Pergamon

0031-9422(95)00573-0

Phytochemistry, Vol. 41, No. 2, pp. 503–512, 1996

0031-9422/96 $15.00 + 0.00

FLAVONOID VARIATION IN EURASIAN *SEDUM* AND *SEMPERVIVUM*

JAN F. STEVENS,*† HENK 't HART,‡§ ELIZABETH T. ELEMA* and ANNABEL BOLCK¶

*Department of Pharmacognosy, University of Groningen, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands; ‡Department of Plant Ecology and Evolutionary Biology, University of Utrecht, Padualaan 8, 3584 CH Utrecht, The Netherlands; ¶Department of Analytical Chemistry, University of Groningen, Ant. Deusinglaan 2, 9713 AW Groningen, The Netherlands

(*Received* 9 *February* 1995)

Key Word Index—*Sedum*; *Sempervivum*: *Jovibarba*; Crassulaceae; flavonoids; herbacetin; sexangularetin; multivariate data analysis; chemotaxonomy; evolution.

Abstract—Flavonoids from vegetative parts of 29 species of Eurasian *Sedum*, *Sedum meyeri-johannis* from central East Africa, 34 species of *Sempervivum*, and *Jovibarba heuffelii* have been identified after acid hydrolysis. Ten flavonoid aglycones were detected, i.e. kaempferol, herbacetin, sexangularetin, quercetin, gossypetin, corniculatusin, isorhamnetin, limocitrin, myricetin, and possibly hibiscetin. Quantitative flavonoid data were obtained by gas chromatography after trimethylsilylation and were studied with multivariate data analysis methods. Principal components analysis of the whole data set distinguishes *Sedum* and *Sempervivum* as separate groups. Flavonoid variation in *Sempervivum* is minimal (kaempferol is the principal flavonol of all species), which reflects the morphological uniformity of the genus and similarity in ecological preference of the species. By contrast, Eurasian *Sedum* contains a much wider range of flavonols and shows a high degree of parallel evolution (notably 8-hydroxylation and 8-*O*-methylation), which seems to mirror the enormous morphological and cytological variation present in this taxon. The recognition of groups in *Sedum* by principal components analysis of flavonoid data partially supports the infrageneric classification based on biosystematic data.

INTRODUCTION

In Europe and adjacent regions of the Near East, northern Africa and the Macaronesian Islands (Eurasia) *Sedum* comprises about 100 species, the majority of which occur predominantly in the Mediterranean climatic zone. *Sedum* is often regarded as the core of the Crassulaceae containing the least advanced as well as some highly derived taxa [1]. *Sempervivum* contains about 50 species which are mainly confined to the mountains of southern Europe, Antaolia and the Caucasus. It is closely related to *Sedum* and thought to have evolved from Eurasian *Sedum* [1, 2]. Species of both genera are succulent herbs and generally occur in dry and exposed habitats.

Species of *Sedum* are known to contain alkaloids, flavonoids, condensed tannins, and cyanogenic compounds [3, 4]. Flavonoids have been reported for about one tenth of the Eurasian *Sedum* species. Of these only *S. acre*, *S. album*, *S. sexangulare*, and *S. sediforme* have been examined in detail. Kaempferol, quercetin, and myricetin glycosides are common as well as their 8-hydroxy and 8-methoxy derivatives [3, 4]. Isorhamnetin and limocitrin glycosides have been isolated from *S. acre* [5–7]. Sedoflorigenin and its monoglucoside sedoflorin, originally isolated from *S. acre* by Nordal in 1947, later appeared to be identical with limocitrin and probably limocitrin-3-glucoside, respectively [8]. *Sedum album* contains glycosides of herbacetin, quercetin, quercetin-3-methyl ether, gossypetin, and isorhamnetin [9]. Glycosides of sexangularetin, corniculatusin, limocitrin, myricetin, and the 8-methoxy derivative of the latter have been reported from *S. sexangulare* [10]. Quercitrin and myricitrin are the principal flavonol glycosides of *Sedum* series *Rupestria* [11]. Frigot [12] reported a glycoside of eriodyctiol and myricetin from *S. sediforme* (= *S. altissimum* Poiret) of this series. Flavan-3-gallates and glycosides of limocitrin, kaempferol, quercetin, and myricetin have been isolated from flowers of *S. sediforme* [13]. Recently, Stevens *et al.* [14] isolated myricetin 3-*O*-arabinofuranoside from leafy shoots of *S. montanum* ssp. *orientale* of *S.* ser. *Rupestria*. Denton and Kerwin [15] found laricitrin, a kaempferol 3,7-diglycoside, and an unidentified flavone in species of the American *S.* sect. *Gormania*. Laricitrin was also found in the Asian *S. crassipes* of *S.* subgenus *Rhodiola* (≡ *Rhodiola*) along with kaempferol 3,7-di-*O*-glucoside [16]. Species of the American section *Ternata* of *Sedum* contain glycosides of kaempferol, quercetin, apigenin, and luteolin [17]. Flavonoid records are available for six species of *Sempervivum*. Gumenyuk *et al.* [18–20] identified glycosides of

†Present address: Oregon State University, Oregon, U.S.A.
§Author to whom correspondence should be addressed.

kaempferol (astragalin), quercetin, isorhamnetin and scutellarein in *Sempervivum ruthenicum*. Combier and Jay [21] found kaempferol in acid hydrolysates of *S. tectorum*, *S. montanum*, *S. arachnoideum*, *S. globiferum*, and *S. arenarium*.

Our previous chemotaxonomic studies of *Sedum* and related genera were focused on alkaloids and epicuticular wax composition [22–24]. In the present paper we report the results of a flavonoid survey of 29 species of *Sedum* originating from Eurasia and one species from central East Africa, 34 species of *Sempervivum*, and one species of *Jovibarba* (≡ *Sempervivum* section *Jovibarba* [25]). The investigations were aimed at determining the taxonomic and evolutionary significance of the flavonoid variation at the aglycone level within and between these taxa.

RESULTS

Ten flavonoid aglycones were detected in the vegetative parts of Eurasian species of *Sedum*, *Sempervivum*, and *Jovibarba* after acid hydrolysis (Tables 1 and 2). The flavonoids were identified by a combination of chromatographic and spectroscopic comparison with authentic samples, i.e. TLC, GC/GC–EIMS, and HPLC with diode-array detection (Table 2). Limocitrin and sexangularetin were not available as standards, and were identified in phenolic extracts of *Sedum acre* and *S. sexangulare* by comparison with spectra in Ref. [26], GC–MS, and conversion to gossypetin and herbacetin, respectively, with pyridinium chloride [27].

For the purpose of multivariate analysis of quantitative flavonoid data (see Discussion), a GC method was developed to quantify flavonoid aglycones in plant extracts after trimethylsilylation, using the internal standard calibration method. Fisetin (3,7,3′,4′-tetrahydroxyflavone) was chosen as the internal standard, because it proved to be absent from species of *Sedum* and *Sempervivum* in preliminary experiments, it does not interfere with other flavonols in the gas chromatogram, and it can be readily purchased from commercial source. The quantitative data of Table 1 refer to flavonoid contents on fresh weight basis. As the moisture content of the species amounts to *ca* 85% for *Sedum* species and to more than 90% for *Sempervivum*, the flavonoid contents listed in Table 1 will be approximately ten times higher when calculated on dry weight basis.

Conversion of the aglycones to their trimethylsilyl or methyl derivatives is generally considered to be necessary prior to GC analysis [28–30], although Schmidt *et al.* [31] reported on the GC analysis of flavonoid aglycones lacking catechol functions on the B-ring without derivatization. We tested a number of silylating agents at different reaction times and temperatures, i.e. HMDS/TMCS, BSTFA containing 1%TMCS, and BSA. Silylation of the flavonoids with BSA at 70° overnight proved to be satisfactory in our study. In two cases flavonoid trimethylsilyl ethers could not be completely separated with GC, i.e. herbacetin/sexangularetin and quercetin/gossypetin. However, GC–MS allowed identification and even quantification of these pairs on the basis of prominent $[M-15]^+$ ions in their electron impact spectra [30] (Table 2).

Sedum urvillei (accession no. 31685) contained a hexahydroxyflavone monomethyl ether (an abundant $[M-15]^+$ ion at *m/z* 677 was observed) after acid hydrolysis, which differed from corniculatusin in GC retention time and R_f-value. The phenolic extract of this plant yielded gossypetin on demethylation with pyridinium chloride. [27]. The principal flavonoid aglycone of this species was thus characterized as a monomethyl ether of gossypetin. Isolation of the compound was not possible due to the limited availability of the plant material.

Two of the three plants of *S. album* contained small amounts of a hepthydroxyflavone (an abundant $[M-15]^+$ ion at *m/z* 823 was observed) in addition to herbacetin, gossypetin, and myricetin. As an 8-hydroxylation enzyme system is apparently present in these plants, the heptahydroxyflavone was tentatively identified as 8-hydroxymyricetin (= hibiscetin). Hibiscetin has not been reported from *Sedum* previously, though its 8-*O*-methyl ether occurs in *S. sexangulare* [10].

The report of an eriodictyol glycoside from *Sedum sediforme* [12] prompted us to examine the species of *S.* ser. *Rupestria* for the presence of flavanones with TLC. Hydrolysed extracts were chromatographed on silica gel with toluene–HCOOEt–HCOOH (5:4:1) against eriodictyol, hesperetin, and naringenin. Developed plates were sprayed with sodium borohydride/aluminium chloride [32], and subsequently fumed with HCl vapour to intensify the colour of the spots. Eriodictyol was found absent from our diploid plant of *S. sediforme* as well as from the other species of *S.* ser. *Rupestria*. Only *S. montanum* ssp. *orientale* and *S. rupestra* ssp. *erectum* showed faint traces of an unidentified flavanone with the specific spray reagent. With GC–MS flavanones could neither be detected in any of the six accessions of *S.* series *Rupestria*.

Special attention was also paid to the report of scutellarein from *Sempervivum ruthenicum* [19], but we were not able to confirm the presence of this 6-hydroxyflavone in *S. ruthenicum* with GC–MS using the single ion monitoring option in the full scan mode. The compound was also found absent from this species by co-TLC with an authentic sample of scutellarein.

DISCUSSION

Of the major flavonoid classes, only the proanthocyanidins [33] and the flavonols are widely distributed in Eurasian *Sedum* and *Sempervivum*. Although we studied the flavonol variation at the aglycone level, our results are in good agreement with literature reports of flavonoid glycosides from species of *Sedum*, *Sempervivum*, and *Jovibarba* (*cf.* Introduction) which indicates that Table 1 gives a good impression of the flavonoid chemistry of the taxa included in the survey. However, several *Sedum* species show considerable infraspecific variation in their flavonoids. Extreme examples are *S. ursi* and *S. urvillei* of *S.* ser. *Alpestria*. Consequently, in *Sedum* the significance of flavonoid variation for assessing

taxonomic and evolutionary relationships loses importance, in particular between species of *S.* ser. *Alpestria.*

Sedum is characterized by the widespread occurrence of 3′-*O*-methylated as well as 8-hydroxy and 8-*O*-methylated flavonols. These derivatives might be considered as specialized compounds relative to kaempferol, quercetin, and myricetin. However, no clear phylogenetic information (that is, consistent with the phylogeny and infrageneric classification according to van Ham [34] and 't Hart [35]) could be inferred from the flavonoid data by cladistic approaches [36–38]. Instead, we used phenetic methods (cluster analysis and principal components analysis) to assess the systematic significance of both the original and transformed flavonoid data (see Experimental), however, did not result in groupings consistent with previous classifications [35] as closely related taxa were scattered through the phenogram.

With principal components analysis (PCA) of the whole data set after transformation (see Experimental) *Sedum* and *Sempervivum* are distinguished as separate groups (Fig. 1). The separation of the two genera is largely due to the overall dominant presence of kaempferol in *Sempervivum* as indicated by the corresponding loadings plot (not shown).

Sempervivum contains a simple array of flavonols, i.e. kaempferol, herbacetin, quercetin, and myricetin. It is also very uniform in its flavonoid composition with kaempferol being the major flavonol. However, the distinct *Sempervivum* cluster seems to consist of two groups, which, on close examination of the data set, differ only in the contents of the minor flavonoids. Here the PCA method employed primarily causes the apparently ideal subdivision, and thus probably lacks any taxonomic significance. On the whole, the flavonoid chemistry of the species *Sempervivum* nicely reflects their similarity in ecological preference as well as the morphological uniformity of the genus. *Sempervivum* species all have succulent, monocarpic rosettes and terminal, cymose inflorescences with 8- to 16-merous flowers. They occur predominantly in rocky places at high altitudes [39].

The three plants of *Jovibarba heuffelii* were placed in a cluster separate from *Sempervivum*, but the separation can be associated with the method used as well, and hence must be regarded as being artificial. The *Jovibarba* plants contain only very small amounts of kaempferol and myricetin (Table 1), and the pattern is similar to that of *Sempervivum* (*cf* e.g. *Sempervivum calcareum* and *Jovibarba heuffelii* in Table 1). *Jovibarba* resembles *Sempervivum* in habit, but differs by its 6-merous flowers with yellowish, fringed, erect petals [39]. The systematic position of *Jovibarba* has been much disputed, and it is often included in *Sempervivum* as *S.* section *Jovibarba* DC. [25, 40, 41]. Our results are in favour of the latter classification, namely the inclusion of *Jovibarba* in *Sempervivum.*

In contrast to *Sempervivum*, the flavonoid compostion of the *Sedum* species is much more diverse. *Sedum* forms a large cluster in the centre of the scatter plot that is well separated from the *Sempervivum* cluster with the exception of *S. brissemoreti* (included in the *Sempervivum* cluster) and *S. tuberosum* (almost included in the *Jovibarba* cluster). Whereas the similarity between *S. brissemoreti* and *Sempervivum* must be due to a parallel development, the resemblance between *S. tuberosum* and *Jovibarba heuffelii* is merely due to their flavonoid poverty.

PCA of the flavonoid data of *Sedum* was performed to distinguish infrageneric groups (Fig. 2). Four groups were separated from the bulk of the species, i.e. *S.* ser. *Rupestria*, a part of *S.* ser. *Alpestria*, *S. album*, and *S. brevifolium.* The latter two species are characterized by the presence of (large amounts) of herbacetin and gossypetin. *Sedum album* has been reported to contain herbacetin, quercetin, gossypetin, and isorhamnetin glycosides [9]. *Sedum brevifolium* has not been investigated previously: it has been classified in *S.* series *Pedicellata* [35], and its distinct flavonoid pattern might be useful to study relationships within this group. Unfortunately, the other species of *S.* ser. *Pedicellata* were not available at the time of the present survey.

The five species of *Sedum* ser. *Rupestria* included in the present survey are characterized by a combination of quercetin and myricetin in addition to minor amounts of limocitrin. In previous phytochemical studies of *Sedum*, *S.* ser. *Rupestria* was found to contain a unique pattern of epicuticular triterpenes [24, 42]. Chemical characters evidently emphasize the distinctiveness of this taxon. Morphologically, the species of *S.* series *Rupestria* differ from the other Eurasian Sedoideae by a unique combination of characters: they have creeping and rooting non-flowering shoots with densely imbricate, linear to oblong, mucronate leaves, long flowering shoots with a dense cymose or corymbose, terminal inflorescence, and 6–9 (– 12)-merous flowers with acuminate or mucronate sepals. The seven species of this group are closely related and can be hybridized in all combinations [43, 44].

The flavonoid patterns of the species of *S.* ser. *Alpestria* show a dichotomy. Six out of the 12 species of this series investigated contained sexangularetin (*S. tuberiferum* was the only species of the series not sampled). These six species formed a distinct cluster, while the remaining species of this series were placed in the centre of the scatter plot along with other taxa of *Sedum.* Sexangularetin was isolated from *S. sexangulare* (belonging to *S.* ser. *Alpestria*) for the first time [45]. Because this rare flavonol is restricted to *S.* ser. *Alpestria* within Eurasian *Sedum*, it could be an important chemical character. However, the presence or absence of sexangularetin in this series could not be correlated either with geographical origin or morphological and cytological features [46]. Moreover, the variable flavonoid patterns contrast with the homogeneous alkaloid profiles of the species of *S.* ser. *Alpestria* [23]. In view of the infraspecific flavonoid variation in *S. ursi*, the species of *S.* ser. *Alpestria* should be more extensively sampled to obtain an adequate conception of the distribution of sexangularetin in this series.

The remaining 18 Eurasian *Sedum* species form a cluster near the centre of the scatter plot (Fig. 2). It contains *S. acre*, *S. meyeri-johannis*, all species of *S.* series *Macaronesia*, half of the species of *S.* ser. *Alpestria*, *S. anglicum*, *S. melananthera*, and *S. lydium.* Kaempferol, quercetin, and limocitrin are commonly encountered in these taxa,

Table 1. Distribution of flavonol aglycones in Eurasian *Sedum* and *Sempervivum*

No.	Genera, sections, series and species*	Origin and accession number	Flavonol aglycones (mg per g fr. wt)†									
			1	2	3	4	5	6	7	8	9	10
	Sedum L.											
1	*S.* sect. *Africana* Fröd.											
	S. meyeri-johannis Engler	Kenya (15515)	—		—	0.06	—	—	0.02	—	0.01	—
	S. sect. *Sedum*											
	S. series *Acria* Berger											
2	*S. acre* L.	Turkey (30869)		—		0.01	—	—	0.02	0.07	—	—
3	*S. acre* L.	France (28589)	—	—		0.05		0.01	0.10	0.03	—	—
	S. series *Macaronesia* Fröd.											
4	*S. brissemoreti* Hamet	Madeira (29000)	0.41	0.01	—	0.03	—	—	—	—	—	—
5	*S. farinosum* Lowe	Madeira (29008)	0.08		—	0.26	—		—	0.01	0.04	—
6	*S. fusiforme* Lowe	Madeira (29011)	—	—	—	0.01	—	0.01	—	0.01	—	
7	*S. nudum* Aiton	Madeira (28995)		—		—	—	—	—	0.01	—	—
	S. series *Anglica* 't Hart											
8	*S. anglicum* Huds.	Fance (21826)	0.10	0.01	—	0.02	—	—		0.01	0.03	—
	S. series *Melananthera* 't Hart											
9	*S. melanantherum* DC.	Spain (29502)	0.09	0.02	—	0.01	—		—	0.07	—	—
	S. sreies *Alpestria* Berger											
10	*S. alpestre* Vill.	Turkey (30915)	0.05		0.59	0.02		0.08	—	0.06	—	—
11	*S. alpestre* Vill.	France (31483)		—	0.06	0.01		0.04	—	0.05	—	—
12	*S. annuum* L.	Italy (31415)				0.01		—		—		—
13	*S. annuum* L.	Turkey (31240)	—	—	—	0.02	—	—	0.01	0.01	—	—
14	*S. apoleipon* 't Hart	Greece (28793)	—	0.01	—	0.13	—	0.01	—	0.04	—	—
15	*S. borissovae* Balk.	Ukraine (7784)	0.44	—	0.10	0.09	—	0.02	—	0.03	—	—
16	*S. euxinum* 't Hart & Alpinar	Turkey (31368)	0.48	—	0.13	0.03	—	—	0.01	0.01	0.01	
17	*S. grisebachii* Boiss. & Heldr.	Turkey (30986)	0.09	—	0.02	0.14	—	0.04	—	0.05	0.03	—
	S. laconicum Boiss. & Heldr.											
18	ssp. *pallidum* 't Hart & Van Ham	Israel 31101)	—	—	—	0.02	—	—		0.01	0.01	—
19	ssp. *laconicum*	Geece (31422)	—	—	—	0.05	—	—	—	0.01	—	—
20	*S. multiceps* Coss. & Dur.	cult., N Africa (26256)	—		—	0.01	—		—	0.01	0.01	—
21	*S. sexangulare* L.	cult., Netherlands (32353)	—	—	0.25	0.01	—	0.01	—		0.01	—
22	*S. sexangulare* L.	Italy (31016)	—	—	0.28	0.02	—	0.06	—	—	0.01	
23	*S. sexangulare* L.	Poland (9630)	—	—	0.29	0.02		0.05	—	—	0.01	—
24	*S. tuberosum* Coss. & Let.	Tunisia (25464)	0.01	—	—	0.01	—	—	—	—		—
25	*S. ursi* 't Hart	Turkey (31724)	0.20	0.12	0.12	0.08	—	0.01	—	0.03	0.03	—
26	*S. ursi* 't Hart	Turkey (30396)	—		—	0.01	—	—	—	0.01	—	—
27	*S. urvillei* DC	Bulgaria (28696)	—	—	—	0.04	—	—	0.01	0.01	0.01	
28	*S. urvillei* DC	Turkey (31685)	—	—	—	—	—	0.13‡	—		—	—
	S. series *Litorea* 't Hart											

29	*S. litoreum* Guss.	Israel (31106)	—	—	—	0.08	—	0.07	—	0.03	0.02	—
	S. series *Samia* 't Hart											
30	*S. samium*	Turkey (32473)	—	—	—	0.01	—	—	—	—	—	—
	S. series *Alba* Berger											
31	*S. album* L.	Spain (29329)	—	—	—	—	—	—	—	—	0.02	—
32	*S. album* L.	Bulgaria (24739)	—	0.01	—	—	0.03	—	—	—	0.03	0.01
33	*S. album* L.	Italy (26052)	—	0.01	—	0.02	0.02	—	—	—	0.03	0.01
	S. series Pedicellata 't Hart											
34	*S. brevifolium* DC.	Portugal (29572)	—	0.70	—	—	0.71	—	—	0.02	0.02	—
35	*S. brevifolium* DC.	Portugal (29595)	—	0.36	—	—	0.26	—	—	—	—	—
36	*S. brevifolium* DC.	Portugal (29532)	—	1.09	—	—	0.08	0.01	—	0.02	0.03	—
	S. series Lydia 't Hart											
37	*S. lydium* Boiss.	Turkey (30939)	—	—	—	0.02	—	—	—	—	—	—
	S. series *Rupestria* Berger											
38	*S. forsterianum* Sm.	Spain (29368)	—	—	—	0.32	—	—	—	0.02	0.53	—
	S. montanum Song. & Perr.											
39	ssp. *montanum*	France (5345)	—	—	—	0.33	—	—	—	0.01	0.60	—
40	ssp. *orientale* 't Hart	Italy (16685)	—	—	—	0.05	—	0.01	—	0.01	0.54	—
41	*S. ochroleucum* Chaix	France (8702)	—	—	—	0.13	—	0.01	—	0.02	0.11	—
42	*S. rupestre* L.											
	ssp. *erectum* 't Hart	Italy (16705)	—	—	—	0.06	—	—	—	0.02	0.41	—
43	*S. sediforme* (Jacq.) Pau	Portugal (15429)	—	—	—	0.16	—	—	—	—	0.23	—
	Sempervivum L.											
44	*S. x adenotrichum*	France (31583)	0.10	0.01	—	0.01	—	—	—	—	0.01	—
45	*S. altum* Turrill	cult., Caucasus (31524)	0.20	—	—	0.02	—	—	—	—	0.01	—
46	*S. arachnoideum* L.	France, Pyrenees (30611)	0.26	—	—	0.02	—	—	—	—	—	—
47	*S. arachnoideum* L.	France, Alps (31516)	0.15	—	—	0.01	—	—	—	—	0.01	—
48	*S. arachnoideum* L.	Switzerland (31518)	0.17	—	—	0.01	—	—	—	—	—	—
49	*S. armenum* Boiss. & Huet	Turkey (31580)	0.05	—	—	0.01	—	—	—	—	—	—
50	*S. atlanticum* Ball	Morocco (31531)	0.32	0.01	—	0.01	—	—	—	—	0.01	—
51	*S. ballsii* Wale	Greece (31533)	0.21	0.01	—	0.01	—	—	—	—	0.01	—
52	*S. borissovae* wale	cult., Caucasus (31534)	0.13	—	—	0.03	—	—	—	—	0.02	—
53	*S. calcareum* Jordan	Italy (31508)	0.02	—	—	—	—	—	—	—	—	—
54	*S. calcareum* Jordan	France (31509)	0.02	—	—	—	—	—	—	—	0.01	—
55	*S. cantabricum* Huber	Spain (31538)	0.09	0.01	—	—	—	—	—	—	—	—
56	*S. cantabricum* Huber	Spain (31539)	0.15	0.01	—	0.01	—	—	—	—	—	—
57	*S. caucasicum* Ruprecht	cult., Caucasus (31545)	0.16	—	—	0.02	—	—	—	—	0.01	—
58	*S. caucasicum* Ruprecht	Russia, Caucasus (31547)	0.24	0.01	—	0.02	—	—	—	—	0.01	—
59	*S. charadzeae* Gurgenidze	cult., Caucasus (31592)	0.03	—	—	—	—	—	—	—	—	—
60	*S. ciliosum* Craib	Greece (30617)	0.17	—	—	0.02	—	—	—	—	0.01	—
61	*S. ciliosum* Craib	Greece (31473)	0.25	0.01	—	0.02	—	—	—	—	0.01	—
62	*S. ciliosum* Craib	Bulgaria (31544)	0.23	0.01	—	0.03	—	—	—	—	0.02	—

Continued Overleaf

Table 1. (continued)

No.	Genera, sections, series and species*	Origin and accession number	Flavonol aglycones (mg per g fr. wt)†									
			1	2	3	4	5	6	7	8	9	10
63	*S. dzhvachischvili* Gurgenidze	cult., Caucasus (31548)	0.22	—		0.02	—	—	—	—	0.01	—
64	*S. erythraeum* Velen.	Bulgaria (31551)	0.05	—	—	0.01	—	—	—	—	0.01	—
65	*S. furseorum* Muirhead	Turkey (31599)	0.09	—	—	0.01	—	—	—	—	0.01	—
66	*S. grandiflorum* Haw.	Italy (31554)	0.16	0.01	—	0.03	—	—	—	—	0.03	—
67	*S. x guiseppii*	Spain (31519)	0.08	—	—	—	—	—	—	—	0.01	—
68	*S. ingwersenii* Wale	Russia, Caucasus (31549)	0.11	—	—	0.01	—	—	—	—	0.01	—
69	*S. italicum* Ricci	Italy (31493)	0.03	—	—	0.01	—	—	—	—	—	—
70	*S. kindingeri* Adamovic	cult., Macedonia (31557)	0.12		—	0.01	—	—	—	—	0.01	
71	*S. kosanini* Praeger	cult., Montenegro (31558)	0.06	—	—	0.01	—	—	—	—	—	—
72	*S. leucanthum* Pancic	cult., Bulgaria (31560)	0.14	—	—	0.01	—	—	—	—	—	—
73	*S. macedonicum* Praeger	Macedonia (31564)	0.10	—	—	0.01	—	—	—	—	—	—
74	*S. macedonicum* Praeger	cult., Macedonia (31565)	0.09	—	—	0.01	—	—	—	—	—	—
75	*S. marmoreum* Griseb.	Greece (31430)	0.13	0.01	—	0.02	—	—	—	—	0.01	—
76	*S. marmoreum* Griseb.	Bulgaria (31527)	0.05		—	0.01	—	—	—	—	—	
77	*S. montanum* L.	Spain (31567)	0.18	—	—	0.04	—	—	—	—	0.01	—
78	*S. montanum* L.	Czechoslovakia (31569)	0.14	0.02	—	0.02	—	—		—	0.03	—
79	*S. nevadense* Wale	Spain (29603)	0.04	—	—	—	—	—	—	—	—	—
80	*S. ossetiense* Wale	cult., Caucasus (31550)	0.15	—	—	0.01	—	—	—	—	0.01	—
81	*S. pumilum* Bieb.	cult., Caucasus (31577)	0.08	—		—	—	—	—	—	0.01	—
82	*S. reginae-amaliae* Halácsy	Greece (31530)	0.09	—	—	0.01	—	—	—	—	0.01	
83	*S. ruthenicum* Schnitt. & Lehmann	Bulgaria (31562)	0.15	—	—	0.01	—	—	—	—	—	—
84	*S. tectorum* L.	France (31489)	0.25	—	—	0.02	—	—		—	—	—
85	*S. tectorum* L.	Spain (31581)	0.08	—	—	—		—	—	—	0.01	—
86	*S. tectorum* L.	Spain, Courgul (31584)	0.20	0.01		0.02	—	—	—	—	0.01	—
87	*S. tectorum* L.	Italy (31586)	0.05	0.01		0.02	—	—	—	—	—	—
88	*S. thompsonianum* Wale	cult., Jugoslavia (31587)	0.15	—	—	0.03	—	—	—	—	0.01	
89	*S. transcaucasicum* Muirhead	Georgia (31588)	0.08	—	—	0.01	—	—			—	—
90	*S. zeleborii* Schott	cult., Montenegro (31591)	0.09	—	—	0.01	—	—		—	—	—
	Jovibarba Opiz.											
91	*J. heuffelii* (Schott) A. & D. Löve	Bulgaria (30612)	0.01	—		—	—	—	—	—	—	—
92	*J. heuffelii* A. & D. Löve	Bulgaria (30613)	0.01	—	—	—	—	—	—	—	0.01	
93	*J. heuffelii* A. & D. Löve	Greece (31467)	0.01	—	—	—	—	—			—	—

*Infrageneric classification of *Sedum* according to 't Hart [35].

†Key to flavonoid identities: see Table 2.

‡Unidentified monomethyl ether of gossypetin.

Table 2. Chromatographic and spectral properties of flavonol aglycones from *Sedum* and *Sempervivum* spp.

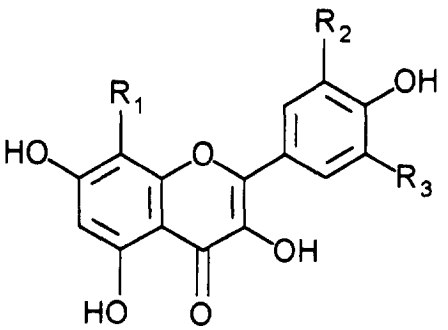
No.	Flavonol	TLC system* ($R_F \times 100$) 1	2	3	Spot colour under UV/NA†	GC RR_T‡	GC-EIMS $[M-15]^+$	HPLC RR_T§	UV max. (nm) with HPLC-DAD
1	Kaempferol	34	40	9	Yellow–green	0.992	559	1.78	266, 295*sh*, 322*sh*, 371
2	Herbacetin	14	30	9	Dull brown	1.015	647	1.57	278, 332, 388
3	Sexangularetin	38	47	21	Brown	1.016	589	1.71	274, 330, 379
4	Quercetin	11	26	3	Yellow–orange	1.070	647	1.65	258, 300*sh*, 377
5	Gossypetin	3	15	4	Dull brown	1.073	735	1.47	264, 275, 345, 386
6	Corniculatusin	15	28	10	Brown	1.097	677	1.57	262, 277*sh*, 336*sh*, 384
7	Isorhamnetin	36	39	10	Yellow–green	1.062	589	1.69	255, 269*sh*, 375
8	Limocitrin	39	42	26	Brown	1.086	619	1.61	260, 273*sh*, 340*sh*, 384
9	Myricetin	2	16	2	Yellow–orange	1.113	735	1.51	255, 300*sh*, 377
10	Hibiscetin	—	—	—	—	1.108	823	—	—

*System 1: Silica gel (Merck art. 5715) run with Bz–Pyr–HCO_2H (72:18:10); system 2; microcrystalline cellulose (Merck art. 5718) run with HOAc–H_2O (60:40); system 3: polyamide 11 F254 (Merck art. 5557) run with Bz–MeCOEt–MeOH (40:30:30).

†Colour under UV light (350 nm) after spraying with a solution of 1% diphenyl–boric acid–ethanolamine complex ('Naturstoffreagenz A') in MeOH–diethylamine (90:10).

‡GC conditions: see Experimental. RR_T of flavonol TMSi-derivatives relative to fisetin–TMSi ether.

§HPLC conditions: see Experimental. RR_T relative to rutin.



	R_1	R_2	R_3
1	H	H	H
2	OH	H	H
3	OCH_3	H	H
4	H	OH	H
5	OH	OH	H
6	OCH_3	OH	H
7	H	OCH_3	H
8	OCH_3	OCH_3	H
9	H	OH	OH
10	OH	OH	OH

albeit in smaller amounts. Though not separated, *Sedum acre* differed from the other species by the presence of isorhamnetin and limocitrin in larger amounts.

In conclusion, *Sedum* and *Sempervivum* (including *Jovibarba heuffelii*) can be delimited on the basis of flavonoid aglycone patterns. Flavonoid variation in *Sempervivum* is minimal, which reflects the uniform morphology and the similarity in ecological preference of the species of this genus. The flavonoid chemistry of *Sedum* is quite diverse and shows a high degree of parallellism (8-hydroxylation and 8-*O*-methylation of common flavonols), which renders any statement as to evolution speculative (see also Refs. [47] and [48]). On the whole, the flavonol aglycone diversity in Eurasian *Sedum* seems to mirror the enormous morphological and cytological variation present in this taxon [35].

EXPERIMENTAL

Plant material. Living plants were collected in the wild and cultivated for further study in the experimental garden at Utrecht. The origins of the cultivated plants are given in Table 1. Voucher specimens, preserved in 70% alcohol, are deposited in the Botanical Institute at Utrecht. Mature shoots of *Sedum* and rosettes of *Sempervivum* of approx. the same age and raised under uniform conditions were either used fr. or kept at −18° prior to phytochemical analysis.

Extraction and hydrolysis of the flavonoids. Approx. 10 g of leafy shoots (*Sedum*) or rosette leaves (*Sempervivum*) were washed with $CHCl_3$ to remove leaf waxes. The air-dried plant material was homogenized in 40 g of acetone with a food processor after addition of the int. st. (2.00 mg fisetin per 10 g of plant material). The mixture was centrifuged, and the clear supernatant concd. on a rotary evaorator. The resulting aq. soln was diluted with H_2O to 10 g, and washed with hexane to remove the majority of lipids and chlorophyll. The aqueous layer was mixed with 10 ml of 2M HCl and kept at 100° for

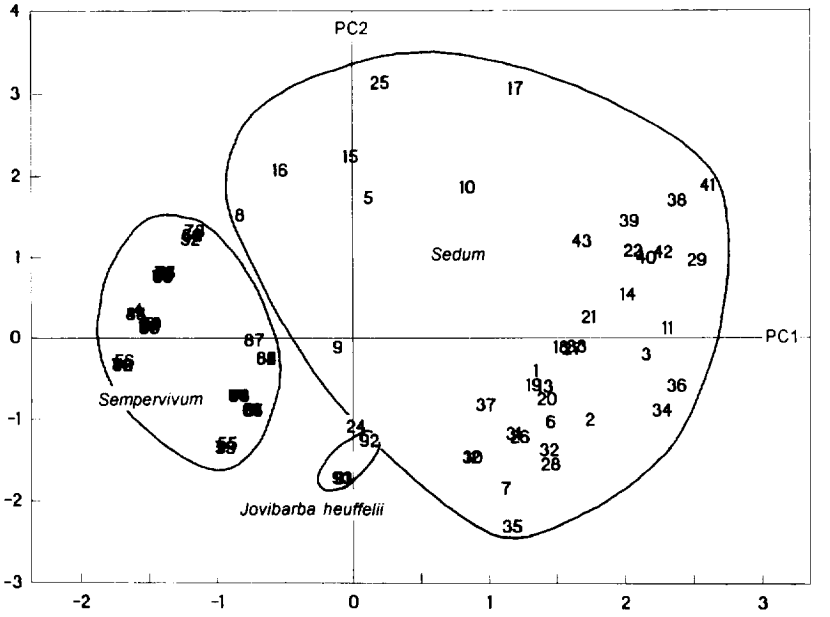


Fig. 1. Principal components analysis (PCA) of transformed flavonoid data (see Experimental) of Eurasian *Sedum* and *Sempervivum*. The first and the second PC account for 33% and 21% of the variance, respectively. Numbers in the scatter plot correspond with the sample numbers in Table 1.

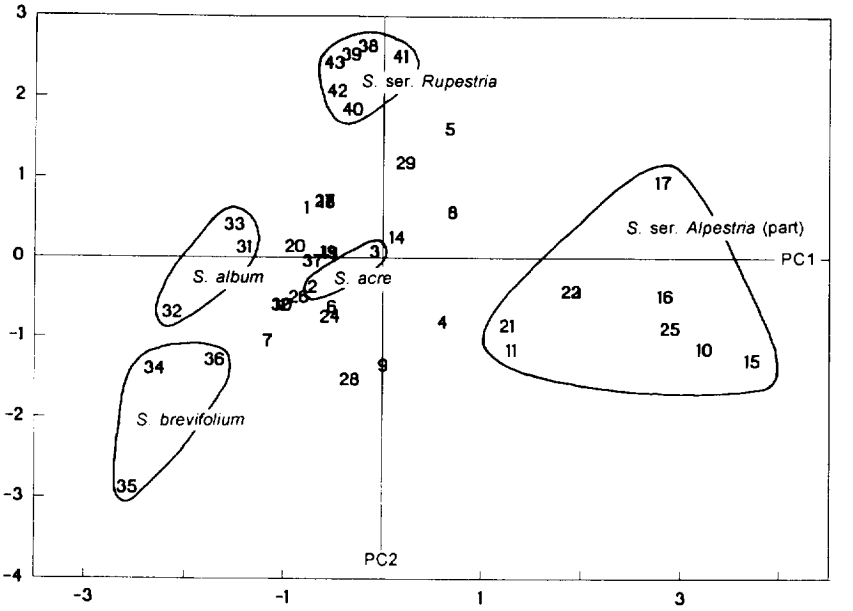


Fig. 2. Principal components analysis (PCA) of transformed flavonoid data (see Experimental) of Eurasian *Sedum*. The first and the second PC account for 27% and 20% of the variance, respectively. Numbers in the scatter plot correspond with the sample numbers in Table 1.

1 hr, after which period the mixture was extracted with Et_2O (3 × 15 ml). The combined ethereal extracts were dried with Na_2SO_4 and evapd in vacuo after addition of 1 ml of dimethoxypropane.

Silylation and quantification of the flavonoid aglycones. Residues of hydrolysed extracts were dissolved in 0.5–1 ml of dry pyridine; 100 μl of this soln was mixed with an equal volume of *bis*-trimethylsilyl acetamide and kept at 70° overnight before GC analysis. An average response ratio flavonol to fisetin for all falvonols was estimated with kaempferol, quercetin, and myricetin standards. Linearity of the FID response was observed in the range 0.01–2.0 mg quercetin per g fr.wt. The coefficient of variation of the analysis method was found to be *ca* 6% ($n = 5$) in plant material of *S. rupestre* (acc.no. 16705).

TLC. Chromatograms of hydrolysed plant extracts (without int. st.) were run on silica gel, microcrystallin cellulose, and polyamide with different solvent systems. Developed plates were sprayed with 'Natrstoffreagenz A' (see Table 2). The species of *S.* series *Rupestria* were checked for the presence of flavanones with silica gel TLC: developed plates were sprayed with solns of $NaBH_4$ (2% in MeOH) and $AlCl_3$ (5% in EtOH) [32], followed by fuming with HCl vapour.

GC and GC–MS. GC was performed under the following conditions: capillary column, fused silica CP Sil 5 CB, 10 m × 0.32 mm i.d., film thickness 0.12 μm; temp programme: 125–325° at 4° min^{-1}, final temp. programme: 125–325° at 4° min^{-1}, final temp, was maintained for 5 min; inj. temp. 250°; FID temp: 300°. Carrier gas and flow: N_2 at 34 cm s^{-1}. Inj. vol.: 1–3 μl; split ratio 1:60. GC–EIMS (70 eV) was performed with a fused silica CP Sil 5 CB (10 m × 0.25 mm i.d., film thickness 0.12 μm) column; temp programme: 125–325° at 4° min^{-1}; inj. temp: 250°; carrier gas and flow: He at 1 ml min^{-1}; inj. vol.: 1–3 μl; split ratio: 1:20.

HPLC. HPLC was performed on a Merck 5 μm LiChrospher 100 RP-18 column (100 × 4 mm) protected by a RP-18 guard column. The solvents were, A, H_2O–HOAc (99:1), and, B, H_2O–THF–HOAc (49:50:1). Elution profile at a flow rate of 2 ml min^{-1}; 10% solvent B in A for 3 min, followed by a gradient in a linear mode to 40% B in A over 6 min and then to 80% B in A over 14 min; isocratic conditions were maintained for a further 5 min. Equilibration of the column was achieved with 10% B in A for 10 min before injection of a new sample. The flavonoids were simultaneously detected with a UV detector operating at 335 nm and a diode array detector operating in the range 240–400 nm.

Demethylation of flavonoid methyl ethers in phenolic extracts. Hydrolysed plant extracts (see above) were covered with 0.5–1 g of pyridinium chloride in a test tube and heated at 150° for at least 6 hr. After cooling, the mixture was dissolved in water, and the phenolic products were extracted with Et_2O.

Multivariate data analysis. Cluster analysis (single-linkage method) and PCA of the flavonoid data were run with the SAS computer package (release 6.03, SAS Institute, Cary, NC, USA) In order to dim the overwhelming influence of the abundantly present flavonoids, the quantitative data were transformed in the following way. Absence was scored 0, traces (0.01 mg g^{-1} fr. wt) were given a value of 1, minor and medium amounts (0.02–0.09 mg g^{-1} fr. wt) a value of 2, and major flavonoids (0.1 mg g^{-1} ft. wt and higher) received a value of 3. PCA (after mean-centring) and CA of the transformed data were carried out without standardization.

Acknowledgements—The authors are indebted to Dr A.P. Bruins and Mrs C.M. Jeronimus-Stratingh for help with the GC–MS work. The investigations were supported by the Foundation for Biological Research (BION), which is subsidized by the Netherlands Organization for Scientific Research (NWO).

REFERENCES

1. Hart, H. 't (1928) *Proc. Kon. Ned. Acad. Wetensch., Ser. C*, **85**, 663.
2. Uhl, C. H. (1961) *Am. J. Bot.* **48**, 114.
3. Hegnauer, R. (1964) *Chemotaxonomie der Pflanzen* (Vol. 3). Birkhäuser, Basel.
4. Hegnauer, R. (1989) *Chemotaxonomie der Pflanzen* (Vol. 8). Birkhäuser, Basel.
5. Krolikowska, M. (1966) *Roczn. Chem.* **40**, 717.
6. Krolikowska, M. (1972) *Roczn. Chem.* **46**, 969.
7. Wolbis, M. and Krolikowska, M. (1988) *Phytochemistry* **27**, 3941.
8. Malterud, K. E. and Nordal, A. (1991) *Acta Pharm. Nord.* **3**, 99.
9. Wolbis, M. (1989) *Phytochemistry* **28**, 2187.
10. Wolbis, M. and Kawalec, M. (1990) *Acta Pol. Pharm.* **47**, 81.
11. Niemann, G. J., Visser-Simons, J. M. J. and Hart, H. 't (1976) *Planta Medica* **30**, 384.
12. Frigot, P. (1960) *Contribution à l'etude chimique de-Quelques Crassulacées et en Particulier de Divers* Sedum. Thesis, Paris.
13. Sakar, M. K., Petereit, F. and Nahrstedt, A. (1993) *Phytochemistry* **33**, 171.
14. Stevens, J. F., Elema, E. T. and Hart, H. 't (1994) *Biochem. Syst. Ecol.* **22**, 861.
15. Denton, M. F. and Kewin, J. L. (1980) *Can. J. Bot.* **58**, 902.
16. Khetwal, K. S., Manral K. and Verma, D. L. (1988) *Planta Medica* **54**, 89.
17. Calie, P. J. (1981) *Brittonia* **33**, 498.
18. Gumenyuk, L. A., Gnedkov, P. A. and Batyuk, V. S. (1971) *Khim. Prir. Soedin.*, 202.
19. Gumenyuk, L. A., Dykhanov, N. N. and Batyuk, V. S. (1972) *Khim. Prir. Soedin.* 391.
20. Gumenyuk, L. A., Golovataya, V. F., Perova, T. V. and Bagrii, A. K. (1982) *Farm. Zh.* 61.
21. Combier, H. and Jay, M. (1967) *Plantes Méd. Phytothérapie* **1**, 165.
22. Stevens, J. F., Hart, H. 't, Hendriks, H. and Malingré T. M. (1992) *Phytochemistry* **31**, 3917.
23. Stevens, J. F., Hart, H. 't, Hendriks, H. and Malingré, T. M. (1993) *Pl. Syst. Evol.* **185**, 207.
24. Stevens, J. F., Hart, H. 't, Block, A., Zwaving, J. H. and Malingré, T. M. (1994) *Phytochemistry* **35**, 389.
25. Eggli, U. and Nyffeler, R. (1992) *Bot. Helv.* **102**, 171.
26. Jay, M., Gonnet, J. F., Wollenweber, E. and Voirin, B. (1975). *Phytochemistry* **14**, 1605.
27. Markham, K. R. (1982) *Techniques of Flavonoid Identification.* Academic Press, London.
28. Schmid, R. D., Mues, R., McReynolds, J. H., Velde, G. Vander, Nakatani, N., Rodriguez, E. and Mabry, T. J. (1973) *Phytochemistry* **12**, 2765.
29. Creaser, C. S., Koupai-Abyazani, M. R. and Stephenson, G. R. (1989) *J. Chromatogr.* **478**, 415.
30. Creaser, C. S., Koupai-Abyazani, M. R. and Stephenson, G. R. (1991) *Organic Mass Spectrom.* **26**, 157.

31. Schmidt, T. J., Merfort, I. and Matthiesen, U. (1993) *J. Chromatogr.* **634**, 350.
32. Grayer, R. J. (1989) in *Methods in Plant Biochemistry*, (vol. 1: Plant Phenolics) (J. B. Harborne, ed.), p. 283. Academic Press, London.
33. Stevens, J. F., Hart, H. 't, Ham, R. C. H. J. van, Elema, E. T., Ent, M. M. V. X van den, Wildeboer, M. and Zwaving, J. H. (1995) *Biochem. Syst. Ecol.* **23**, 157.
34. Ham, R. C. H. J. van (1994) PhD thesis, Utrecht University, Utrecht.
35. Hart, H. 't (1991) *Flora Mediterranea* **1**, 31.
36. Humphries, C. J. and Richardson, P. M. (1980) in *Chemosystematics: Principles and Practice* (Bisby, F. A., Vaughan, J. G. and Wright, C. A. eds), 353. Academic Press, London.
37. Richardson, P. M. and Young, D. A. (1982) *Biochem. Syst. Ecol.* **10**, 251.
38. Stuessy, T. F. and Crawford, D. J. (1983). *Plant Syst. Evol.* **143**, 83.
39. Webb, D. A. (1964) in *Flora Europaea* (vol. 1) (Tutin, T. G., Heywood, V. H., Burges, N., Valentine, D. H., Walters, S. M. and Webb, D. A. eds), p. 350. Cambridge University Press, Cambridge.
40. DeCandolle, A. P. (1828) *Prodromus Systematis Naturalis Regni Vegetabilis* (Vol. 3). Treuttel and Wurtz, Paris.
41. Berger, A. (1930) in *Die natürlichen Pflanzenfamilien*, (2nd edn, Vol. 18a) (Engler, A. and Prantl, K. eds), p. 352. Verlag Wilhelm Engelmann, Leipzig.
42. Stevens, J. F., Hart, H. 't, Pouw, A. J. A., Bolck, A. and Zwaving, J. H. (1994b) *Phytochemistry* **36**, 341.
43. Hart, H. 't (1978) PhD thesis, Utrecht University, Utrecht.
44. Ham, R. C. H. J. van and Hart, H. 't. (1994) *Plant Syst. Evol.* **190**, 1.
45. Combier, H., Markham, K., Audier, H., Lebreton, P., Mabry, T. and Jay, M. (1968) *Compt. Rend. Acad. Sci., Ser D* **266**, 2495.
46. Hart, H. 't (1995) *Flora Mediterranea* (in press).
47. Gornall, R. J. and Bohm, B. A. (1978) *Syst. Bot.* **3**, 353.
48. Crawford, D. J. (1979) *Bot. Rev.* **44**, 431.