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DIFFERENCES IN FLAVONOID CONTENT BETWEEN FRESH AND HERBARIUM LEAF TISSUE IN *DILLENIA*

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Abstract—While acid-hydrolysed extracts of herbarium leaf of *Dillenia indica* showed only the presence of kaempferide (kaempferol 4'-methyl ether), similar extracts of fresh tissue contained in addition dihydrokaempferide and naringenin. Examination of the glycosides in fresh leaves showed the two principal constituents to be 7-diglucosides of naringenin and dihydrokaempferide together with kaempferide 3-diglucoside. These results suggest that dihydroflavonols are easily oxidized to the corresponding flavonols during the drying process of leaves, so that their presence in herbarium tissue may well be missed during surveys. Examination of fresh tissue of six other *Dillenia* species again showed some discrepancies from the results previously reported using herbarium leaf. In particular, leucoanthocyanidin content was much higher in fresh leaf and thus much more readily identified. It is concluded that the use of herbarium tissue for flavonoid surveys should whenever possible be checked with fresh tissue.

INTRODUCTION

Dillenia (Dilleniaceae), a genus of some 50 species of tropical and subtropical trees,[1] has been extensively surveyed for leaf flavonoids mainly on the basis of herbarium tissue.[2] The flavonoids of *D. indica* have been examined by Kubitzki and by Lebreton and Bouchez[3] who both reported the rare kaempferol 4'-methyl ether, kaempferide, as the major constituent. As part of a study of the phytogeographical significance of flavonoids in *Dillenia*, fresh leaves of *D. indica* supplied by Kew gardens were examined in our laboratories for flavonoids. While kaempferide was indeed present, the main flavonoids as judged by u.v. absorption and colour properties, were flavanones and/or dihydroflavonols. A more detailed examination of the flavonoids in this species was therefore undertaken.

RESULTS

An alcoholic extract of fresh leaves of *D. indica* had the characteristic u.v. absorption of flavanones (main peak 284 nm, shoulder at 330 nm) and their presence was confirmed by the facts that on alkaline treatment, a deep chalcone yellow colour developed and that on reduction with $NaBH_4$ and subsequent treatment with conc. mineral acid, a deep rose-purple colour was produced (cf. Bate-Smith).[4] Acid hydrolysis of this extract gave aglycones which

[1] R. D. HOOGLAND, *Blumea* **7**, 1 (1952).
[2] K. KUBITZKI, *Ber. Deut. Botan. Ges.* **81**, 238 (1968).
[3] P. LEBRETON and M. P. BOUCHEZ, *Phytochem.* **6**, 1601 (1967).
[4] E. C. BATE-SMITH, *Phytochem.* **8**, 1803 (1969).

again showed all the colour and u.v. properties of flavanones. Thus, it was clear that kaempferide, reported previously in herbarium tissue, was not the major constituent of fresh leaf.

Two-dimensional paper chromatography of these alcoholic extracts showed three main components. Two had the colour reactions of flavanones in u.v. light and were identified by standard procedures as 7-diglucosides of dihydrokaempferide and naringenin. The third component, which had dark brown absorbing colour in u.v. light, was identified as kaempferide 3-diglucoside. The derivatives of kaempferide and dihydrokaempferide were very susceptible to aerial oxidation since on keeping the spots on the chromatograms changed colour, becoming bright yellow. Also, on hydrolysing the 7-diglucoside of dihydrokaempferide with acid, the major product was kaempferide.

A check on herbarium tissue of *D. indica* from a number of different sources confirmed that they all lacked the above flavanonol. It is clear, therefore, that the flavanonol in fresh tissue is converted during drying or subsequent storage to the corresponding flavonol. Whether naringenin glycosides are similarly converted to apigenin in herbarium tissue is not apparent from our results, but under laboratory conditions, this conversion is much slower than in the case of dihydrokaempferide.

The micro-scale identification of kaempferide in *Dillenia indica* and in other plant tissues depends on the assumption that it can be distinguished from the closely similar isomer rhamnocitrin (kaempferol 7-methyl ether). In our experience, it was impossible to separate these two isomers by chromatography using a wide range of paper and TLC solvents and also paper electrophoresis. Even the system of Egger, mentioned by Kubitzki,[1] does not give a completely satisfactory separation. Distinction has to be based, therefore, on spectral differences. While the neutral spectra of the 7- and 4′-methyl ethers are identical, there are

TABLE 1. SPECTRAL IDENTIFICATION OF KAEMPFERIDE IN *Dillenia indica*

Spectral solvent	Spectral max. (nm) of Kaempferol 7-methyl ether*	Kaempferol 4′-methyl ether†	*Dillenia* flavonol
95% EtOH	268, 325, 367	268, 323, 366	268, 325, 366
EtOH–NaOH	273, 442	287, 432	289, 436
$E_{\text{Band I}}/E_{\text{Band II}}$ of alkaline spectrum	76%	140%	136%
EtOH–NaOAc	268 ($\Delta\lambda$ 0)	272, 277 ($\Delta\lambda$ + 11)	275 ($\Delta\lambda$ + 9)

* Obtained by controlled demethylation of kaempferol 3,7,4′-Trimethyl ether.
† Kaempferide, as supplied by Fluka A. G.

differences in the alkaline spectra, particularly with regard to the relative intensities of the maxima (see Table 1), which are sufficient to separate the compounds. Furthermore, in contrast to the data of Kubitzki,[1] the sodium acetate spectra were also distinctive (Table 1).

Examination of six other *Dillenia* species available as fresh leaves (Table 2) showed again differences in flavonoid content from those reported earlier from a herbarium tissue survey. As reported by Kubitzki,[2] leuco-anthocyanins were present in all the species examined, but we found them in much greater quantity in the fresh leaves than in those of herbarium specimens. In the latter, the amount of anthocyanidin formed was insufficient for detection on chromatographic papers, but in the fresh leaves it was easily possible to identify

TABLE 2. FLAVONOID AGLYCONES OF *Dillenia* LEAVES

Plant species	Flavonoid analyses of Fresh tissue*	Herbarium tissue†
Dillenia indica L.	Kaempferide, dihydrokaempferide naringenin, Qu & Km; LCy	Isorhamnetin, kaempferide, rhamnetin, glycoflavone and Km
D. excelsa (Jack) Gilg. var *tomentella* (Martelli) Hoogl.	kaempferide, Qu and Km; LCy	
D. suffruticosa (Griff.) Martelli	Qu, Km and a trace of azaleatin (?); LCy	
D. ovata Wall. ex Hook f. & Thoms	Glycoflavone (+++) and Km (tr); LD	Qu, Km, glycoflavone
D. pulchella (Jack) Gilg.	My and Qu; LD and LCy	Qu and Km
D. reticulata King	Qu and Km; LCy	—
D. scortechinii (King) Ridl. (*D. eximia* Miq)	Qu and Km; LD and LCy	Qu and Km

Key: My = myricetin; Qu = quercetin; Km = kaempferol; LD = Leucodelphinidin; LCy = leucocyanidin.

* Sources: *D. indica* Kew and Singapore B.G.; *D. suffruticosa* Singapore B.G. and Selangor (via J. B. Lowry); *D. reticulata and D. scortechinii* Selangor; remainder Singapore B.G.

† Results in the main of Kubitzki,[2] checked in the case of *D. indica* by us on material from the University of Cambridge herbarium. All specimens recorded simply as "leucoanthocyanidin positive" by Kubitzki.

delphinidin and cyanidin when present in the hydrolysate. These results also are recorded in the table.

Ellagitannin was present in all the species as disclosed by the nitrite reaction, but ellagic acid could not as a rule be detected on the chromatograms of the hydrolysates because of the formation of a brown trail in the relevant region. It was, however, easily recognizable on the chromatograms of the herbarium specimens, except in the case of *D. indica*.

DISCUSSION

In chemotaxonomic surveys, herbarium tissue is frequently used to supplement the supply of fresh tissue available, since it usefully extends the scope of surveys to plants not readily accessible in the living state, e.g. particularly tropical plants. Such procedures are especially valuable in the flavonoid field, since only small amounts of tissue are required and experience has shown that the results are usually very reliable; even the more highly reactive flavonols such as gossypetin and quercetagetin can be readily detected in such tissues.[5,6]

The present study of *Dillenia indica* indicates however, that serious discrepancies can sometimes occur between flavonoid results from fresh and dried tissue. While dihydrokaempferide may be especially easily lost from herbarium tissue, these results do suggest that when surveying plants for dihydroflavonols as a class fresh tissue should always be used.

[5] E. C. BATE-SMITH, *Phytochem.* **4**, 535 (1965).
[6] J. B. HARBORNE, *Phytochem.* **8**, 177 (1969).

EXPERIMENTAL

Flavonoid aglycones were surveyed in hydrolysed leaf extracts using standard procedures.

The three main flavonoids in ethanolic extracts of fresh *Dillenia indica* leaf were separated and purified by preparative paper chromatography in BAW, 5% HOAc and BEW. They were identified as follows:

(1) *Naringenin 7-diglucoside* had λ_{max} in EtOH 227, 283 (main peak) and 338 nm; λ_{max} + $AlCl_3$ 309, 389 nm; λ_{max} + NaOH 243, 287 and 370 changing with time (a deep yellow colour developed). It gave naringenin and glucose on hydrolysis. Naringenin was identified by its conversion with $NaBH_4$ and acid to apigeninidin (λ_{max} 535 nm; required 533 nm). R_f data in comparison with naringenin 7-glucoside and 7-rutinoside (values in parentheses) were as follows: R_f (×100) in BAW 52 (64, 47); in BEW 67 (73, 58); in H_2O 58 (49, 69); and in PhOH 38 (81, 75). These data indicate the presence of two glucose units in the 7-position.

(2) *Dihydrokaempferide 7-diglucoside* had λ_{max} 225, 283 (main peak) and 332 nm; λ_{max} + $AlCl_3$ 311, 390; and λ_{max} + NaOH 285, 371 nm (stable). Its colour reactions in u.v. light (dark to intense light yellow green with NH_3) were typical of dihydroflavonol 7-glycosides. On acid hydrolysis, it gave glucose, dihydrokaempferide and kaempferide. R_f data (×100) in BAW 19, in BEW 24, in H_2O 84 and in PhOH 09.

(3) *Kaempferide 3-diglucoside* had λ_{max} 268, 340 nm; + NaOAc 268, 275, 383 nm; and + NaOH 290, 380 nm. It gave glucose and kaempferide on acid hydrolysis. R_fs (× 100) were 48 in BAW, 60 in BEW, 37 in H_2O and 49 in PhOH. Kaempferide was identified by spectral means as indicated in Table 1. The spectral properties of the glycoside show that the sugar is attached in the 3-position and its R_f values (e.g. 37 in H_2O is comparable with the lit. value of 29 for kaempferol 3-sophoroside) and its failure to undergo β-glucosidase hydrolysis indicates that it is a 3-diglucoside.

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