Flavonoid Constituents of *Rhamnus lycioides* L.*

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From the aerial part of *Rhamnus lycioides*, which is used in folk medicine in eastern Spain as an antihypertensive drug, seven free flavonoid aglycones were isolated. They were structurally elucidated as rhamnazin, rhamnocitrin, isorhamnetin, kaempferol, quercetin, quercetin-3-methyl-ether and taxifolin, by applying the principal spectroscopic methods and TLC with authentic markers.

Introduction

The genus *Rhamnus* is characterized from a phytochemical point of view by the abundance of phenolic substances like anthraquinones, tannins and flavonoids [1]. One of its more typical taxa in the Mediterranean vegetation is *Rhamnus lycioides* L., a bush that appears frequently in southern and eastern Spain. It is used in folk medicine as an antihypertensive drug and has never been chemically studied. Our research on the pharmacological activity of this species has demonstrated that its butanolic extract possesses hypotensive action [2] which may explain its traditional use. Lower activity was found in the ethyl acetate extract.

The present paper deals with the isolation and identification of the free flavonoid aglycones that are present in the plant, particularly in chloroformic and ethyl acetate extracts. Most of them are flavonols, often methyl ethers, but also a dihydroflavonol (taxifolin) is reported. Further studies on the isolation and characterization of the flavonoid glycosides from the butanolic extract, which has a high content of this kind of principles, are now in progress.

Materials and Methods

Plant material and extraction: *Rhamnus lycioides* aerial parts were collected in Torrent (Valencia, Spain) in June 1984 and a voucher specimen was deposited in the Herbarium of the Faculty of Pharmacy (Univ. of Valencia). Air dried leaves and stems (5.56 kg) were cut off, crushed and extracted with 70% aq. MeOH (4×6 l). Liquors were combined and the methanol was removed under reduced pressure. The aqueous fraction was successively treated with $CHCl_3$ to obtain extract I (46 g), EtOAc, extract II (87 g) and BuOH, extract III (97 g).

Separation and identification: A portion of extract I (3.7 g) was chromatographed on a silica gel 60 Merck column with $CHCl_3$–MeOH (95:5), and this mixture was progressively enriched in MeOH. Three flavonoids, **A** (20 mg), **B** (5 mg), and **C** (60 mg) were obtained from fractions 5, 8–11 and 12, respectively. Separation of substance **B** by PTLC on silica gel PF_{254} Merck plates (0.5 mm thick; solvent $CHCl_3$–MeOH (9:1)) proved difficult. 10 g of extract II were chromatographed in the same way as I, but the elution began with $CHCl_3$–MeOH (9:1). From fractions 7–8 (620 mg), substance **E** (80 mg) was isolated in another silica gel 60 column, solvent C_6H_{14}–EtOAc (1:1); another aglycone (substance **D**) whose R_f was very similar to that of **E** could not be purified since it was found in trace amounts. Fraction 9 furnished substance **F** by means of PTLC on silica gel, solvent C_6H_{14}–EtOAc (1:1), and from fractions 12–13 (1 g) it was possible to obtain **G** (80 mg) and **H** (120 mg) by cellulose CC, elution with MeOH–H_2O (7:3). General detection of flavonoids and anthraquinones in fresh plant material was done by applying the Shinoda (Mg/aq. HCl) and Bornträger (aq. KOH) tests. TLC determinations were carried out with silica gel G 60 and microcrystalline cellulose Merck aluminium sheets (0.2 mm) and polyamide RS Carlo Erba (0.25 mm), using 365 nm UV light and aminoethyl ester of diphenylboric acid 1% in MeOH (Neu's reagent) as revealers.

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UV spectra were run on a Pye Unicam SP 8-100 spectrophotometer, in methanolic solution. ^{1}H NMR spectra were determined on Varian 360 EM and Hitachi P. Elmer R-24B spectrometers (60 MHz) and ^{13}C NMR spectrum on a Bruker WP 8054 spectrometer (80 MHz); δ-values are given in ppm (TMS = 0).

Results and Discussion

Preliminary tests seemed to show that flavonoid substances were quite abundant in the plant, whereas anthraquinones were absent. The aglycones were obtained as explained above and their polarities covered a wide range. R_f values on silica gel TLC (Table I) illustrate this matter.

Table I. TLC data of the detected flavonoids on silica gel solvent $CHCl_3$–MeOH (9:1). Colour: I = UV light (365 nm), II = Neu's reagent + I.

Compound	R_f	Colour	
		I	II
A	0.88	ochre	yellow
B	0.64	ochre	bright yellow
C	0.60	ochre	yellow
D	0.56	ochre	orange
E	0.50	ochre	bright yellow
F	0.32	dark brown	orange
G	0.28	ochre	orange
H	0.24	violet	faint yellow

The main tool to establish hydroxyl substitution patterns was the UV spectral analysis. Peak wavelengths of the spectra measured with the usual shift reagents [3] are shown in Table II.

Substances **C**, **E**, and **G** were identified as the widespread flavonols isorhamnetin, kaempferol and quercetin, respectively. This conclusion was reached on the basis of the coincidence of all TLC and UV results with those of the authentic markers.

Substance **A** is a flavonol with a free hydroxyl group at C-4′, as is demonstrated by the 433 nm peak with increased intensity in NaOMe spectrum. No *ortho* dihydroxyl substitution at B ring was observed, since addition of HCl did not modify the $AlCl_3$ spectrum. However the peak at 370 nm suggests a dioxygenation pattern. The A ring possesses a free hydroxyl group at C-5 but not at C-7 as $AlCl_3$ and NaOAc spectra showed, respectively. Methoxyl groups at C-7 and C-3′ were determined by ^{1}H NMR according to the following results (DMSO-d_6 sol.): 7.68 (1H, d, J = 2.5 Hz, C-2′), 7.62 (1H, dd, J = 8 and 2.5 Hz, H-6′), 6.91 (1H, d, J = 8.4 Hz, C-5′), 6.73 (1H, d, J = 2.5 Hz, C-8), 6.31 (1H, d, J = 2 Hz, C-6), 3.90 (6H, s, $-OCH_3$, C-7 and C-3′). Thus substance **A** was identified as 3,5,4′-trihydroxy-7,3′-dimethoxyflavone (rhamnazin).

Substance **B** gave UV spectra practically identical to those of kaempferol except the NaOAc spectrum that indicated an 7-O-substituted structure on the basis of the lack of any shift in band II. It was established that the radical was a methyl group because of

Table II. UV spectral data of the isolated flavonoids. Maxima in nm, dec. = decomposition. Values in brackets correspond to shoulders.

	MeOH		$AlCl_3$		$AlCl_3$ + HCl		NaOMe		NaOAc		NaOAc + H_3BO_3	
A	370	(324)	426	(362)	425	360	433	267	(410)	385	367	(324)
	(270)	254	302	267	302	266	247		256		(270)	253
B	364	(286)	423	355	421	352	447	354	(410)	376	366	(290)
	266		303	270	301	268	(295)	270	263		267	
C	368	(320)	428	360	428	358	330	271	390	323	368	(320)
	(270)	254	305	263	302	263	dec.		273		(270)	254
E	365	(323)	425	352	423	350	418	318	397	319	367	323
	(294)	266	306	268	306	268	278		390	310	298	263
F	358	293	435	323	396	362	408	319	397	319	368	(320)
	255		304	274	(274)	260	273		304	273	(270)	254
G	368	(300)	440	302	423	355	325	248	386	326	386	259
	(270)	255	270		302	258	dec.		275	(257)		
H	323	289	370	314	370	312	325	(243)	325	(285)	(323)	290

its complete chromatographic coincidence with kaempferol-7-methyl-ether obtained from *Anthyllis onobrychioides* [4]. With these data we propose the structure of 3,5,4′-trihydroxy-7-methoxyflavone (rhamnocitrin) for substance **B**.

Substance **F**, dark brown when observed under UV light, showed in its MeOH UV spectrum a band I at 358 nm, the lowest value if compared with the other flavonols examined. This immediately suggests the existence of a substituted hydroxyl group at C-3. Positions of band I in $AlCl_3$ and $AlCl_3 + HCl$ as well as in $NaOAc + H_3BO_3$ demonstrated the presence of free *ortho* dihydroxylation in B ring, and band II in NaOAc indicates a hydroxyl group at C-7. Shape and situation of the peaks and shoulders coincide perfectly with the spectra given for quercetin-3-methyl-ether by Voirin [5]. This structure was also supported by ^1H NMR data (acetone-d_6 sol): 7.65 (1H, dd, J = 9 and 2.5 Hz, C-6′), 7.58 (1H, d, J = 2.5 Hz), 7.06 (1H, d, J = 8.5 Hz, C-5′), 6.53 (1H, d, J = 2.5 Hz, C-8), 6.27 (1H, d, J = 2 Hz, C-6), 3.89 (3H, s, $-OCH_3$, C-3). Substance **F** was therefore identified as 5,7,3′,4′-tetrahydroxy-3-methoxyflavone.

Substance **H** had some properties that differentiated it from the other aglycones: white colour, ease in crystallizing, notable R_f with 15 and 30% AcOH over cellulose and a very sharp peak at 289 nm in its UV spectrum in MeOH. We therefore thought that may possesses a flavanone structure. From the UV data no noteworthy information was obtained; only free hydroxyl groups at C-5 and C-7 were determined with the $AlCl_3$ and NaOAc spectra. From ^{13}C NMR analysis the following signals were obtained (methanol-d_4, sol.): 198.15 (C-4), 168.76 (C-7), 165.16 (C-5), 164.40 (C-9), 147.02 (C-4′), 146.20 (C-3′), 129.88 (C-1′), 120.93 (C-6′), 116.20 (C-2′), 115.97 (C-5′), 101.81 (C-10), 97.44 (C-6), 96.40 (C-8), 84.96 (C-2) and 73.60 (C-3). Carbon assignments were made on the basis of the comparison with the values reported in the literature [6]. Peaks at 73.60 and 84.96 ppm indicate clearly that substance **H** is a dihydroflavonol, since they correspond unambiguously with alcoholic C-3, and C-2 respectively. Phenolic substitution was established at 5,7,3′ and 4′ positions, leading to the structure of 3,5,7,3′,4′-pentahydroxyflavanone (taxifolin). Signals attributed to C-5 and C-7 may be reversed if the suggestions of Agrawal *et al.* [7] are accepted.

The flavonoid aglycones reported here are closely related to those previously observed in the genus *Rhamnus*, but a few special considerations must be taken into account: 1) The relatively high content of taxifolin, a compound already described in *Rhamnus pallasii* [8] and *Zizyphus nummularia* (Rhamnaceae) [9] but certainly not very widespread in the family. 2) The absence or, at best, the low content of rhamnetin, if it is identified as substance **D**. That flavonol is fairly typical of the genus and it was to be expected that it would appear as it did in *Rhamnus cathartica, R. dahurica, R. frangula* and *R. erythroxylon*. 3) The presence of quercetin-3-methyl-ether, that is now cited for the first time in the Rhamnaceae. Flavonoid glycosides present in butanolic extract are in the way to be isolated for chemical and pharmacological studies.

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