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QUANTITATIVE ANALYSIS OF GLUCOFRANGULINS AND PHENOLIC COMPOUNDS IN CROATIAN *RHAMNUS* AND *FRANGULA* SPECIES

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We determined the content of biologically active compounds in the bark of seven *Rhamnus* L. and two *Frangula* Mill. species growing in Croatia. All taxa tested had high content of total polyphenols (from 2.68% in *R. orbiculata* Bornm. to 8.50% in *R. pumila* Turra), moderate content of glucofrangulins (from 0.22% in *R. pumila* to 9.26% in *R. fallax* Boiss.), nontannic polyphenols (from 0.73% in *R. orbiculata* to 5.92% in *F. alnus* Mill.) and tannins (from 1.10% in *R. saxatilis* Jacq. to 4.92% in *R. alaternus* L.), and low content of phenolic acids (from 0.44% in *R. orbiculata* to 1.81% in *R. intermedia* Steud. & Hochst.) and flavonoids (from 0.02% in *F. alnus* to 1.44% in *R. pumila*). By ANOVA, variability was highest for glucofrangulin content, less for flavonoids, phenolic acids and nontannic polyphenols, and least for total polyphenols and tannins.

Key words: *Rhamnus*, *Frangula*, glucofrangulins, phenolic compounds, quantitative analysis.

INTRODUCTION

The genus *Rhamnus* L. 1753 (family Rhamnaceae Juss. 1789) comprises 125 (or ~200) species distributed throughout the temperate Northern Hemisphere southward to Brazil and South Africa (Wielgorskaya, 1995; Erhardt et al., 2002). The current classification separates 25 species in the genus *Frangula* Mill. (Erhardt et al., 2002). In Europe there are 13 *Rhamnus* and 3 *Frangula* species (Tutin, 1978a,b) and, according to Domac (2002), 8 *Rhamnus* and 2 *Frangula* species commonly grow in Croatia.

For centuries the fruit and bark of *Rhamnus* and *Frangula* species [especially of *R. cathartica*, *F. alnus* and *F. purshiana* (DC.) J. G. Cooper (syn. *Rhamnus purshianus* DC.)] have been used in folk and standard medicine as purgatives (Hiller and Melzig, 2003). Previous chemical studies of *Rhamnus* and *Frangula* species have examined mainly anthranoides, the most interesting medicinal substances from these plants. *R. cathartica* fruits contain anthraquinone derivates, flavonols and bitter substances (Hiller and Melzig, 2003). The bark of *F. alnus* contains a mixture of anthraquinone derivates, flavonoids, tannins and peptide alkaloids (Wichtl, 1994), while the bark of *F. purshiana* also contains a significant quantity of anthraquinone derivates (Hiller and Melzig, 2003). There are no published data on the chemical composition, application and therapeutic effects or in vitro cultures of *F. rupestris* (Sajc et al., 1999), *R. intermedia*, *R. orbiculata* or *R. pumila*.

The aim of this study was to determine the content of major groups of chemical compounds (glucofrangulins, flavonoids, phenolic acids, total polyphenols, nontannic polyphenols and tannins) in bark of *Rhamnus* and *Frangula* species growing in Croatia. This study is the first attempt to assess the phytochemical content of *F. rupestris* and two endemic Illyric-Balkan species, *R. intermedia* and *R. orbiculata*.

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TABLE 1. Geographical location and altitude of studied *Rhamnus* and *Frangula* species

Species (Abbreviation)	Locality	Latitude; Longitude	Altitude (m a.s.l.)	Collection date
R. alaternus L. (Ra)	Bivio (Rijeka)	45°20' N; 14°21' E	5	15–10–2008
R. cathartica L. (Rc)	Fran Kušan Pharmaceutical Botanical Garden (Zagreb)	45°50' N; 15°59' E	195	29–07–2008
R. fallax Boiss. – VK (Rf-VK)	Veliki Kozjak (Mt. Velebit)	44°73' N; 15°04' E	1500	26–07–2008
R. fallax Boiss. – Vo (Rf-Vo)	Vošac (Mt. Biokovo)	43°18' N; 17°02' E	1300	20–06–2008
F. alnus Mill. – CO (Fa-CO)	Commercial origin (Croatia)	–	–	2006
F. alnus Mill. – FBG (Fa-FBG)	Fran Kušan Pharmaceutical Botanical Garden (Zagreb)	45°50' N; 15°59' E	195	29–07–2008
F. alnus Mill. – H (Fa-H)	Hrastenica (Lonjsko Polje)	46°38' N; 16°43' E	164	02–09–2008
F. alnus Mill. – R (Fa-R)	Rječina – Kukuljani (Rijeka)	45°24' N; 14°25' E	295	15–10–2008
R. intermedia Steud. et Hochst. (Ri)	Sv. Križ above Martinšćica (Rijeka)	45°19' N; 14°29' E	90	15–10–2008
R. orbiculata Bornm. (Ro)	Mt. Sniježnica	42°34' N; 18°21' E	600	25–06–2008
R. pumila Turra (Rp)	Pakleno (Mt. Obruč)	45°27' N; 14°28' E	1260	30–05–2009
F. rupestris (Scop.) Schur – Va (Fr-Va)	Vaganac (Mt. Velebit)	44°19' N; 15°28' E	700	20–06–2008
F. rupestris (Scop.) Schur – S (Fr-S)	Mt. Sniježnica	42°34' N; 18°21' E	600	23–06–2008
R. saxatilis Jacq. (Rs-Va)	Vaganac (Mt. Velebit)	44°19' N; 15°28' E	700	20–06–2008
R. saxatilis Jacq. (Rs-Vo)	Vošac (Mt. Biokovo)	43°18' N; 17°02' E	1300	25–06–2008

MATERIAL AND METHODS

HERBAL MATERIAL AND EXTRACTION

Randomly selected samples of 9 *Rhamnus* L. and *Frangula* Mill. species were collected at different locations in Croatia (Tab. 1). Plant material was dried for three weeks in a well-ventilated room, in a single layer, protected from direct solar light. To limit oxidation and photo-oxidation, air-dried bark was placed in double paper bags, closed in a dark container and stored in a dry place protected from light until analysis.

Voucher specimens are deposited in the Herbarium of the Department of Pharmaceutical Botany and Fran Kušan Pharmaceutical Botanical Garden, Faculty of Pharmacy and Biochemistry, University of Zagreb, Zagreb, Croatia.

QUANTITATIVE ANALYSIS

Loss on drying was measured and spectrophotometric determination of all investigated compounds was done according to the *European Pharmacopoeia* (2007). The quantity of glucofrangulins was determined spectrometrically at 515 nm after the reaction between glucofrangulins and magnesium acetate. After acid hydrolysis (with 25% hydrochloric acid in acetone for 30 min at 100°C), the liberated flavonoid aglycones form a complex with aluminum chloride in a methanol-ethyl acetate-acetic acid medium, which is determined spectrometrically at 425 nm. Total phenolic acid content was determined by measuring the absorbance of the complex formed between phenolic acids and sodium nitrite-sodium molybdate at 505 nm. Determination of total polyphenols, polyphenols unadsorbed on hide powder (nontannin polyphenols) and tannins was done spectrophotometrically with phosphorous-wolframic acid and hide powder, using a Varian Cary 50 Bio spectrophotometer (Varian Inc., U.S.A.).

STATISTICAL ANALYSIS

The experiments were performed in triplicate and data are expressed as means ±SD. Statistical comparisons between and within species employed one-way ANOVA followed by Scheffe's post-hoc test at $p \leq 0.05$. Since the data for glucofrangulin and flavonoid content did not follow a normal distribution, the data were $1/\sqrt{x}$ transformed. The results were also assessed by Principal Component Analysis (PCA). To confirm the PCA results, the unweighted pair-group method with arithmetic mean (UPGMA) with Euclidean distance (DE) was applied (Miller and Miller, 2000). Statistical analyses were performed using Statistica 7 (StatSoft Inc., Tulsa, OK, U.S.A.).

TABLE 2. Loss on drying (LD) and dry-weight content of glucofrangulins (G), flavonoids (F), phenolic acids (PA), total polyphenols (TP), nontannic polyphenols (NTP) and tannins (T) in bark of studied *Rhamnus* and *Frangula* species (%)[a]. For abbreviations see Table 1

Locality	LD	G	F	PA	TP	NTP	T
R. alaternus	9.30	2.96 ± 0.01	0.08 ± 0.01	1.48 ± 0.03	7.95 ± 0.58	3.03 ± 0.48	4.92 ± 0.10
R. cathartica	8.17	0.95 ± 0.01	0.10 ± 0.00	1.62 ± 0.01	7.29 ± 0.58	2.93 ± 0.44	4.36 ± 0.13
R. fallax – VK	7.91	7.96 ± 0.01	0.99 ± 0.01	0.84 ± 0.08	6.29 ± 0.78	2.75 ± 0.04	3.54 ± 0.81
R. fallax – Vo	7.78	9.26 ± 0.01	0.40 ± 0.02	0.57 ± 0.01	8.35 ± 1.07	5.66 ± 0.04	2.69 ± 0.01
F. alnus – CO	8.03	7.63 ± 0.01	0.02 ± 0.00	0.91 ± 0.06	7.33 ± 0.97	4.91 ± 0.43	2.42 ± 0.54
F. alnus – FBG	8.97	6.43 ± 0.01	0.05 ±0.00	1.21 ± 0.02	5.57 ± 0.83	4.13 ± 0.02	1.44 ± 0.04
F. alnus – H	7.94	3.72 ± 0.01	0.08 ± 0.00	1.44 ± 0.03	8.30 ± 0.78	5.92 ± 0.54	2.38 ± 0.24
F. alnus – R	8.65	5.74 ± 0.00	0.08 ± 0.00	1.28 ± 0.16	6.15 ± 0.01	4.16 ± 0.52	1.99 ± 0.54
R. intermedia	6.67	0.40 ± 0.00	0.10 ± 0.00	1.81 ± 0.08	3.82 ± 0.61	2.26 ± 0.20	1.56 ± 0.41
R. orbiculata	5.79	0.89 ± 0.04	0.04 ± 0.00	0.44 ± 0.11	2.68 ± 0.29	0.73 ± 0.51	1.95 ± 0.22
R. pumila	8.10	0.22 ± 0.00	1.44 ± 0.09	0.67 ± 0.04	8.50 ± 0.45	4.09 ± 0.01	4.41 ± 0.44
F. rupestris – Va	7.26	0.26 ± 0.00	0.06 ± 0.00	0.99 ± 0.17	5.24 ± 0.27	2.55 ± 0.03	2.69 ± 0.24
F. rupestris – S	7.75	0.54 ± 0.01	0.06 ± 0.01	0.62 ± 0.04	4.09 ± 0.31	1.64 ± 0.48	2.45 ± 0.17
R. saxatilis – Va	7.18	0.54 ± 0.01	0.08 ± 0.01	1.10 ± 0.13	7.45 ± 0.13	5.60 ± 0.52	1.85 ± 0.65
R. saxatilis – Vo	4.54	0.50 ± 0.01	0.07 ± 0.00	0.83 ± 0.09	4.24 ± 0.22	3.14 ± 0.26	1.10 ± 0.48

[a] = m/m (Mean value ± SD; n = 3)

RESULTS AND DISCUSSION

The content of glucofrangulins, flavonoids, phenolic acids, total polyphenols, nontannic polyphenols and tannins in the bark of *Rhamnus* and *Frangula* species collected in Croatia are reported in Table 2. Glucofrangulin content ranged from 0.22% (*R. pumila*) to 9.26% (*R. fallax* – Vo). Only the samples of *R. fallax* and *F. alnus* showed high content of anthraquinone derivates. Glucofrangulin content in these two species was similar to that in *F. purshiana* (6–9%) (Newall et al., 1996). According to Locatelli et al. (2009) the total content of five anthraquinones in bark is 0.49 mg/g in *R. saxatilis* and 2.42 mg/g in *R. alpinus*.

Flavonoid content ranged from 0.02% (*F. alnus* – CO) to 1.44% (*R. pumila*). *R. fallax* collected at two localities also contained significant flavonoid content (0.99% and 0.40%). Flavonoid content was similar and very low in all other investigated species (from 0.04% in *R. orbiculata* to 0.10% in *R. cathartica* and *R. intermedia*). Content of phenolic acids was found to be from 0.44% (*R. orbiculata*) to 1.81% (*R. intermedia*). *R. pumila* and *R. fallax* – Vo showed the highest content of total polyphenols (8.50% and 8.35%, respectively), and *R. orbiculata* showed the lowest content of total polyphenols (2.68%) and nontannic polyphenols (0.73%). The sample of *F. alnus* – H contained the highest content of nontannic polyphenols (5.92%). Tannins varied from 1.10% (*R. saxatilis* – Vo) to 4.92% (*R. alaternus*).

The ANOVA results for interspecific and intraspecific variability of the analyzed substances in bark are given in Table 3. ANOVA showed variability to be highest for glucofrangulin content, less for content of flavonoids, phenolic acids and nontannic polyphenols, and least for content of total polyphenols and tannins.

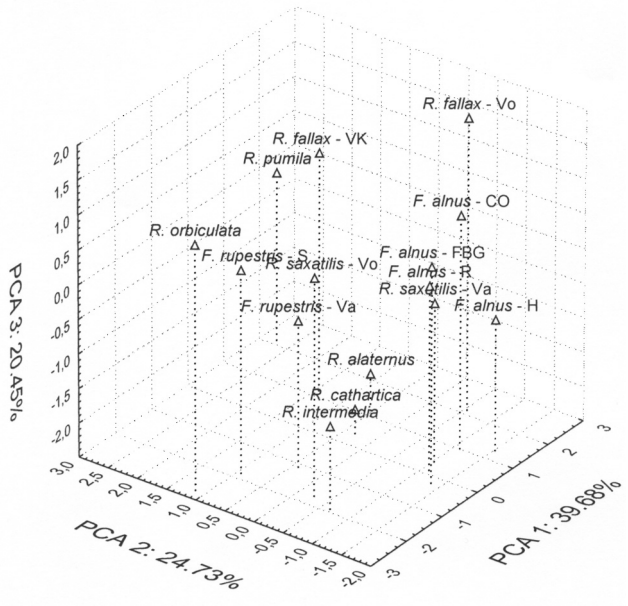

Fig. 1. PCA of studied Croatian *Rhamnus* and *Frangula* species. For abbreviations see Table 1.

TABLE 3. Interspecific and intraspecific variability of content of glucofrangulins (G), flavonoids (F), phenolic acids (Pa), total polyphenols (Tp), nontannic polyphenols (Np) and tannins (T) in studied *Rhamnus* and *Frangula* species. Asterisk behind the letter indicates significant difference at $p \leq 0.05$. For abbreviations see Table 1.

Species	Ra	Rc	Rf-VK	Rf-Vo	Fa-CO	Fa-FBG	Fa-H
Ra							
Rc	G*FPaTpNp*T						
Rf-VK	G*F*Pa*TpNpT	G*F*Pa*TpNpT					
Rf-Vo	G*F*Pa*TpNp*T*	G*F*Pa*TpNp*T*	GFPaTpNp*T				
Fa-CO	G*F*Pa*TpNp*T*	G*F*Pa*TpNp*T*	GF*PaTpNp*T	GF*PaTpNpT			
Fa-FBG	G*F*PaTpNpT*	G*F*Pa*TpNpT*	GF*PaTpNpT*	G*F*Pa*Tp*NpT	GF*PaTpNpT		
Fa-H	G*FpaTpNp*T*	G*FPaTpNp*T*	G*F*Pa*TpNp*T	G*F*Pa*TpNpT	G*F*Pa*TpNpT	G*F*PaTp*Np*T	
Fa-R	G*FPaTpNpT*	G*FPaTpNpT*	G*F*Pa*TpNpT	G*F*Pa*TpNpT	G*F*PaTpNpT	GFPaTpNpT	G*FPaTpNp*T
Ri	G*FpaTp*NpT*	G*FPaTp*NpT*	G*F*Pa*TpNpT*	G*F*Pa*Tp*Np*T	G*F*Pa*Tp*Np*T	G*F*Pa*TpNp*T	G*FPaTp*Np*T
Ro	G*F*Pa*Tp*Np*T*	GF*Pa*Tp*Np*T*	G*F*Pa*Tp*Np*T	G*F*PaTp*Np*T	G*F*Pa*Tp*Np*T	G*FPa*Tp*Np*T	G*F*Pa*Tp*Np*T
Rp	G*F*Pa*TpNpT	G*F*Pa*TpNpT	G*FPaTpNpT	G*FPaTpNpT	G*F*PaTpNpT*	G*F*Pa*Tp*NpT*	G*F*Pa*TpNp*T*
Fr-Va	G*Fpa*Tp*NpT*	G*FPa*TpNpT	G*F*PaTpNpT	G*F*Pa*Tp*Np*T	G*F*PaTpNp*T	G*FPaTpNpT	G*FPa*Tp*Np*T
Fr-S	G*Fpa*Tp*NpT*	G*FPa*Tp*NpT*	G*F*PaTpNpT	G*F*PaTp*Np*T	G*F*PaTp*Np*T	G*FPa*TpNp*T	G*FPa*Tp*Np*T
Rs-Va	G*Fpa*TpNp*T*	G*FPa*TpNp*T*	G*F*PaTpNp*T	G*F*Pa*TpNpT	G*F*PaTpNpT	G*F*PaTpNpT	G*FPaTpNpT
Rs-Vo	G*Fpa*Tp*NpT*	G*FPa*Tp*NpT*	G*F*PaTpNpT*	G*F*PaTp*Np*T	G*F*PaTp*Np*T	G*FPa*TpNpT	G*FPa*Tp*Np*T

TABLE 3. *cont.*

Species	Fa-R	Ri	Ro	Rp	Fr-Va	Fr-S	Rs-Va	Rs-Vo
Ra								
Rc								
Rf-VK								
Rf-Vo								
Fa-CO								
Fa-FBG								
Fa-H								
Fa-R								
Ri	G*FPa*TpNp*T							
Ro	G*F*Pa*Tp*Np*T	G*F*Pa*TpNpT						
Rp	G*F*Pa*TpNpT*	G*F*Pa*Tp*Np*T*	G*F*PaTp*Np*T*					
Fr-Va	G*FPaTpNp*T	G*FPa*TpNpT	G*FPa*TpNp*T	G*F*PaTp*NpT				
Fr-S	G*FPa*TpNp*T	G*FPa*TpNpT	G*F*PaTpNpT	G*F*PaTp*Np*T*	G*FPaTpNpT			
Rs-Va	G*FPaTpNpT	G*FPaTp*Np*T	G*F*Pa*Tp*Np*T	G*F*Pa*TpNpT*	G*FPaTpNp*T	GFPa*Tp*Np*T		
Rs-Vo	G*FPa*TpNpT	G*FPa*TpNpT	G*F*Pa*TpNp*T	G*F*PaTp*NpT*	G*FPaTpNpT	G*FPaTpNpT	GFPaTp*Np*T	

TABLE 4. Eigenvectors of the principal components. Bold values indicate the highest contribution to each PC axis

Variable	PC 1	PC 2	PC 3	PC 4	PC 5
Glucofrangulins	0.355870	-0.282667	0.464540	**-0.745961**	-0.145606
Flavonoids	0.344462	**0.575770**	0.163597	0.184531	**-0.699299**
Phenolic acids	0.029023	-0.379647	**-0.736243**	-0.198704	-0.522962
Total polyphenols	**0.621566**	-0.071441	-0.165187	0.171174	0.253875
Nontannic polyphenols	0.483730	-0.452999	0.114229	0.470406	0.016152
Tannins	0.365407	0.483885	-0.418390	-0.345579	0.389330

PCA of the analyzed substances separated the investigated species as shown in Figure 1. The most similar samples were those of *F. alnus* – FBG, *F. alnus* – R, and *R. saxatilis* – Va. Higher separation was seen for samples of *R. fallax* – Vo, *R. fallax* – VK and *R. pumila*, which had higher flavonoid content. *R. orbiculata* is quite different from the other species and has low content of biologically active compounds. The eigenvector matrix with the loading of each variable in each principal component is presented in Table 4.

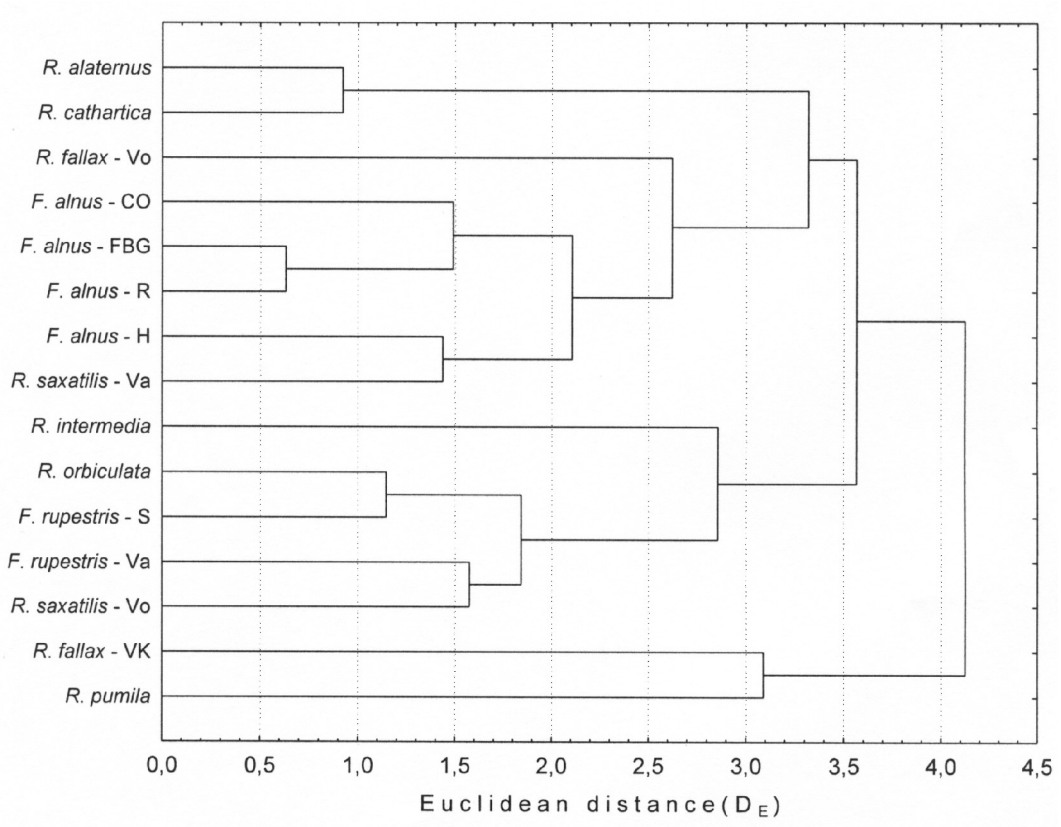

Fig. 2. UPGMA dendrogram of studied Croatian *Rhamnus* and *Frangula* species. For abbreviations see Table 1.

Generally, UPGMA confirmed the PCA results (Fig. 2). The most dissimilar samples were *R. fallax* – VK and *R. pumila*, which form one cluster at D_E 3.09. Those two samples were connected to the other samples at D_E 4.12 distance. The other samples are separated into two large groups connected at D_E 3.57. The most similar samples were *F. alnus* – FBG and *F. alnus* – R (DE 0.53).

This paper is the first report of a quantitative analysis of biologically active compounds (glucofrangulins, flavonoids, phenolic acids, total polyphenols, nontannic polyphenols and tannins) in the species *Frangula rupestris*, *Rhamnus intermedia*, *R. orbiculata* and *R. pumila*. The results show that only *R. fallax* and *F. alnus* contain high amounts of glucofrangulins and could be used as laxatives. *R. pumila* had the highest content of total polyphenols and flavonoids, while *R. intermedia* had the highest content of phenolic acids. These two species could be of interest for the study of other biologically active compounds and, for example, for their antioxidant and antimicrobial activity. Our findings on the content of biologically active compounds did not separate the examined species into the two currently known genera, *Rhamnus* and *Frangula*.

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REFERENCES

Domac R. 2002. *Flora Hrvatske*. Školska knjiga, Zagreb.

Erhardt W, Götz E, Bödeker N, and Seybold S. 2002. *Zander – Handwörterbuch der Pflanzennamen.* 17. Aufl., Eugen Ulmer GmbH und Co., Stuttgart.

European Pharmacopoeia. 2007. 6th ed. Loss on drying, vol. 1, 53; *Polyphenols*, vol. 1, 255; *Glucofrangulins*, vol. 2, 1950; *Phenolic acids*, vol. 2, 2355; *Flavonoids*, vol. 2, 2448. Council of Europe, Strasbourg Cedex.

Hiller K, and Melzig MF. 2003. *Lexikon der Arzneipflanzen und Drogen*, band 2, 220–222. Elsevier GmbH, Spectrum Akademischer Verlag, Heidelberg.

Locatelli M, Tammaro F, Menghini L, Carlucci G, Epifano F, and Genovese S. 2009. Anthraquinone profile and chemical fingerprint of *Rhamnus saxatilis* L. from Italy. *Phytochemistry Letters* 2: 223–226.

MILLER JN, and MILLER JC. 2000. *Statistics and Chemometrics for Analytical Chemistry*. Pearson Education Limited, Essex.

NEWALL CA, ANDERSON LA, and PHILLIPSON JD. 1996. *Herbal Medicines: A Guide for Health-Care Professionals*. The Pharmaceutical Press, London.

SAJC L, KOVAČEVIĆ N, GRUBIŠIĆ D, and VUNJAK-NOVAKOVIĆ G. 1999. *Frangula* Species: *In Vitro* Culture and the Production of Anthraquinones. In: Bajaj YPS [ed.], *Biotechnology in Agriculture and Forestry 43 – Medicinal and Aromatic Plants XI*, 157–176. Springer-Verlag, Berlin, Heidelberg, New York.

TUTIN TG. 1978a. *Frangula* Mill. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, and Webb DA [eds.], *Flora Europaea*, vol. 5, 245. Cambridge Univ. Press, Cambridge.

TUTIN TG. 1978b. *Rhamnus* L. In: Tutin TG, Heywood VH, Burges NA, Moore DM, Valentine DH, Walters SM, and Webb DA [eds.], *Flora Europaea*, vol. 5, 244–245. Cambridge Univ. Press, Cambridge.

WICHTL M. 1994. *Herbal Drugs and Phytopharmaceuticals*. Medpharm Scientific Publishers, Stuttgart.

WIELGORSKAYA T. 1995. *Dictionary of Generic Names of Seed Plants*. Columbia Univ. Press, New York.