Externally Accumulated Flavonoids in Three Mediterranean *Ononis* Species

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The Mediterranean *Ononis* species, *O. fruticosa*, *O. natrix* subsp. *ramosissima* and *O. tridentata*, have been analyzed for their exudate flavonoids. More than 20 flavonoid aglycones were identified, some of which are rather rare natural compounds. One of them, namely hypolaetin-8,3′,4′-trimethyl ether, had been found only once before. The results are presented in a table along with literature data, and the chemotaxonomic impact of the flavonoid patterns is discussed.

Key words: Ononis, Epicuticular Flavonoids, Chemosystematics

Introduction

Ononis is a Fabaceae genus (tribe Trifolieae) that comprises some 75 species, occurring in the Canaries, the Mediterranean region, Europe to Central Asia (Willis,1973). Many species bear glandular trichomes that produce a more or less viscid excretion, which in several cases has been reported to contain flavonoids. In the scope of our ongoing studies on the occurrence and distribution of externally accumulated flavonoid aglycones in higher plants we now studied three species: *O. fruticosa*, *O. natrix* subsp. *ramosissima* and *O. tridentata.*

Material and Methods

Plant material

Ononis tridentata L. was field collected in July, 2000 at Los Castaños (Sorbas, Almeria, Spain) by D. Rivera Nuñez (DRN) and C. Obón. *Ononis fruticosa* L. was collected in May, 1990 near Salida de Tibi direccion a Jijona (Alicante, Spain). *Ononis natrix* ssp. *ramosissima* Desf. comes from a) Molino Rio Aguas (DRN, 04/1990) and b) Los Castaños (Sorbas, Almeria; DRN and C. Obón, 07/2000). Voucher specimens are deposited at the Herbarium of Murcia University.

Extraction and isolation

Fresh material was briefly rinsed with acetone to dissolve the lipophilic material accumulated on the plant surfaces and the solutions were evaporated to dryness. Amounts of air-dried plant material and resinous exudates were as follows: *O. tridentata* 97 g/0.85 g, *O. fruticosa* 54 g/1.33 g, *O. natrix* a) 57 g/3.26 g, and b) 170 g/12.3 g. The resinous residues were dissolved in a small volume of hot methanol, cooled to – 10 °C, and the precipitating material was eliminated by centrifugation. The thus defatted solutions were passed over Sephadex LH-20 (Pharmacia), eluted with MeOH, to separate the flavonoids from the overwhelming terpenoids. In the cases of *O. fruticosa* and *O. tridentata*, the flavonoid aglycones were then identified directly by TLC comparisons with authentic samples. The flavonoid fractions of the two different collections of *O. natrix* subsp. *ramosissima* were essentially identical and were therefore combined. They were further chromatographed over acetylated polyamide (Macherey-Nagel), eluted with toluene and increasing quantities of methylethyl ketone and methanol.

Fractions were monitored and comparisons with markers were done by TLC on polyamide (DC 11, Macherey-Nagel) with the solvents i) $PE_{100-140}$/toluene/MeCOEt/MeOH (12:6:1:1 v/v/v/v), ii) to-

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luene/PE100–140/MeCOEt/MeOH (12:6:2:1 v/v/v/v), and iii) toluene/MeCOEt/MeOH (12:5:3 v/v/v), and on silica gel with solvent iv) toluene/MeCOEt (9:1 v/v). Chromatograms were viewed under UV light (366 nm) before and after spraying with "Naturstoffreagenz A" (1% of diphenyl-boric acid ethanolamine complex in MeOH). Authentic samples of flavonoids were available in E. W.'s lab.

Some flavonoids were isolated (purified) after a further passage over Sephadex LH-20, eluted with $CHCl_3$/MeOH (99:1 v/v), followed by CTLC on the Chromatotron with benzene/2-propanol (95:5).

NMR and MS

Carbon and proton NMR spectra were recorded in DMSO-d_6 on a Bruker AMX 400 spectrometer at 100 MHz and 400 MHz, respectively. Electron impact mass spectra were obtained on a Varian MAT 212 Spectrometer at 70 eV. The mass and NMR spectra confirmed the structures of the following flavones: acerosin, agecorynin D, desmethylsudachitin, hymenoxin, nevadensin, pectolinarigenin, sideritiflavone, xanthomicrol and 5,7-dihydroxy-8,3′,4′-trimethoxyflavone. In the following we report the NMR data of the less common compounds [DMSO-d_6, δ (ppm) downfield from tetramethylsilane].

Sideritiflavone: ^{13}C NMR: δ_C = 164.3 (C-2), 102.5 (C-3), 182.3 (C-4), 148.4 (C-5), 135.7 (C-6), 152.3 (C-7), 132.6 (C-8), 145.1 (C-9), 106.1 (C-10), 121.3 (C-1′), 113.3 (C-2′), 145.7 (C-3′), 150.0 (C-4′), 116.1 (C-5′), 119.1 (C-6′) 60.5 (6-OMe), 61.9 (7-OMe), 61.4 (8-OMe). – ^{1}H NMR: δ_H = 6.77 (s, H-3), 7.47 (d, J = 2.4 Hz, H-2′), 6.92 (d, J = 8 Hz, H-5′), 7.46 (dd, J = 8, 2.4 Hz, H-6′), 12.79 (s, 5-OH), 3.82 (s, OMe), 3.93 (s, OMe), 4.02 (s, OMe).

Acerosin: ^{13}C NMR: δ_C = 163.3 (C-2), 102.9 (C-3), 182.1 (C-4), 148.3 (C-5), 131.5 (C-6), 150.8 (C-7), 127.9 (C-8), 145.3 (C-9), 102.9 (C-10), 123.1 (C-1′), 112.8* (C-2′), 146.8 (C-3′), 151.2 (C-4′), 112.3* (C-5′), 118.5 (C-6′), 60.1 (6-OMe), 61.2 (8-OMe), 55.7 (4′-OMe). – ^{1}H NMR: δ_H = 6.77 (s, H-3), 7.12 (d, J = 8.4 Hz, H-5′), 7.55 (dd, J = 2.4, 8.4 Hz, H-6′), 12.77 (s, 5-OH), 9.52 (s, 7-OH), 10.37 (s, 3′-OH), 3.79 (s, OMe), 3.87 (s, OMe), 3.89 (s, OMe).

Xanthomicrol: ^{13}C NMR: δ_C = 164.1 (C-2), 102.6 (C-3), 182.5 (C-4), 145.5* (C-5), 135.8 (C-6), 152.4 (C-7), 132.6 (C-8), 145.1*, (C-9),106.1 (C-10), 121.0 (C-1′), 128.4 (C-2′ and C-6′), 116.1 (C-3′ and C-5′), 161.4 (C-4′), 60.5, (6-OMe), 61.8 (7-OMe), 61.4 (8-OMe). – ^{1}H NMR: δ_H = 6.88 (s, H-3), 7.95 (d, J = 8.8 Hz, H-2′ and H-6′), 6.97 (d, J = 8.8 Hz, H-3′ and H-5′), 12.77 (s, 5-OH), 10.41 (s, 4′-OH), 3.82 (s, OMe), 3.92 (s, OMe), 4.02 (s, OMe).

Agecorynin D: ^{13}C NMR: δ_C = 161.9 (C-2), 104.3 (C-3), 182.4 (C-4), 148.4 (C-5), 135.5 (C-6), 152.2 (C-7), 132.4 (C-8), 145 (C-9), 106.6 (C-10), 105.9 (C-1′), 153.3 (C-2′), 106.5 (C-3′), 151.9 (C-4′), 141.6 (C-5′), 110.6 (C-6′), 60.4 (6-OMe), 61.6 (7-OMe), 61.4 (8-OMe), 55.9 (5′-OMe). – ^{1}H NMR: δ_H = 6.58 (s, H-3), 7.12 (s, H-3′), 7.41 (s, H-6′), 12.85 (s, 5-OH), 10.50 (s, 2′-OH)*, 10.08 (s, 4′-OH)*, 3.80 (s, 5′-OMe), 3.82 (s, OMe), 3.93 (s, OMe), 4.02 (s, OMe).

5,4′-Dihydroxy-6,7,8,3′-tetramethoxyflavone: ^{13}C NMR: δ_C = 164.0 (C-2), 102.9 (C-3), 182.5 (C-4), 148.4 (C-5), 135.8 (C-6), 152.4 (C-7), 132.5 (C-8), 145.1 (C-9), 106.1 (C-10), 121.3 (C-1′), 110.0 (C-2′), 148.0 (C-3′), 151.0 (C-4′), 115.9 (C-5′), 120.3 (C6′), 60.5 (6-OMe), 61.8 (7-OMe), 61.4 (8-OMe), 55.8 (3′-OMe). – ^{1}H NMR: δ_H = 7.00 (s, H-3), 6.98 (d, J = 8.8 Hz, H-5′), 7.59 (m, H-2′ and H-6′), 12.76 (s, 5-OH), 10.02 (s, 4′-OH), 3.82 (s, OMe), 3.90 (s, OMe), 3.93 (s, OMe), 4.02 (s, OMe).

Results and Discussion

Structure elucidation

A good number of flavonoid aglycones were found in the lipophilic material accumulated on aerial parts of *Ononis fruticosa*, *O. natrix*, and *O. tridentata.* The following compounds from *O. natrix* were identified by their MS, NMR, and UV spectra: scutellarein-6,4′-di-O-methyl ether (pectolinarigenin), 5,7,4′-trihydroxy-6,8-dimethoxy flavone (desmethylsudachitin), 5,4′-dihydroxy-6,7,8-trimethoxy flavone (xanthomicrol), 5,7-dihydroxy-6,8,4′-trimethoxy flavone (nevadensin), 5-hydroxy-6,7,8,4′-tetramethoxy flavone (gardenin B), 5,7-dihydroxy-8,3′,4′-trimethoxy flavone (hypolaetin-8,3′,4′-tri-O-methyl ether), 5,3′,4′-trihydroxy-6,7,8-trimethoxy flavone (sideritiflavone), 5,7,3′-trihydroxy-6,8,4′-trimethoxy flavone (acerosin), 5,4′-dihydroxy-6,7,8,3′-tetramethoxy flavone, 5,7-dihydroxy-6,8,3′,4′-tetramethoxy flavone (hymenoxin) and 5,2′,4′-trihxdroxy-6,7,8,5′-tetrame-

Table I. Exudate Flavonoids of *Ononis* species (Me, methyl ether; OMe, methoxy substituent).

	Ononis fruticosa	*Ononis natrix* ssp. *hispanica*	*Ononis natrix* ssp. *natrix*	*Ononis natrix* ssp. *ramosissima*	*Ononis natrix* ssp. *ramosissima*	*Ononis natrix (2 coll)*	*Ononis rotundifolia*	*Ononis sicula*	*Ononis speciosa*	*Ononis spinosa*	*Ononis tridentata*	*Ononis vaginalis*
References	1	2	3	4	1	5	5	6	7	5	1	8
Flavone												
Chrysin												X
Apigenin	X				X	X	X	X		X		X
Ap-7-Me (Genkwanin)	X					X	X			X		
Ap-4′-Me (Acacetin)	X						X			X		
Ap-7,4′-diMe	X									X		
Scutellarein-6-Me (Hispidulin)						X	X	X		X		
Scut-6,7-diMe (Cirsimaritin)								X			X	X
Scut-6,4′-diMe (Pectolinarigenin)	X				X		X					
5- Scut-6,7,4′-triMe (Salvigenin)	X						X				X	
Luteolin	X					X				X	X	
Lut-7-Me	X				X	(x)					X	
Lut-3′-Me (Chrysoeriol)					X	X				X	X	
Lut-7,3′-diMe (Velutin)						X				X	X	
Lut-3′,4′-diMe												X
Lut-7,3′,4′-triMe										X		
5,7,4′-triOH-6,8-diOMe (Desmethylsudachitin)						X						
5,4′-diOH-6,7,8-triOMe (Xanthomicrol)			X		X							
5,7-diOH-6,8,4′-triOMe (Nevadensin)		X			X	X						
5-OH-6,7,8,4′-tetraOMe (Gardenin B)			X									
6-OH-Luteolin-6-Me (Nepetin)					X						X	
6-OH-Lut-6,3′-diMe (Jaceosidin)						X	X			X	X	
6-OH-Lut-6,7,3′-triMe (Cirsilineol)											X	
6-OH-Lut-6,3′,4′-triMe (Eupatilin)												X
6-OH-Lut-6,7,3′,4′-tetraMe											X	
Hypolaetin –8,3′,4′-triMe					X							
5,3′,4′-triOH-6,7,8-triOMe (Sideritiflavone)					X							
5,7,3′-triOH-6,8,4′-triOMe (Acerosin)					X							
5,4′-diOH-6,7,8,3′-tetraOMe					X						X	
5,7-diOH-6,8,3′,4′-tetraOMe (Hymenoxin)			X		X							
5,2′,4′-triOH-6,7,8,5′-tetraOMe (Agecorynin D)					X							
Flavonole												
Quercetin	X							X		X		
Quercetin-3-glykosid	X											
Quercetagetin-3,6,7-triMe (Penduletin)								X				
5,7,4′-triOH-3,6,8-triOMe (Sarothrin)							X					
5,7,4′-triOH-3,6,8,3′-tetraOMe							X					
Flavanones, chalcones, dihydrocchalcones				X								

References: 1) Present study. 2) Barrero *et al.*, 1990. 3) Al-Khalil *et al.*, 1995. 4) Barrero *et al.*, 1997. 5) Wollenweber, 1990. 6) Barrero *et al.*, 1998. 7) Barrero *et al.*, 1989. 8) Amer *et al.*, 1989.

thoxy flavone (agecorynin D). The structures of these products (except for 5,4′-dihydroxy-6,7,8,3′-tetramethoxy flavone) and the remaining compounds were confirmed by co-TLC with authentic markers.

In Table I, the flavonoids herein identified are listed together with those reported earlier for various collections of *O. natrix* and for further species found to exhibit flavonoid aglycones.

Among the flavones identified in the present study, hypolaetin-8,3′,4′-tri-O-methyl ether is an extremely rare compound. It has thus far been reported only from the resin of *Gardenia gummifera* (dikamali gum; Krishnamurti *et al.*, 1972) and from the leaf of *Gardenia lucida* (another source of dikamali gum; Kumari, 1989). 5,2′,4′-trihydroxy-6,7,8,5′-tetramethoxy flavone (agecorynin D) is also a rare compound, previously reported from *Ageratum corymbosum* (Quijano *et al.*, 1980) and from *Gutierrezia microcephala* (Fang *et al.*, 1986), and 5,7,3′-trihydroxy-6,8,4′-trimethoxy flavone (acerosin) is an uncommon flavone, too, that has been found only five or six times, thus far.

Flavonoid distribution

Wollenweber (1990) found a series of methylated flavones to be exudate constitutents of *O. natrix*, *O. rotundifolia* and *O. spinosa*, and so they are in the species now studied. It is hence assumed that the flavonoid aglycones reported for *O. natrix* ssp. *hispanica* (Barrero *et al.*, 1990), ssp. *natrix* (Al-Khalil *et al.*, 1995) and ssp. *ramosissima* (Barrero *et al.*, 1997) are also accumulated externally, although they were analysed from the extracts of aerial parts. It may be mentioned in this context that, for *O. natrix*, dihydroisocoumarins have also been reported as lipophilic constituents (San Feliciano *et al.*, 1990).

Ononis natrix is a polytypic taxon for which 11 subspecies have been described (Sirjaev, 1932). It has recently been split into different species (Devesa, 2000). Barrero *et al.* (1997) pointed out that the flavonoid profiles of the three *O. natrix* subspecies they studied were clearly different. In subspecies *hispanica* they found only one flavone, but several resorcinol derivatives (Barrero *et al.*, 1990), and Al-Khalil *et al.* (1995) found only three flavones in subspecies *natrix*. [Note that for Devesa (2000), subspecies *hispanica* does not exist and is either *O. natrix* spp. *ramosissima or O. natrix sensu stricto*.] However Barrero *et al.* (1997) detected two flavanones, four chalcones and six dihydrochalcones in *O. natrix* spp. *ramosissima,* along with alkylresorcinol. Flavanones, chalcones and dihydrochalcones have not been reported for any other *Ononis* species than *natrix*; however our examination of the exudate from *O. natrix* spp. *ramosissima* revealed none of these types of compounds, a discrepancy for which we presently have no explanation. It is striking that a study of the glandulose *O. viscosa* did not reveal any flavonoid aglycones. Alkylresorcinols and pterocarpans seem to be predominant in its exudate (Barrero *et al.*, 1991, 1998). Unfortunatelly, material of *O. viscosa* was not available for our present studies.

With the exception of those flavanones, chalcones and dihydrochalcones previously reported for *O. natrix* spp. *ramosissima* (Barrero *et al.*, 1997), the majority of the flavonoid aglycones found on aerial parts of *Ononis* species are flavones; flavonols occur only occasionally. Furthermore the presence of 6- and 6,8-O-substitution seems to be a characteristic feature of these *Ononis* flavones and flavonols.

While more or less lipophilic flavonoid aglycones have been reported from root, stem, bark, and seed of a number of genera (*Dalbergia*, *Derris*, *Distemonanthus*, *Flemingia*, *Glycyrrhiza*, *Lonchocarpus*, *Millettia*, *Pongamia*, *Prosopis*, *Sophora*, *Tephrosia*, to give some prominent examples), the occurrence of externally accumulated flavonoid aglycones is a rather rare phenomenon within the Fabaceae. It was reported from *Acacia neovernicosa* (Wollenweber and Seigler, 1982), from *Zuccagnia punctata* (Pederiva and Giordano, 1984) and from the above cited *Ononis* species. In some further cases, where leaves or aerial parts were reported as the source of flavonoid aglycones, it is well possible that they also occured externally: so *e.g.* in *Anthyllis* (Pistelli *et al.*, 1996), *Crotalaria* (Krohn *et al.*, 2002), *Dalea* (Dominguez *et al.*, 1982), *Lonchocarpus* (Roussis *et al.*, 1987), *Lupinus* (Nicholls and Bohm, 1983), *Millettia* (Ganapaty *et al.*, 1998), and *Parkia* (Lemmich *et al.*, 1996).

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