PERGAMON

Phytochemistry 50 (1999) 1175–1180

PHYTOCHEMISTRY

Chemotaxonomical investigation in the genus *Viburnum*

Annelise Lobstein*, Gisèle Haan-Archipoff, Jürgen Englert, Jean-Georges Kuhry, Robert Anton

Department of Pharmacognosy, Faculty of Pharmacy of Strasbourg, University Louis Pasteur, B.P. 24, 67401 Illkirch Cedex, France

Received 29 July 1998

Abstract

A chemical examination of the leaves and branchlets of *Viburnum* sp. (Adoxaceae) has been carried out in order to find chemical clues of possible taxonomic value. Precise quantitative data of the amentoflavone content were obtained for about sixty representative taxa of the genus. A great heterogeneity of distribution of this biflavone was observed between the sections. The highest average concentration (more than 0.5%) was found in the section Lantana.

Keywords: *Viburnum*; Adoxaceae; Amentoflavone; Biflavonoids; Chemotaxonomy

1. Introduction

The systematic classification of the genus *Viburnum* has led to many controversial arguments. Phylogenetic studies of Dipsacales, based both on morphological and molecular characters imply that *Viburnum* together with *Sambucus* should no longer be related to the traditional Caprifoliaceae (Hutchinson, 1969) but to Viburnaceae (Dahlgren, 1980) and more recently to Adoxaceae (Judd, Sanders, & Donoghue, 1994; Donoghue, 1995). *Viburnum* was subdivided first in seven, then in nine and now in up to eleven distinct sections according to morphological characters and geographical origins (Nicholson, 1992). Moreover, phenetical and cladistical analyses concerning a collection of 190 individual samples, confirmed the extreme diversity of the genus (Malecot, 1997).

The taxonomical determination of *Viburnum* remains difficult, not only because of its large distribution in all the northern hemisphere, but also because of the existence of numerous wild and horticultural hybridations (Hoch, 1995). Several phytochemical investigations (Thomas, Daniel, & Sabnis, 1988; Glasby, 1991; Plouvier, 1992) have shown that *Viburnum* biosynthezises diterpenoid, iridoid, coumarin and flavonoid glycosides and biflavonoid. The distribution of the latter polyphenolic compounds proved to be useful in recognizing taxonomic discontinuities, in several instances in gymnosperms; on the contrary, biflavonoid production in angiosperms is limited and also recognized as rather than a primitive character (Geiger, 1994). Our purpose in this work was to develop a chemotaxonomical investigation in the genus *Viburnum*, with the help of this potential marker.

This genus comprises more than 230 species distributed from South America (Peru) to South-East Asia (Philippines, Malaysia), the majority of them being endemic (Eglof, 1962). This is the reason why exhaustive phytochemical studies are virtually impossible. However, using some plant material available in France and after careful authentification of their taxonomic identities, we were able to examine the biflavonoid content of about sixty different samples, representing eight of the eleven sections of *Viburnum*. With this material, we developed and validated a standard procedure based on chromatographic analyses, allowing to quantify, with a high degree of accuracy, the concentration of dimeric flavonoids present in leaves and branchlets of *Viburnum* sp.

2. Results and discussion

Our chromatographic conditions allowed not only biflavonoid identification in crude plant extracts (Fig. 1) but also their quantitative determination in a lot of samples (Table 2). In that way, they appeared to be mostly suitable for chemotaxonomical investigations, with an easy and fast chemical screening procedure.

As already mentioned, this study was not exhaustive,

* Corresponding author.

0031-9422/99/$ - see front matter
PII: S0031-9422(98)00681-5

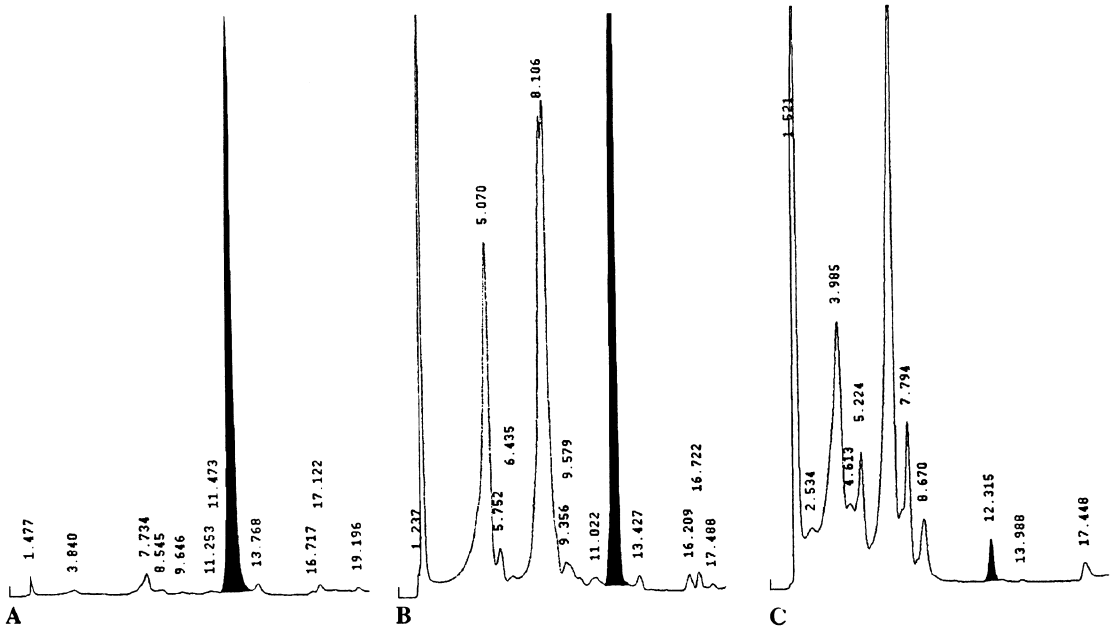

Fig. 1. Comparative HPLC chromatograms of a standard solution (A), leaf extract from *V. lantana* L. (B) and from *V. opulus* L. (C) (for chromatographic conditions, see Section 3).

because of the limited availability of the plant material. Nevertheless, with this reserve, our results clearly indicate that (i) amentoflavone is the unique biflavone synthesized in the genus *Viburnum*; (ii) its content displays important variations between the analyzed samples and (iii) its concentration is always higher in leaves than in branchlets.

Until now, the presence of amentoflavone in *Viburnum* was only mentioned in following species: *V. x burkwoodii* Burkw., folium (Bohm & Glennie, 1971), *V. carlesii* Hemsl., folium (Bohm & Glennie, 1971), *V. coriaceum* Bl., folium (Khan, Kamil, Ahmad, & Ilyas, 1983), *V. lantana* L., folium (Godeau, Pelissier, Sors, & Fouraste, 1978), *V. rhytidophyllum* Hemsl., folium and cortex (Pelissier, Godeau, & Fouraste, 1979) and *V. prunifolium* L., cortex (Hörhammer, Wagner, & Reinhardt, 1965). Except for the last one, all of them are classified in the section Lantana. With a higher accuracy, our results confirm the particular high amentoflavone content in this section.

Moreover, according to the distribution of this taxonomic marker, we are now able to propose a subdivision of the genus *Viburnum* into three distinct groups: the first, comprising the four sections Lentago (Raf.) D.C., Megalotinus (Maxim.) Rehd., Viburnum Nicholson D.C. (=Tinus (Borkh.) Maxim.=Tinus (Miller) C.B. Clarke) and Solenotinus D.C. (=Thyrsoma (Rafin) Rehder) with a particular low amentoflavone average content (0.05 to 0.15 mg/g in the leaves and 0.01 to 0.04 mg/g in the branchlets); the second, including three other sections Opulus D.C., Pseudopulus Dipp and Odontotinus Rehd., with only slight amounts (0.42 to 0.98 mg/g in the leaves and 0.02 to 0.1 mg/g in the branchlets) and the third, limited to the single section Lantana Spach. which possesses the highest concentrations in the leaves as well as in the branchlets (5.46 and 0.23 mg/g, respectively).

The relative heterogeneity of the biflavone average content within some sections (Table 2) and the corresponding high standard deviation, could be explained for instance by heterogeneities in sample's age or by horticultural crossings. This could also raise questions about the present classification system of some species, mainly based on morphological characters and/or geographical origins (Nicholson, 1992). This could particularly be the case for *V. betulifolium* Batal and *V. bracteatum* Rehder (samples No. 24 and 25, respectively) classified today in the section Odontotinus, or for *V. buddleifolium* C.H. Wright and *V. glomeratum* Maxim. (samples No. 2 and 9, respectively) now classified in the section Lantana.

The knowledge of the biochemistry of biflavonoids (i.e. their natural occurrence within the plant, their biosynthesis and factors controlling their formation, accumulation and turn-over) is so far very limited. Nevertheless, the research of possible correlations between amentoflavone content and some anatomical leaf characters appears to be useful to explain the biosynthetic potentiality of some particular *Viburnum* species. It could be also justified in order to confirm or to invalidate the present subdivisions of the genus. The extension of our chemical screening procedure to other genera could be even well advised to clarify the taxonomical relationships

Table 1
List of the examinated plants (botanical classification according to Nicholson (1992))

Sample No.	Section, genus (V.) taxon (' '), species, variety (v.), hybride (X)	Crossing	Origin
Section Lantana Spach. (=Viburnum Act. non Nicholson)			
1	*V. 'Anne Russel'*	*V. burkwoodii* × *V. carlesii*	horticulture
2	*V. buddleifolium* C.H. Wright		Asia
3	*V. burejaeticum* Regel and Herd.		Asia
4	*V. carlesii* Hemsl.		Asia
5	*V. carlesii v. bitchiuense* (Makino) Nakai		Asia
6	*V. carlesii 'Aurora'*		horticulture
7	*V. 'Chesapeake'*	*V. 'Cayuga'* × *V. utile*	horticulture
8	*V. cotinifolium* D. Don.		Asia
9	*V. glomeratum* Maxim.		Asia
10	*V. lantana* L.		Europe
11	*V. lantana 'Aureum'*		horticulture
12	*V. 'Pragense'* (Vik.)	*V. rhytidophyllum* × *V. utile*	horticulture
13	*V. rhytidophyllum* Hemsl.		Asia
14	*V. utile* Hemsl.		Asia
15	*V. X burkwoodii* Burkw and Skip. ex Anon.	*V. utile* × *V. carlesii*	horticulture
16	*V. X burkwoodii 'Chenault' Chenault*		horticulture
17	*V. X carlcephalum* Burkw. and Ship. ex A. V. Pike	*V. carlesii* × *V. macrocephalum* f. keteleeri	horticulture
18	*V. X rhytidophylloides* Suring	*V. lantana* × *V. rhytidophyllum*	horticulture
Section Lentago (Raf.) D.C.			
19	*V. lentago* L.		North America
20	*V. nudum* L.		North America
21	*V. nudum 'Pink Beauty'*		horticulture
22	*V. prunifolium* L.		North America
Section Megalotinus (Maxim.) Rehd.			
23	*V. cylindricum* Buch.- Ham. ex D. Don		Asia
Section Odontotinus Rehd.			
24	*V. betulifolium* Batal		Asia
25	*V. bracteatum* Rehder		North America
26	*V. dentatum* L.		North America
27	*V. orientale* Pall.		Europe
28	*V. setigerum* Hance		Asia
Section Opulus D.C.			
29	*V. opulus* L.		Europe
30	*V. opulus v. sargentii* (Kœhne) Takeda		Asia
31	*V. opulus v. sargentii 'Onondaga'*		horticulture
32	*V. opulus 'Aureum'*		horticulture
33	*V. opulus 'Compactum'*		horticulture
34	*V. opulus 'Roseum'*		horticulture
35	*V. opulus 'Xanthocarpum'*		horticulture
Section Pseudopulus Dipp.			
36	*V. plicatum v. plicatum* (Thumb.)		horticulture
37	*V. plicatum v. plicatum 'Rotundifolium'*		horticulture
38	*V. plicatum v. tomentosum 'Lanarth'*		horticulture
39	*V. plicatum v. tomentosum 'Mariesi'*		horticulture
40	*V. plicatum v. tomentosum 'Rowallane'*		horticulture
41	*V. plicatum v. tomentosum 'St. Keverne'*		horticulture
Section Solenotinus D.C. (=Thyrsoma (Rafin) Rehder)			
42	*V. farreri* W. T. Stearn		Asia
43	*V. farreri 'Candidissimum'*		horticulture
44	*V. farreri 'Nanum'* Cl Elliot		horticulture
45	*V. grandiflorum* Wallich ex D.C.		Asia
46	*V. henryi* Hemsl.		Asia

(continued on next page)

Table 1 *(continued)*

Sample No.	Section, genus (V.) taxon (' '), species, variety (v.), hybride (X)	Crossing	Origin
47	*V. odoratissimum* Ker-Gawler		Asia
48	*V. sieboldii* Miq.		Asia
49	*V. sieboldii 'Seneca'*	*V. sieboldii* × *V. sieboldii*	horticulture
50	*V. suspensum* Lindley		Asia
51	*V. X bodnantense* Aberconway	*V. farreri* × *V. grandiflorum*	horticulture
52	*V. X hillieri* Stearn	*V. erubescens* × *V. henryi*	horticulture
53	*V. X hillieri 'Winton'* Hillier		horticulture
Section Viburnum Nicholson D.C (=Tinus (Borkh.) Maxim.)			
54	*V. cinnamomifolium* Rehder		Asia
55	*V. davidii* Franchet		Asia
56	*V. propinquum* Hemsl.		Asia
57	*V. tinus* L.		Europe

between *Viburnum*, *Sambucus* and *Adoxa*, until now based on morphological characters rather than on chemical criteria.

3. Experimental

3.1. Plant material

The used plants were from botanical gardens of France (Mulhouse, Nancy, Strasbourg) or from private French collection (St. Romain) where voucher specimens were kept. They were all collected in Spring, between 1992 and 1995. The origin country and the nature of horticultural crossings are listed in Table 1. Their complete botanical descriptions and accession number was checked by the authors.

3.2. Sample preparation

Fresh plant material (leaves or branchlets) was exhaustively extracted with boiling 95% EtOH (1:10 w/v). Comparison between initial and repeated extracts of the same material revealed no significant differences in the biflavonoid content after 15 min of hot extraction. This was confirmed by determination of the recovery level, calculated from the added and found quantities (method accuracy). After filtration, the volume of the alcoholic extract was adjusted to 20 ml. An aliquot was filtered through a 0.45 μm filter and then 20 μl of this aliquot was submitted to HPLC analysis.

3.3. Analysis of the biflavonoid content

HPLC analysis was carried out with a multigradient pump (Varian 2510, CA) connected to a photodiode-array detector (Varian Polychrom 9065, CA) operating at 330 nm. Chromatographic assays were performed on a Superspher 100 RP-8, 5 μM, 125–4 mm column (Merck, Darmstadt). The methanol/0.5% phosphoric acid elution gradient was established so as to elute all dimeric flavonoids between 10 to 25 min, with a flow rate of 1 ml/min. Photodiode array detection (190–375 nm) and co-injection with commercial standard (Carl Roth, Ref. 5255.1) allowed precise identification of the 12 min eluted peak as amentoflavone to be achieved (Fig. 1).

3.4. Validation of the chromatographic conditions (Lobstein, 1995)

The linearity of the HPLC method was checked in the range 0.02–0.1 mg/ml. Data for least-squares regression analysis of the calibration graph were $y = 17991 + 13992x$ ($R = 0.9985$; RSD = 1.72%) where y = peak area, x = concentration in mg/ml, R = correlation coefficient and RSD = relative standard deviation. At a 2:1 signal-to-noise ratio, the detection of amentoflavone was 0.5 μg/ml. The reproducibility of the method was established from six assays of the same extract (RSD = 1.82%). The reproducibility of amentoflavone standard preparation was tested by assaying ten solutions at a concentration of 0.1 mg/ml (RSD = 0.36%). Six different extracts from the same plant material were analysed for the reproducibility of the extraction procedure (RSD = 0.97%). The quantification limit, determined with ten successive injections, was 10 μg amentoflavone per ml crude extract (RSD = 2.85%).

3.5. Amentoflavone quantification

Quantitative determination of amentoflavone was obtained with external calibration. The results are the average of three separate determinations. They were expressed in percent with reference to the weight of the dry plant (Table 2).

Table 2
Average amentoflavone content in the different plant materials

Sample number	Plant examined	*Amentoflavone content* (mg/g, dry wt) in leaves	in branchlets
Section Lantana Spach		5.49±2.47	0.23±0.37
1	*V. 'Anne Russel'*	2.43	0.04
2	*V. buddleifolium* C.H. Wright	1.72	[a]
3	*V. burejaeticum* Regel and Herd.	9.91	0.20
4	*V. carlesii* Hemsl.	4.20	–
5	*V. carlesii v. bitchiuense* (Makino) Nakai	2.40	[a]
6	*V. carlesii 'Aurora'*	3.53	0.06
7	*V. 'Chesapeake'*	5.40	0.27
8	*V. cotinifolium* D. Don.	3.31	–
9	*V. glomeratum* Maxim.	10.32	0.10
10	*V. lantana* L.	5.78	0.26
11	*V. lantana 'Aureum'*	5.09	0.93
12	*V. 'Pragense'* (Vik.)	7.12	0.19
13	*V. rhytidophyllum* Hemsl.	8.85	1.50
14	*V. utile* Hemsl.	7.35	0.15
15	*V. X burkwoodii* Burkw and Skip. ex Anon.	4.20	0.26
16	*V. X burkwoodii 'Chenault' Chenault*	6.51	[a]
17	*V. X carlcephalum* Burkw. and Ship. ex A. V. Pike	4.02	0.03
18	*V. X rhytidophylloides* Suring	6.63	0.15
Section Lentago (Raf.) D.C.		0.05±0.09	0.04±0.05
19	*V. lentago* L.	–	0.05
20	*V. nudum* L.	–	–
21	*V. nudum 'Pink Beauty'*	0.20	[a]
22	*V. prunifolium* L.	[a]	0.12
Section Megalotinus (Maxim.) Rehd.		0.06	–
23	*V. cylindricum* Buch.– Ham. ex D. Don	0.06	–
Section Odontotinus Rehd.		0.98±0.87	0.02±0.02
24	*V. betulifolium* Batal	1.51	–
25	*V. bracteatum* Rehder	2.12	0.02
26	*V. dentatum* L.	0.30	0.04
27	*V. orientale* Pall.	nd	0.05
28	*V. setigerum* Hance	[a]	
Section Opulus D.C.		0.42±0.28	0.10±0.14
29	*V. opulus* L.	0.72	–
30	*V. opulus v. sargentii* (Kœhne) Takeda	0.09	0.09
31	*V. opulus v. sargentii 'Onondaga'*	0.07	0.44
32	*V. opulus 'Aureum'*	0.88	0.05
33	*V. opulus 'Compactum'*	0.32	0.09
34	*V. opulus 'Roseum'*	0.31	0.02
35	*V. opulus 'Xanthocarpum'*	0.53	0.04
Section Pseudopulus Dipp.		0.90±0.68	–
36	*V. plicatum v. plicatum* (Thumb.)	0.30	–
37	*V. plicatum v. plicatum 'Rotundifolium'*	1.62	–
38	*V. plicatum v. tomentosum 'Lanarth'*	2.01	[a]
39	*V. plicatum v. tomentosum 'Mariesi'*	0.44	[a]
40	*V. plicatum v. tomentosum 'Rowallane'*	0.80	–
41	*V. plicatum v. tomentosum 'St. Keverne'*	0.25	–
Section Solenotinus D.C.		0.15±0.18	0.03±0.04
42	*V. farreri* W. T. Stearn	0.15	0.04
43	*V. farreri 'Candidissimum'*	0.10	[a]
44	*V. farreri 'Nanum'* Cl Elliot	0.58	[a]
45	*V. grandiflorum* Wallich ex D.C.	0.20	0.02
46	*V. henryi* Hemsl.	[a]	–

(continued on next page)

Table 2 *(continued)*

Sample number	Plant examined	*Amentoflavone content* (mg/g, dry wt) in leaves	in branchlets
47	*V. odoratissimum* Ker-Gawler	–	0.02
48	*V. sieboldii* Miq.	0.20	0.05
49	*V. sieboldii 'Seneca'*	0.41	–
50	*V. suspensum* Lindley	[a]	0.04
51	*V. X bodnantense* Aberconway	[a]	–
52	*V. X hillieri* Stearn	0.10	0.10
53	*V. X hillieri 'Winton'* Hillier	[a]	0.14
Section Viburnum Nicholson D.C		0.08 ± 0.01	–
54	*V. cinnamomifolium* Rehder	0.08	–
55	*V. davidii* Franchet	0.10	0.01
56	*V. propinquum* Hemsl.	0.07	[a]
57	*V. tinus* L.	0.07	–

nd: Not determined.
–: Not detected (<0.005%).
[a]: Trace amount (<0.01%).

Acknowledgements

We are grateful to the nursery gardener M. Laurent and to V. Turlot, P. Valck and A. Zeiler for their kind supply of living samples. We would also like to sincerely thank V. Kremer, E. Wund and S. Le Blévec for their technical assistance.

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