with petunidin, but on co-chromatography, it is clearly distinct from it. On demethylation with pyridinium chloride at 120–140° for 1 hr, it yields delphinidin (λ_{max} 276, 546 nm E_{440}/E_{max} 15%). The 3-glucosides and 3-rhamnosides of delphinidin accompanying the pulchellidin glycosides were identified by co-chromatography with authentic samples, delphinidin 3-rhamnoside being obtained from *Lathyrus odoratus* petals and the 3-glucoside being a synthetic specimen (for R_f and spectral details, see Ref. 27).

Europinidin was isolated from *Ceratostigma plumbaginoides* petals as the 3-galactoside and from *P. europea* petals as the 3-glucoside. These glycosides have the same spectra (λ_{max} 276 and 536 nm, E_{440}/E_{max} 10%) and the same R_f values (0·27 in BAW, 0·19 in BuHCl, 0·06 in 1% HCl and 0·31 in HOAc–HCl) and give europinidin (bluish-black needles) and galactose or glucose on hydrolysis. The properties of europinidin are shown in Table 1. On demethylation, it yields petunidin and delphinidin.

Apart from pulchellidin and europinidin glycosides, all the other anthocyanins found in the Plumbaginaceae are known pigments and were identified by direct comparison with authentic specimens. Malvidin 3,5-diglucoside (malvin) isolated from *Armeria pseudarmeria* petals had λ_{max} 274 and 535 nm, E_{440}/E_{max} 11%, R_f values 0·30, 0·05, 0·13 and 0·50 (in BAW, BuHCl, 1% HCl and HOAc–HCl respectively) and gave malvidin and glucose on hydrolysis. It did not separate chromatographically from added malvin isolated from *Clarkia elegans* petals. Malvin was identified in other *Armeria* species by co-chromatography, colour reactions and by isolating malvidin after hydrolysis of petal tissue. The rare petunidin 3-rhamnoside-5-glucoside isolated from *Limonium binervosum* had λ_{max} 272 and 535 nm, E_{440}/E_{max} ratio 10 per cent and R_fs 0·32, 0·13, 0·26 and 0·52 and gave glucose, rhamnose and petunidin on hydrolysis. It did not separate from added authentic pigment obtained from *Lathyrus odoratus* petal. Cyanidin and delphinidin 3-galactosides isolated from autumnal leaves of *Ceratostigma plumbaginoides* had the following properties: λ_{max} 529 and 536 nm, E_{440}/E_{max} both 26 per cent, R_fs 0·35, 0·29, 0·07, 0·26 and 0·20, 0·17, 0·03, 0·18 respectively. These two pigments and also the cyanidin 3-glucoside and delphinidin 3-glucoside and 3,5-diglucoside isolated from other members of the Plumbaginaceae (Tables 2 and 4) were identified by co-chromatography with authentic markers.

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

Flavonol Glycosides

Europetin was isolated from leaves of *P. europea* as the 3-rhamnoside, a yellow crystalline solid, λ_{max}^{EtOH} 257 and 356 nm, $\lambda_{max}^{alc.\ NaOAc}$ 256 nm, $\lambda_{max}^{alc.\ NaOEt}$ 270 and 380 nm (slow decomposition), $\lambda_{max}^{alc.\ H_3BO_3}$ 380 nm and $\lambda_{max}^{alc.\ AlCl_3}$ 395 and 420 nm. The R_f values are (rutin for comparison in parentheses): 0·64 (0·43) in BAW, 0·22 (0·30) in H_2O and 0·60 (0·45) in PhOH. On acid hydrolysis, the glycoside yields rhamnose and europetin as yellow needles, m.p. 278°. Molecular weight by mass spectrum is 332 ($C_{16}H_{12}O_8$ required 332). The R_fs and spectral maxima are shown in Table 1. Europetin has the same colour on paper as myricetin. On demethylation with pyridinium chloride, it slowly yields myricetin (20 per cent was recovered unchanged after 4 hr heating at 130°; rhamnetin behaves similarly). On reductive acetylation followed by acid treatment, europetin yields an anthocyanidin with the properties of 7-*O*-methyldelphinidin (Table 1). On reductive cleavage with sodium amalgam, europetin yields phloroglucinol monomethyl ether and 3,4,5-trihydroxyphenylpropionic acid.[28] 7-*O*-methylmyricetin was synthesized by methylating myricetin 3-rhamnoside under N_2 for 4 hr with dimethyl sulphate, dry K_2CO_3 and acetone. The product was hydrolysed with 2 N HCl and then demethylated for 3 hr with pyridinium chloride. The mixture of flavonoids produced was separated by chromatography on No. 3 paper in 50% HOAc. A major band corresponded to europetin in R_f and colour reaction and was cut out and eluted. This synthetic material did not separate from europetin when co-chromatographed in five solvent systems.

Annulatin was isolated from leaves of *Aegialitis annulata* as a glycoside with λ_{max} 254, 270 (inflection) and 362 nm, $\lambda_{max}^{alc.\ AlCl_3}$ 273 and 425 nm, $\lambda_{max}^{alc.\ NaOEt}$ 268 and 425 nm and $\lambda_{max}^{alc.\ H_3BO_3}$ 392 nm and with R_f values of 0·39 in BAW, 0·28 in H_2O and 0·56 in BEW. The properties of the aglycone are shown in Table 1. On demethylation, it gave myricetin and on reductive cleavage with sodium amalgam it gave phloroglucinol and 3,4,5-trihydroxyphenylpropionic acid. Insufficient material of annulatin was available for further study. Quercetin 3-methyl ether, isolated as a glycoside from the same source, was identified by direct comparison with synthetic material (cf. Ref. 29).

Known flavonol glycosides found in the Plumbaginaceae (the 3-rhamnosides of kaempferol, quercetin, myricetin and azaleatin) were identified by direct comparison with authentic specimens. Myricetin 3-galactoside has recently been isolated for the first time in crystalline form from *Oenothera lavandulaefolia*.[30] Material from *Limonium serbicum* and *Ceratostigma plumbaginoides* leaf was identified by the fact that it was identical in spectrum, R_f and colour reactions with the known 3-glucoside but that it gave myricetin and galactose, not glucose, on acid hydrolysis. Azaleatin 3-galactoside has recently been isolated from *Eucryphia*[25] and materials from both plant sources had identical properties, giving in each case azaleatin and galactose on hydrolysis. 5-*O*-methylmyricetin has previously been obtained only as an aglycone from *Rhododendron*.[31] It was identified as the 3-galactoside in *C. plumbaginoides* and had R_f 0·28 in BAW, 0·12 in 5% HOAc. The glycoside band had λ_{max} 257, 263 and 358 nm, gave a borate shift (+20 nm) but no $AlCl_3$ shift and was rapidly decomposed in dilute alkali. On hydrolysis, it gave galactose and 5-*O*-methylmyricetin, identified by direct comparison with authentic material from *Rhododendron*, kindly supplied by Dr. K. Egger, University of Heidelberg. The luteolin 7-glycoside present in *Limonium sinuatum* had a spectrum and shifts identical to that of luteolin 7-glucoside.

Plumbagin was identified in members of the Plumbagineae by direct comparison with a synthetic sample kindly supplied by Professor R. H. Thomson, University of Aberdeen. The spectral properties are as follows: λ_{max}^{EtOH} 270, 404 and 419 nm; $\lambda_{max}^{alc.\ AlCl_3}$ 440 nm; and λ_{max}^{NaOEt} 526 nm. The R_f on silica gel plates is 0·76 in petroleum ether (b.p. 60–80°)–ethyl acetate (7:3) and thin-layer chromatography proved to be the most sensitive technique for determining its presence or absence. On paper chromatograms, it appeared as a dull yellow spot in visible light with R_f 0·91 in BAW, 0·95 in PhOH and 0·91 in Forestal.

Galloylglucoses

Two compounds A and B, with intense purple colours on paper in u.v. and ammonia, were isolated by chromatography in BAW of a petal extract of *Plumbago rosea*. The R_f values were 0·31 in BAW and 0·21 in H_2O for A, 0·28 in BAW and 0·66 in H_2O for B. A was obtained as a colourless solid, crystallized from water, with m.p. 215°. Both A and B have the same u.v. spectrum λ_{min}^{EtOH} 242, λ_{max}^{EtOH} 281 nm, $\lambda_{max}^{EtOH-NaOH}$ 250, 290 and 360 nm (unstable) and both give an intense blue-black colour with aqueous ferric chloride. On acid or esterase hydrolysis, both yield glucose and gallic acid as the only components. B is hydrolysable by β-glucosidase to glucose and gallic acid and appears to be identical to 1-β-D-galloylglucose, glucogallin (literature R_fs 0·30 in *iso* Bu–HOAc–H_2O (4:1:5) and 0·75 in 6% aq. HOAc). A is hydrolysed by β-glucosidase or acid to a monogalloylglucose, R_f 0·20 in BAW and 0·58 in H_2O. The mass spectrum of B by negative ionization was complex, much degradation occurring at $T_v = 160°$, but the results fit those for a digalloylglucose.

[28] H. M. Hurst and J. B. Harborne, *Phytochem.* 6, 1111 (1967).
[29] J. B. Harborne and E. Hall, *Phytochem.* 3, 453 (1964).
[30] J. Kagan, *Phytochem.* 6, 317 (1967).
[31] K. Egger, *Z. Naturforsch.* 17b, 489 (1962).

Peaks were observed at 466 (digalloylglucose–H_2O) 314 (monogalloylglucose–$1H_2O$), 296 (monogalloylglucose–$2H_2O$), 169 (gallic acid–1) and 125 (glucose–$3H_2O$–1).

Phenolics of Anisadenia

Acid hydrolysis of leaf of *A. saxitalis* and *A. pubescens* (taken from herbarium sheets) gave caffeic acid and some flavone (possibly luteolin). Chromatography of direct alcoholic extracts of the leaves showed the presence of a flavone glycoside and of two caffeic esters, both with different R_f values from those of the common esters such as chlorogenic acid. Chromatography of the extract of a single petal of *A. pubescens* showed the presence of the same caffeic esters and of two flavonol (quercetin?) glycosides, R_f values in BAW and H_2O of 0·67/0·19 and 0·44/0·69.