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TWELVE 6-OXYGENATED FLAVONE SULPHATES FROM *LIPPIA NODIFLORA* AND *L. CANESCENS*

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Key Word Index—*Lippia nodiflora*; Verbenaceae; 6-oxygenated flavone sulphates; geographical variation; FAB-MS; HPLC.

Abstract—From the aerial parts of the maritime plant *Lippia nodiflora*, 15 flavonoids, 3 flavone aglycones and 12 new flavone sulphates, have been isolated and identified. The new flavone sulphates are mono- and disulphates of nepetin, jaceosidin, hispidulin, 6-hydroxyluteolin and nodifloretin present as the sodium salts. These sulphates are the only flavone conjugates detected in this plant. Flavone trisulphates are additionally present in populations of this species from Malaysia and Saudi Arabia, but lack of plant material prevented their complete characterization. Analysis of the closely related species *Lippia canescens* showed that it has the same flavonoid pattern. By contrast, a third species *L. triphylla* showed a flavonoid pattern lacking flavonoid sulphates, but characterized by the presence of 7-glucuronylglucosides of luteolin, diosmetin and apigenin. This is the first finding of flavonoid sulphates in the Verbenaceae.

INTRODUCTION

In Europe, the neotropical genus *Lippia* of the Verbenaceae is represented by the cosmopolitan species *Lippia nodiflora* (L.) Michx and the closely related *L. canescens* Kunth and by *L. triphylla* (L' Hér) O. Kuntze (syn. *L. citriodora*), a South American species cultivated in Mediterranean areas for its lemon scent.

Lippia nodiflora characteristically grows in maritime areas or near rivers, showing a strong preference for wet grassy places. In view of the recognised association between a salt or fresh water habitat and the conjugation in plants of flavonoids with the sulphate anion [1, 2], it was of interest to re-examine this plant for its flavonoids and compare the pattern with that of the other two species.

Flavonoid sulphates have not previously been reported in *Lippia*, although analysis of *L. nodiflora* growing in India has revealed the presence of nodifloretin (6-hydroxyluteolin 3'-methyl ether) [3] and of two 6-hydroxyluteolin glycosides, the 7-arabinoside and the 7-arabinoside-4'-rhamnoside [4]. We here report a populational study of the flavonoids of *L. nodiflora*, together with an account of the flavonoids of the other two European species.

RESULTS AND DISCUSSION

The aerial parts of a Spanish collection of *L. nodiflora* contained 15 flavonoids; 3 flavone aglycones and 12 new flavone sulphates. No flavonoid glycoside was detected. The free aglycones nepetin (6-methoxyluteolin), jaceosidin (6-methoxyluteolin 3'-methyl ether) and hispidulin (6-methoxyapigenin) were identified by their UV spectral properties and by chromatographic comparisons against authentic markers.

The flavone monosulphates jaceosidin 7-sulphate, nepetin 7-sulphate, hispidulin 7-sulphate, hispidulin 4'-sulphate, 6-hydroxyluteolin 7-sulphate, 6-hydroxyluteolin 6-sulphate and nodifloretin 7-sulphate were identified by standard procedures [1, 5, 6]. The UV studies, before and after acid hydrolysis at room temperature, allowed the location of the free hydroxyls and the position of the sulphate in the natural products. No sugar was detected in any of these compounds. These substances migrated towards the anode on paper electrophoresis under the specific conditions for sulphates (Table 1) with similar mobilities to that of a marker of quercetin 3'-sulphate. The presence of sulphate was confirmed after acid hydrolysis by precipitation with barium chloride. These flavonoid sulphates were also readily hydrolysed by the enzyme aryl sulphatase to yield their corresponding aglycones. The aglycones were identified by chromatographic comparisons against authentic markers isolated in previous work on Labiatae species, and by UV studies in methanol (MeOH) and after addition of the classical shift reagents. Under controlled acid hydrolysis at room temperature, all seven compounds were completely hydrolysed to the corresponding aglycone within the first 5 min of hydrolysis, without giving any intermediate products; thus all seven are monosulphates. The UV, chromatographic and electrophoretic behaviour of these compounds are shown in Table 1.

In order to confirm these structures, the two major compounds were submitted to negative ion fast atom bombardment mass spectrometry (FAB-MS). Thus, jaceosidin 7-sulphate gave a quasimolecular ion at m/z 409 ($[M-H]^-$) and other significant ions at m/z 329 ($[M-SO_3-H]^-$) and m/z 431 ($[M+Na-H]^-$) showing the presence of sodium in the sample. Sulphates are normally found in plants as the potassium salts [2]. So the

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Table 1. R_f, electrophoretic mobility and spectral properties of the flavonoid compounds isolated and identified from *Lippia nodiflora*

Flavone structures	‡R_f × 100 15% HOAc	‡R_f × 100 BAW	Electrophoretic mobility†	λ_{max}^{MeOH} (nm)§
5,7,3',4'-Tetrahydroxy-6-methoxyflavone (nepetin)	05	81	0	349, 272, 256
5,7,4'-Trihydroxy-6,3'-dimethoxyflavone (jaceosidin)	05	86	0	345, 274, 245 sh
5,7,4'-Trihydroxy-6-methoxyflavone (hispidulin)	08	91	0	335, 275
Hispidulin 7-sulphate	52	63	1.8	332, 276
Hispidulin 4'-sulphate	52	53	1.7	330, 276
Nepetin 7-sulphate	35	48	1.0	345, 275, 256, 240 sh
Jaceosidin 7-sulphate	39	54	1.8	342, 274, 252 sh
6-hydroxyluteolin 7-sulphate	17	43	1.1	347, 284, 254 sh
6-Hydroxyluteolin 6-sulphate	15	40	0.8	348, 272, 255
Nodifloretin 7-sulphate*	17	46	1.2	347, 284, 254
6-Hydroxyluteolin 6,7-disulphate	54	14	3.1	345, 275, 255, 240 sh
Nodifloretin 6,7-disulphate	68	21	4.4	343, 274, 250 sh
Nepetin 3',4'-disulphate	75	36	4.4	332, 275, 240 sh
Jaceosidin 7,4'-disulphate	59	43	3.5	340, 275, 252 sh
Hispidulin 7,4'-disulphate	73	34	4.4	330, 276

*Nodifloretin = 5,6,7,4'-tetrahydroxy-3'-methoxyflavone.

†Mobilities are relative to quercetin 3'-sulphate, run at pH 2.2 for 1 hr at 400 V/cm.

‡TLC on cellulose, BAW = *n*-BuOH–HOAc–H_2O (4:1:5, upper phase).

§Spectral maxima measured in the presence of added NaOH, NaOAc, NaOAc/H_3BO_3 and $AlCl_3$ were in close accord with lit. values.

discovery here of sodium salts is of some interest. The only other report of sodium in this connection is the detection of the ions Na^+, K^+ and Ca^{2+} in samples of flavonoid sulphates from *Flaveria* by atomic absorption spectroscopy [6]. Nodifloretin 7-sulphate gave similar ions at *m/z* 417 ($[M + Na - H]^-$), 395 ($[M - H]^-$) and 315 ($[M - SO_3 - H]^-$).

The five flavone disulphates 6-hydroxyluteolin and nodifloretin 6,7-disulphates, nepetin 3',4'-disulphate, hispidulin 7,4'-disulphate and jaceosidin 7,4'-disulphate were also identified in *Lippia nodiflora*. These compounds showed higher electrophoretic mobility, lower R_f values in BAW and higher R_f values in 15% acetic acid than the flavone monosulphates in keeping with their disulphate character. After acid hydrolysis at room temperature and after enzymic hydrolysis with aryl sulphatase, they yielded the respective aglycones that were identified by their UV properties and by chromatographic comparisons with authentic markers. Under controlled acid hydrolysis at room temperature the monosulphates were detected in a very small amount, and the sulphated character of these intermediate products was established by paper electrophoresis. The positions of the sulphate attachments were established by UV studies of the naturally occurring compounds and the aglycones obtained after acid hydrolysis. No sugar was detected.

The major disulphate, nodifloretin 6,7-disulphate, was submitted to negative FAB-MS, giving a quasimolecular ion at *m/z* 475 ($[M - H]^-$) and other significant ions at *m/z* 519($[M + 2Na - H]^-$),497($[M + Na - H']^-$), 417($[M + Na - SO_3 - H]^-$), 395 ($[M - SO_3 - H]^-$) and 315

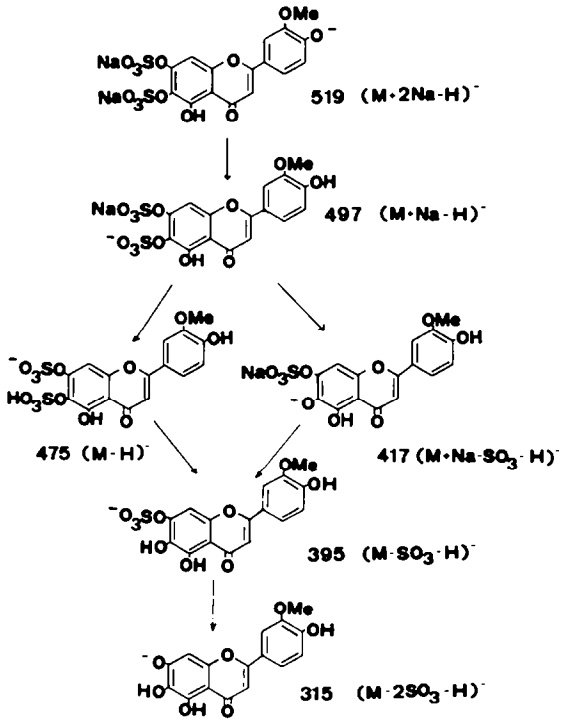

Fig. 1. FAB-MS fragmentation pattern of nodifloretin 6,7-disulphate.

($[M-2SO_3-H]^-$). The fragmentation pattern of this compound is shown in Fig. 1.

The most unusual compound was nepetin 3',4'-disulphate, by reason of its novel substitution pattern. Its electrophoretic mobility and chromatographic behaviour suggested that it was a disulphate. Its UV spectrum in MeOH (332, 275, 240 sh) and after addition of sodium hydroxide (NaOH) (370, 320, 275) suggested that the 4'-hydroxyl was substituted (bathochromic shift in band I after addition of NaOH with a decrease in absorbance relative to the MeOH spectrum) and the presence of a band III at 320 nm in the NaOH spectrum supported the presence of a free hydroxyl at the 7-position. This was confirmed by a small bathochromic shift in band II relative to the MeOH spectrum after addition of sodium acetate (365, 278). After acid hydrolysis this compound yielded nepetin which was identified by UV properties and by chromatographic comparison against an authentic marker.

In the last few years reversed-phase HPLC has proved to be very useful for the separation of flavonoid methyl ethers [7] and flavonoid sulphates [8]. The flavonoids isolated in the present work constitute a very interesting system to study the influence of sulphate and methyl groups on the reversed-phase HPLC behaviour of flavonoid compounds. As a general rule, the flavonoid disulphates, 6-hydroxyluteolin 6,7-disulphate (R_t 12.87), nodifloretin 6,7-disulphate (R_t 14.12), hispidulin 7,4'-disulphate (R_t 13.70) and jaceosidin 7,4'-disulphate (R_t 16.74) elute earlier than the respective monosulphates, 6-hydroxyluteolin 7-sulphate (R_t 15.21), nodifloretin 7-sulphate (R_t 16.52), nepetin 7-sulphate (R_t 16.79), hispidulin 7-sulphate (R_t 17.64) and jaceosidin 7-sulphate (R_t 18.05 min). The monosulphates elute with sharper peaks than the disulphates, this being explained by the ionization of the sulphate groups. The introduction of a second sulphate at the 6-position decreases the retention time by 2.40 and 2.34 min but when this is introduced at the 4'-position, the decrease in retention time depends on the substitution pattern on the B ring, being only 1.31 min if there is already a methyl ether at the 3'-position and being as great as 3.94 min in flavones with a monosubstituted B ring. By contrast, the introduction of a methyl ether increases the retention time by about 1.5 min with a slightly greater increase when this is in the 6-position. This could be explained by internal hydrogen bonding between the hydroxy groups at C-6 and C-5, which decreases the interaction between the latter hydroxy and the 4-keto group and decreases the retention time as previously described [7].

Since the discovery of flavone sulphates in a Spanish population of *L. nodiflora* is at variance with the earlier report of two flavone glycosides in an Indian population [3], it was decided to examine other available specimens of this plant. Four samples, collected variously in Israel, Egypt, Saudi Arabia and Malaysia, were analysed by 2D chromatography and all showed almost identical patterns to the Spanish sample, with jaceosidin and nodifloretin 7-sulphates being the major components. The samples from Saudi Arabia and Malaysia differed slightly in the relative concentrations of the various sulphates and also contained in addition small amounts of flavone trisulphates detected by electrophoretic and chromatographic techniques; insufficient material was available for their further characterization.

To complete this study, the two other European species of this genus were analysed; i.e. *L. canescens* and *L. triphylla*. The first one showed a flavonoid pattern very similar to that of *L. nodiflora*, supporting the fact that these two species are very closely related in morphology [9]. On the other hand, *L. triphylla* showed a very simple flavonoid pattern which was completely different from those of *L. nodiflora* and *L. canescens* in lacking flavonoid sulphates. The three flavonoids detected migrated on electrophoresis towards the anode at pH 4.4 and remained immobile at pH 2.2, suggesting that they were flavonoid glucuronides. The UV study of the natural glycosides and of the aglycones obtained after acid hydrolysis revealed that these compounds were 7-glycosides of luteolin, apigenin and diosmetin. Glucose and glucuronic acid were detected after acid hydrolysis of the three compounds. Under controlled hydrolysis, intermediate products were found which were immobile on electrophoresis and were identified as the 7-monoglucosides, supporting the view that the original compounds were luteolin, diosmetin and apigenin 7-glucuronylglucosides.

The present results suggest that in the genus *Lippia* there is a correlation between habitat and the synthesis of flavone conjugates. Thus two species which occupy maritime or inland moist habitats contain flavone sulphates, whereas a third species of drier soils lacks these salts and contains ordinary flavone glycosides in the aerial parts. Further species in the genus need to be examined to confirm that this pattern is a general one in these plants. The discrepancy between our results with the cosmopolitan species *L. nodiflora* and those obtained earlier on an Indian population [4] cannot readily be explained unless there is marked infraspecific variation present, possibly determined by soil type. The earlier failure to detect sulphates could be due to the lability of these conjugates, but their identification of two glycosides in this plant could not be confirmed by our analyses. Further populations need to be sampled, before these differences can be resolved.

EXPERIMENTAL

Plant material. Aerial parts of *L. nodiflora* were collected at flowering in the banks close to the Albufera lake in Valencia and a voucher specimen is deposited in the Herbarium of the University of Reading. Plant material deposited in the same Herbarium was used for the analysis of flavonoids from *L. canescens* Kunth collected near Jerez (Spain), and four populations of *L. nodiflora* collected on the banks of Yarkon river in Tel-Aviv (Israel), Alexandria (Egypt), Wadi kholb at khoba (Saudi Arabia) and Bukit Damansara (Malaysia). The aerial parts of *L. triphylla* were obtained from a plant growing in the Botanical Garden of Reading University.

Extraction and isolation of flavonoids. Air dried aerial parts were extracted with EtOH–H_2O (7:3) and these extracts concd under red pres. The different flavonoids were isolated by means of PC on Whatman 3 and 1 with 15% and 2% HOAc (the last one for disulphates) and *n*-BuOH–HOAc–H_2O (4:1:5, upper layer).

Identification of flavonoids. The flavonoid sulphate structures were established by UV studies (in MeOH and after addition of the classical shift reagents) of the naturally occurring sulphates and of the aglycones obtained after acid hydrolysis at room temp. (2N HCl). The aglycones obtained were identified by chromatographic comparisons against authentic samples (TLC on cellulose 30% and 50% HOAc).

Enzymic hydrolysis of the flavonoid sulphates. This was ac-

hieved by means of aryl sulphatase (Sigma) in 0.1 M acetate buffer pH 4.5 at 30°.

Acidic hydrolysis of the flavonoid sulphates. This was carried out at room temp. with 2N HCl, and samples were taken every 5 min.

HPLC analyses. The isolated flavonoid sulphates were dissolved in MeOH–H_2O (1:1) and samples of 2–10 μl were injected in a Waters 600 apparatus. A Spherisorb S5 ODS-2 (Hichrom, Reading, U.K.) column 250 × 5 mm I.D. was used, the solvents being A, water; and B, MeOH–HOAc–H_2O (18:1:1). The initial solvent composition was 25% B in A and gradient elution was linear with a 6% increase of B in A per min for 10 min. Then the system was maintained isocratic (85% of B in A) for a further 10 min. Flow rate was 1 ml/min, the column was kept at 25° and peaks were detected at 254 nm.

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