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# FLAVONOID BISULPHATES AND THEIR CO-OCCURRENCES WITH ELLAGIC ACID IN THE BIXACEAE, FRANKENIACEAE AND RELATED FAMILIES*

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**Key Word Index**—*Bixa orellana*; Bixaceae; *Frankenia pulverulenta*; Frankeniaceae; Parietales; flavonoid bisulphates; apigenin and luteolin 7-bisulphates; hypolaetin 8-bisulphate; kaempferol and isorhamnetin 7-bisulphates; flavonol 3-glucuronide-7-bisulphates.

**Abstract**—Three new flavone bisulphates have been found in the leaves of *Bixa orellana*. They have been identified as the 7-bisulphates of apigenin and luteolin and the 8-bisulphate of hypolaetin; the identities of the first two have been confirmed by synthesis. Related flavonol bisulphates have been detected universally in the Tamaricaceae and Frankeniaceae and occasionally in the Guttiferae and Cistaceae. More detailed examination of *Frankenia pulverulenta* yielded five new flavonol conjugates: the 7-bisulphates of kaempferol and isorhamnetin and the 7-bisulphate-3-glucuronides of kaempferol, quercetin and isorhamnetin. By contrast, bisulphates were not detected in other families of the same order, notably in Cochlospermaceae, Violaceae, Turneraceae and Flacourtiaceae. The occurrence of bisulphates seems to be correlated at the family level, with one exception, with the presence of ellagic acid. In relation to the various existing classifications of the above families, the chemical data generally fit in closer with the arrangement of Cronquist rather than with any other. However, Cronquist's suggestion that *Cochlospermum* and *Bixa* should be placed close together does not accord with the chemical evidence.

## INTRODUCTION

The tree *Bixa orellana*, apparently the only known representative of the family Bixaceae, is notable phytochemically for the production in the fruit of the unique $C_{25}$ carotenoid bixin, used as an orange colouring in food [1]. Taxonomically, the affinities of *Bixa* are a matter of some conjecture but it is usually placed close to the Cochlospermaceae in the order Parietales [2]. In an earlier leaf survey of the angiosperms by Bate-Smith [3], this plant was shown to contain ellagic acid and two unidentified flavonoids one of which appeared from its colour reactions to be a 6- or 8-hydroxylated flavone or flavonol. In view of the relative rarity of such compounds in nature and of our own interest in their systematic distribution [4, 5], a more detailed examination of the leaf flavonoids was initiated.

A preliminary study of the leaf aglycones of *Bixa* indeed revealed the presence of small amounts of hypolaetin (8-hydroxyluteolin) but the major constituents were apigenin and luteolin. Examination of the direct leaf extracts then indicated that each of these two common flavones occurred in two combined forms, as the 7-glucoside and as a new type which lacked sugar. Electrophoresis at pH 2 revealed that this second form was a conjugate with potassium bisulphate. Since such flavonoid bisulphates as a class are relatively rare, at least in the dicotyledons [6] (their frequent occurrence in two monocotyledonous families has recently been established [7–9]), a detailed search was made for

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* Dedicated to Dr. E. C. Bate-Smith, C.B.E., on the occasion of his 75th birthday.

these compounds in families related to *Bixa*. The present paper therefore records the structures of the three new flavone bisulphates of *Bixa* and the discovery of related flavonol derivatives in *Frankenia* and several other related plants. A brief account of some aspects of this work have already been reviewed elsewhere [10].

## RESULTS

### *Flavone bisulphates of* Bixa

In the earlier leaf survey, *Bixa orellana* was reported to contain leucocyanidin, ellagic acid and two unidentified flavonoids with dark colours in UV light changing to dark olive with ammonia, of $R_f$ values 0·44 and 0·64 [3]. The occurrence of ellagic acid was later confirmed by Lebreton and Bouchez [11] and also in the present study.

Two dimensional chromatography in butanol-acetic acid-water and 5% acetic acid of a direct leaf extract showed the presence of only two major flavonoids, with a third minor component with a dark colour in UV unchanged by ammonia vapour. On purification, the two major components, with the usual flavone colour on paper, each further separated into two. Two of the four were readily identified as the 7-glucosides of apigenin and luteolin from the results of acid hydrolysis, spectral analysis and by direct comparison with authentic markers.

The other two components were apparently new derivatives of apigenin and luteolin but failed to give any sugar on acid hydrolysis. However, their structures became clear when they were discovered to be anionic at pH 2 on paper electrophoresis and also they gave bisulphate on hydrolysis. Spectral measurements showed they were clearly substituted only in the 7-position so they were immediately formulated as the 7-bisulphates of apigenin and luteolin. These identities were confirmed by comparison with synthetic samples, prepared by reacting the parent flavones with sulphamic acid in pyridine. The major products of synthesis were the 4′-bisulphates as expected from previous studies of sulphation in the flavonol series [12], but small amounts of the 7-bisulphates were readily separated from the reaction mixtures; significant amounts of the 7,4′-*bis*-bisulphates were also formed (see Table 1 and Experimental).

The properties of the new natural and synthetic flavone bisulphates are listed in Table 1. The close similarity in $R_f$ values in many of the solvents between the pairs of 7-glucosides and 7-bisulphates should be noted. The best separation is given in water or in phenol–water. By contrast, the *bis*-bisulphates can be readily distinguished from monobisulphates in all solvents; furthermore, they migrate much further from the origin when subjected to electrophoresis at pH 2.

Table 1. $R_f$ and spectral properties of natural and synthetic flavone bisulphates

| | $R_f$ (× 100) in | | | | | | |
|---|---|---|---|---|---|---|---|
| Flavone* | BAW | BEW | $H_2O$ | 15HOAc | PhOH | Electrophoretic mobility† | $\lambda_{max}$ MeOH‡ (in nm) |
| Apigenin | | | | | | | |
| 7-glucoside§ | 58 | 71 | 05 | 24 | 85 | 0·00 | 268, 335 |
| 7-bisulphate§ | 52 | 74 | 34 | 24 | 58 | 0·70 | 269, 335 |
| 4′-bisulphate | 55 | 72 | 34 | 28 | 58 | 0·80 | 271, 335 inf |
| 7,4′-*bis*-bisulphate | 14 | 40 | 76 | 68 | 12 | 2·20 | 270, 325 |
| Luteolin | | | | | | | |
| 7-glucoside§ | 41 | 55 | 05 | 15 | 64 | 0·0 | 255, 268 inf, 353 |
| 7-bisulphate§ | 41 | 62 | 25 | 17 | 34 | 0·56 | 257, 267 inf, 352 |
| 3′- and 4′-bisulphate | 53 | 57 | — | 32 | 35 | 0·75 | 269, 333, 347 inf |
| 7,4′-*bis*-bisulphate | 25 | 21 | — | 64 | 13 | 3·5 | 269, 318, 348 inf |
| 7,3′-*bis*-bisulphate | 19 | 22 | — | 50 | 11 | 3·0 | 269, 333, 350 inf |
| 8-Hydroxyluteolin | | | | | | | |
| 7-bisulphate§ | 30 | 26 | 12 | 29 | 17 | 0·52 | 260, 271, 360 |

* Apigenin and luteolin 7-glucosides are included for comparative purposes. Colour reactions of flavone bisulphates are similar to those of the corresponding glycosides, e.g. apigenin 7-bisulphate is the same as the 7-glucoside, the 7,4′-*bis*-bisulphate as the 7,4′-diglucoside, etc.

† Mobilities are relative to quercetin 3-bisulphate, run at pH 2·2 for 2 hr at 400 V/cm.

‡ Flavone bisulphates give the same shifts in the presence of NaOAc, NaOAc–$H_3BO_3$, $AlCl_3$ and NaOEt as the corresponding glycosides.

§ Natural compounds isolated from *Bixa orellana*; other samples are synthetic.

The fifth and minor flavonoid in *Bixa* was only found in the leaves in any quantity during certain times of the year. On acid hydrolysis, it yielded a mixture of 6- and 8-hydroxyluteolin and was clearly based on 8-hydroxyluteolin (hypolaetin) since acid treatment of this compound is known to give increasing amounts of the 6-isomer [13]. No sugar was found in the hydrolysate and formulation as a bisulphate followed from its mobility on electrophoresis at pH 2 and from its yielding bisulphate ion on acid treatment. Spectral analysis (see Experimental) showed that the bisulphate was attached, not in the expected 7-position, but to the 8-hydroxyl group. Thus, it was identified as hypolaetin 8-bisulphate.

The discovery of these three bisulphates in *Bixa* represents the first report of simple flavone bisulphates in nature. All the flavone bisulphates recently reported in the Palmae and Gramineae [7–9] are more complex in having either *O*- or *C*-glycosidic sugars in their structures. The luteolin 7-bisulphate has however been more recently detected (J. B. Harborne, unpublished results) in two members of the Umbelliferae, *Daucus carota* and *Oenanthe fistulosa*.

### *Flavonol bisulphates of the Tamaricaceae and Frankeniaceae*

In his order Violales (≡Parietales), Cronquist [14] places the Bixaceae close to the Tamaricaceae and Frankeniaceae, although in some other treatments [15] these two families are referred to a separate order, the Tamaricales. That there is a chemical link between the Bixaceae and Tamaricaceae is apparent from the above discovery of bisulphates in *Bixa* and from two earlier reports of flavonol bisulphates in the latter family. Thus tamarixetin (quercetin 4′-methyl ether) 3-bisulphate has been isolated from *Tamarix laxa* [16] and a rhamnetin (quercetin 7-methyl ether) 3′-glucuronide tris-bisulphate has recently been reported in leaves of *Tamarix aphylla* [17]. That flavonol bisulphates are characteristically present in Tamaricaceae was amply clear when an electrophoretic survey of six *Tamarix* species and one *Myricaria* species showed that all contained from four to five bisulphate conjugates.

The species studied were: *Tamarix africana* Poiret, *T. aphylla* L., *T. canariensis* Willd., *T. gallica* L., *T. hispida* Willd. var. *aestivalis*, *T. pentandra* Pall. var. *rubra*, *T. smyrnensis* Bge. and *Myricaria germanica* (L.) Desv. On hydrolysis of leaf tissue, all gave ellagic acid, anthocyanidin (from proanthocyanidin), quercetin, kaempferol and at least two or more methyl ethers of quercetin. From colour reactions and electrophoretic mobility, components in the direct extracts were provisionally identified as the corresponding 3-bisulphates and 7-bisulphates of the above flavonols; more highly substituted bisulphates were also present. Further work on the separation and identification of these compounds is in progress.

With regard to the Frankeniaceae, no previous detailed studies of the flavonoids appear to have been carried out [18]. Electrophoresis again showed that all available species contained flavonoid components mobile at pH 2. The six taxa surveyed were *Frankenia corymbosa* Desf., *F. ericifolia* Chr. Sm. ex DC., *F. laevis* L., *F. laevis* L. var. *capitata*, *F. pulverulenta* L. and *F. thymifolia* Desf. In order to confirm this finding, the flavonoids of *F. pulverulenta* were examined in more detail. Five new flavonol conjugates were identified: the 7-bisulphates of kaempferol and isorhamnetin and the 7-bisulphate-3-glucuronides of kaempferol, quercetin and isorhamnetin. The properties of these various compounds are given in Table 2. Two uncharged flavonoids were also detected, namely isorhamnetin 3-glucoside and quercetin 3-glucuronide. Two-dimensional chromatography of the other five *Frankenia* species suggest that the same or closely related conjugates occur throughout the genus. In addition, some species contain a presumed *bis*-bisulphate. Each species has a slightly different complement of conjugates but in all cases most of the flavonoid in the leaf appears to be bound as bisulphate.

### *Bisulphates in other families of the Parietales*

The Guttiferae have been shown by Lebreton and Bouchez [11] to contain proanthocyanidins and the common flavonols; in addition two of 24 species examined were found to have ellagic acid. A survey of 12 *Hypericum* species showed the presence of bisulphates in three: *Hypericum coadnatum* Chr. Sm., *H. elodes* L. and *H. grandiflorum* Choisy. Negative results were recorded from leaf tissue of *H. aegyptiacum* L., *H. canariense* L., *H. caprifolium* Boiss., *H. elegans* Stephan ex Willd., *H. empetrifolium* Willd., *H. glandulosum* Ait, *H. hirsutum* L., *H.*

Table 2. $R_f$ and spectral properties of flavonol bisulphates of *Frankenia pulverulenta*

| Flavonol | $R_f$ (× 100) in BAW | $H_2O$ | 15% HOAc | PhOH | Electrophoretic mobility* | $\lambda_{max}$ MeOH† (in nm) |
|---|---|---|---|---|---|---|
| 7-Bisulphate of‡ | | | | | | |
| Kaempferol | 53 | 21 | 11 | 30 | 0·34 | 267, 372 |
| isorhamnetin | 44 | 12 | 08 | 40 | 0·25 | 254, 269 inf, 374 |
| quercetin | 32 | 05 | 08 | 11 | 0·18 | – |
| 7-Bisulphate-3-glucuronide of | | | | | | |
| kaempferol | 33 | 90 | 65 | 08 | 1·44 | 267, 352 |
| isorhamnetin | 28 | 90 | 54 | 13 | 1·28 | 253, 267 inf, 356 |
| quercetin | 17 | 87 | 52 | 05 | 1·22 | 256, 266 inf, 360 |
| Quercetin 3-bisulphate§ | 26 | 67 | 33 | 13 | 1·00 | 256, 266, 354 |
| Quercetin 3′-bisulphate | 44 | 26 | 16 | 16 | 0·66 | 250, 266, 367 |

* Relative to quercetin 3-bisulphate, run at pH 2·2 for 2 hr at 400 V/cm on Whatman 1 paper.

† All compounds gave the expected shifts or lack of shifts in the presence of NaOAc, NaOAc–$H_3BO_3$, $AlCl_3$ and NaOEt.

‡ Colours in UV light: 7-bisulphates, very bright green, yellow–green and yellow respectively; 7-bisulphate-3-glucuronides, dark colours changing to intense yellow–green, yellow and yellow brown in the presence of $NH_3$.

§ Data for these two compounds given for comparative purposes. The 3-bisulphate has been obtained from *Oenanthe crocata* and also synthetically; the 3′-bisulphate is a synthetic specimen.

Table 3. Plants in which flavonoid bisulphates could not be detected

| Plant examined | Source* | Flavonoid aglycones† | Other phenols |
|---|---|---|---|
| Cochlospermaceae | | | |
| *Cochlospermum insigne* St. Hil. (2 samples) | K | Qu, Km | Ellagic |
| *C. orinocense* (HBK.) Steud | J | Qu | Ellagic |
| *C. planchonii* Hook f. (3 samples) | K | My, Qu, Km L-Cy | Ellagic |
| *C. tinctorium* A. Rich (2 samples) | K | My, Qu, Km L-Cy | Ellagic |
| *C. vetifolium* (Willd.) Spreng | J | N.D. | Ellagic |
| Flacourtiaceae | | | |
| *Aberia caffra* Harv. et Sond. | E | Qu | — |
| *Azara lanceolata* Hook f. | R | N.D. | — |
| *Carpotroche brasiliensis* Endl. | J | Lu, Ap | — |
| *Casearia arborea* (L. C. Rich) Urb. | J | N.D. | — |
| *Flacourtia cataphracta* Roxb. ex Willd. | E | N.D. | Many $C_9$ compounds |
| *Idesia polycarpa* Maxim. | E | Lu, Ap, Qu (tr) | — |
| *Oncoba spinosa* Forsk. | K | N.D. | Many $C_9$ compounds |
| *Xylosma racemosa* Miq. subsp. *pubescens* | E | N.D. | — |
| *X. salzmanni* (Clos) Eichl. | J | N.D. | — |
| Turneraceae | | | |
| *Piriqueta capensis* (Harv.) Urb. | R | Lu | — |
| *P. cistoides* (L.) Griseb. | J | N.D. | — |
| *Turnera lilaireana* Urb. var. *oblongifolia* (Camb.) Urb. | J | Unidentified flavonols | |
| *Wormskioldia longipedunculata* Mast. | R | Qu | |
| Violaceae | | | |
| *Hybanthus parviflorus* (Mut.) Baillon | R | Qu | — |
| 14 *Viola* spp. | R | Qu, Km, Lu, Ap, L-Cy (variously) | — |

* Sources: E, Edinburgh Botanic Garden; J, Rio de Janeiro Herbarium; K, Kew Herbarium; R, Reading University Herbarium.

† Key: Qu, quercetin; Km, kaempferol; My, myricetin; Lu, luteolin; Ap, apigenin; L-Cy, procyanidin.

*perfoliatum* L., *H. reflexum* L. fil and *H. tetrapterum* Fries. Further study of two of the three positive species indicated that *H. coadnatum* contains quercetin 3-bisulphate, while *H. elodes* has quercetin 7-bisulphate and another compound which from its electrophoretic mobility must be a flavone *bis*-bisulphate.

The Cistaceae are similar to the Guttiferae in having proanthocyanidins and flavonols in their leaves, but differ in that ellagic acid is consistently present [11]. An electrophoretic survey of 22 species from five genera showed only one positive result. This was from *Helianthemum squamatum* (L.) Pers., a plant endemic to Spain. Five other *Helianthemum* species were negative: *H. caput-felix* Boiss., *H. croceum* (Desf.) Pers., *H. hirtum* (L.) Miller, *H. lavandufolium* Miller and *H. viscarium* Boiss. et Reut. Also negative were 7 spp. of *Cistus*, 2 of *Fumana*, 5 of *Halimium* and 2 of *Tuberaria*. The bisulphates of *H. squamatum* were further investigated and two were identified as isorhamnetin and quercetin 3-bisulphates. The 7-bisulphate of isorhamnetin was provisionally identified and a fourth unknown bisulphate was also noted.

Comparable sample surveys were carried out in four other families of the Parietales where material was available and the results for bisulphates were uniformly negative. The data are tabulated in Table 3, since flavonoid analyses of many of these taxa have not been previously published. In the case of Cochlospermaceae, a tropical family of three genera and 25 spp., only material of *Cochlospermum* could be obtained. Analyses of two South American and three African species showed flavonols in most and ellagic acid in all samples. These data significantly supplement the similar results of the earlier survey of a single species, *C. religiosum* (L.) Alston, by Bate-Smith [3]. In the case of the Flacourtiaceae, another tropical family, 9 species representing eight genera were surveyed. Only three had detectable flavonoids, the remainder being especially rich in cinnamic acid derivatives instead. There results agree with those of Lebreton and Bouchez [11], who surveyed eight species and recorded flavonoids (luteolin) in only three.

## DISCUSSION

The present discovery of flavonoid bisulphates in four families of the Parietales considerably extends the known occurrence of these conjugates in the Dicotyledons. They have been detected here in 18 of 80 species surveyed, which represents a frequency of 22%. How reliable the bisulphate character is as a taxonomic marker is difficult to assess, because little is known as yet whether it is affected by environmental factors. Since the synthesis in the leaf must depend to some extent on supply of inorganic sulphate, it could be regulated by the availability of sulphate in the soil or within the plant. Considering the relative chemical lability of the flavonoid–bisulphate linkage, particularly that in the flavonol-3-bisulphates, it is remarkable that these compounds are so readily detected in herbarium samples, as they have been during the present work.

The results obtained here do suggest that bisulphates may be useful systematic markers. At least, the nine families surveyed fall clearly into three groups: those which have bisulphate consistently; those in which it is occasionally present; and those where it appears to be absent. Perhaps the most surprising feature about the bisulphates of the Parietales is that their distribution at the family level is apparently correlated, with the exception of the Cochlospermaceae, with the presence of ellagic acid. This correlation in distribution of two biosynthetically unrelated chemical characters may be a more general feature within the dicotyledons. Thus, in unpublished surveys, we have recorded bisulphates in the Rosaceae, but only in the Rosoideae, the one subfamily which also has ellagic acid [3].

The present flavonoid data, when combined with the results of earlier investigations [3, 11], can be used as a measure of similarity within those families of the Parietales which have been sufficiently investigated (Table 4). Obviously, wider surveys particularly of the larger tropical families are needed to confirm these findings. However, using the relatively meagre data available (seven characters) for calculating coefficients of association, one obtains by cluster analysis two main groupings: the Cistaceae, Guttiferae, Frankeniaceae and Tamaricaceae with 50–83% similarity; and the Turneraceae, Flacourtiaceae and Violaceae with 50–75% similarity. The Cochlospermaceae and the Bixaceae are somewhat isolated but show the greatest similarity with the first group. Thus, the Cochlospermaceae show 60% similarity

Table 4. Flavonoid profiles in some families of the parietales

| Family and no. of genera/species surveyed* | Presence/absence of† | | | | | | |
|---|---|---|---|---|---|---|---|
| | Bisulphates | Ellagic acid | Proantho-cyanidins | Kaempferol, quercetin | Myricetin | Flavonol methyl ethers | Flavones |
| Cistaceae (5/22) | (+) | + | + | + | (+) | + | – |
| Guttiferae (1/13) | (+) | (+) | + | + | (+) | – | – |
| Frankeniaceae (1/6) | + | + | (+) | + | – | + | – |
| Tamaricaceae (2/8) | + | + | – | + | – | + | – |
| Cochlospermaceae (1/5) | – | + | – | + | + | – | – |
| Turneraceae (3/4) | – | – | – | (+) | – | – | (+) |
| Flacourtiaceae (8/9) | – | – | + | (+) | – | – | (+) |
| Violaceae (2/15) | – | – | + | + | – | + | + |
| Bixaceae (1/1) | + | + | + | – | – | – | + |

* Arranged in order of clusters, from a simple numerical analysis of similarities.

† +, Present in all or majority of species; (+), present in one or a few species; –, not detected, presumably absent.

to the Guttiferae, 50% to the Cistaceae, while the Bixaceae show 50% similarity to both the Guttiferae and Frankeniaceae. However, when compared with each other, these two families only show 17% similarity. This is a very low figure, considering that *Bixa* was originally included within the Cochlospermaceae; thus there is overwhelming chemical support for the correctness of the decision by van Tieghem [19] to separate them into two families. In the light of this figure, there can be no chemical justification for Cronquist's recently expressed wish (with no reason given) for merging *Bixa* back into Cochlospermaceae.

How far this or any other chemical data [20] can be used to assess the validity of the many competing taxonomic arrangements of families within the Parietales *sensu lato* remains for the future. It is worth noting here that the results so far fit in rather better with Cronquist's arrangement than with any others. Thus, his Violales has one sequence Flacourtiaceae–Violaceae–Turneraceae and another Bixaceae–Cistaceae–Tamaricaceae–Frankeniaceae, which correspond approximately with the two chemical groupings mentioned above (see Table 4). Finally, it is satisfying to find that the morphological and ecological similarities between the Tamaricaceae and Frankeniaceae (members of both families have a heath-like appearance and prefer an arid or saline habitat) are reflected in their chemistry, since closely related flavonol bisulphates and bisulphate–glucuronides have been noted in both groups.

## EXPERIMENTAL

*Plant material.* Leaves were collected from *Bixa orellana* growing under glass at Reading. *Frankenia pulverulenta* was grown from seed and a voucher specimen has been deposited in the herbarium. For survey work, material was mainly from herbarium sheets at Reading, Kew or Rio de Janeiro and these have been appropriately labelled to indicate their use in this work.

*Methods of identification.* Ordinary flavonoids were detected and identified by standard procedures. Bisulphates were detected in the first instance by their anionic mobility on paper electrophoresis in formate-acetate buffer, pH 2·2 for 1–2 hr at 400 V/cm. On 2D-chromatograms, flavonol bisulphates could often be distinguished as elliptical spots of relatively low $R_f$ in BAW and high $R_f$ in aq solvents. The compounds were purified by PC and by preparative electrophoresis. They were identified as bisulphates by detection of the bisulphate ion after acid hydrolysis [8, 9] and by their ready hydrolysis when treated with sulphatase. The properties of all the new bisulphates are given in Tables 2 and 3. Flavone bisulphates were synthesized by heating luteolin or apigenin with sulphamic acid and pyridine at the b.p. for 1·5 hr. After pouring the reaction mixture into aq KOAc, the products were separated and purified by PC and identified from their $R_f$ and spectral properties.

Hypolaetin 8-bisulphate had $\lambda_{max}$ in MeOH at 260, 271 and 360 nm and showed the following shifts: $\Delta\lambda$ alk + 56 nm (free 4′-OH), $\Delta\lambda$ NaOAc + 10 nm (free 7-OH), $\Delta\lambda$ NaOAc–$H_3BO_3$ + 18 nm (free 3′,4′-diOH) and $\Delta\lambda$ $AlCl_3$ + 48 nm (free 5-OH). It appeared as a dark spot in UV light, unchanged ammonia. On acid hydrolysis, it gave 8-hydroxyluteolin accompanied by increasing amounts (with time) of the isomeric 6-hydroxyluteolin. These aglycones were identified by direct PC and spectral comparison with authentic specimens.

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