Article

Chemophenetic approach to selected Senecioneae species, combining morphometric and UHPLC-HRMS analyses

Yulian Voynikov[1]*†, Vessela Balabanova[2]†, Reneta Gevrenova[2], Dimitrina Zheleva-Dimitrova[2]

[1]*Medical University, Faculty of Pharmacy, Department of Chemistry, 2 Dunav str., 1000 Sofia, Bulgaria*
[2]*Medical University, Faculty of Pharmacy, Department of Pharmacognosy, 2 Dunav str., 1000 Sofia, Bulgaria*
†Authors with equal contributions
* Correspondence: y_voynikov@pharmfac.mu-sofia.bg

Abstract: Herein, a chemophenetic significance, based on phenolic metabolite profiling of three *Senecio* (*S. hercynicus, S. ovatus* and *S. rupestris*) and two *Jacobaea* species (*J. pancicii* and *J. maritima*) coupled to morphometric data, is presented. A set of twelve morphometric characters were recorded from each plant species and used as predictor variables in a Linear Discriminant Analysis (LDA) model. From a total 75 observations (15 from each of the five species), the model correctly assumed their species' membership, except 2 observations. Among the studied species, *S. hercynicus* and *S. ovatus* presented the greatest morphological similarity. A phytochemical profiling of phenolic specialized metabolites by UHPLC-Orbitrap-HRMS revealed 46 hydroxybenzoic, hydroxycinnamic, acylquinic acids and their derivatives, 1 coumarin, and 21 flavonoids. Hierarchical and PCA clustering applied to the phytochemical data corroborated the similarity of *S. hercynicus* and *S. ovatus*, observed in the morphometric analysis. This study contributes to the phylogenetic relationships between the tribe Senecioneae taxa and highlights the chemophenetic similarity/dissimilarity of the studied species belonging to *Senecio* and *Jacobaea* genera.

Keywords: *Senecio*; *Jacoboea*; Orbitrap; chemophenetic; clustering; R;

1. Introduction

The tribe Senecioneae (Asteraceae) encompasses more than 150 genera and 3 000 species; approximately half of its species belong to genus *Senecio* L., considering it one of the largest genera of flowering plants [1]. *Senecio* species have a wide distribution and they occur in various habitats - from low altitudes to high mountain communities, and from arctic regions to hot tropical areas [1]. Although phylogenetic studies have been carried out classifying the taxa, the intergeneric relations are still vague [1, 2]; some *Senecio* species have been recently transferred to a separate genus *Jacobaea* Mill. [3]. Within the genus *Senecio*, hybridization has been observed e.g. *S. hercynicus* × *S. ovatus* [4]. Most taxa in the tribe can be identified by the existence of capitula (flower heads) with a typically uniseriate involucre. However, some species are poorly differentiated morphologically and there is still uncertainty about recognition of their taxa [2, 5-7]. *Senecio* species are reported to accumulate sesquiterpenoids (eremophilanes, furanoeremophilanes, cacalols, eudesmanes, oplopanes, germacranes, etc.) and pyrrolizidine alkaloids (PAs) [1, 8, 9], phenolic compounds [10-15], and various other secondary metabolites [4, 9, 16]. *Senecio* species have been described to possess analgesic [17] and hypoglycemic [18] activity, related to the typical for the genus sesquiterpene lactones, and insecticidal properties [19] related to the presence of PAs. Additionally, the taxa are reported to express strong antioxidant, cytotoxic and antimicrobial activity due to the presence of phenolics [11-13, 15].

In the Bulgarian flora, *S. hercynicus* Herborg., *S. ovatus* (G. Gaertn. & Al.) Willd., *S. rupestris* Waldst. & Kit.) and *Jacobaea pancicii* (Degen) Vladimirov & Raab-Straube are perennial plants distributed in the mountain regions up to 1500 (2200) m a.s.l., while *J. maritima* (L.) Pelser & Meijden is a shrub spread on the Black Sea coast [20]. Presently, *S. hercynicus* and *S. ovatus* are included in *S. nemorensis* group. Formerly, *S. hercynicus* had been recognized as *S. nemorensis* L.; *S. ovatus* as *Jacobaea ovata* G. Gaertn.; *J. maritima* as *Senecio*

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maritima (L.) Rchb.; *J. pancicii* as *Senecio pancicii* Degen [20]. Phytochemical studies on some *Senecio* and *Jacobaea* species with Bulgarian origin were based on the characterization of PAs [19, 21, 22], phenolic and flavonoid derivatives [14]. Although the studied species are distributed in other European floras, including the floras of neighboring countries [2, 23], up to now there has been no study focused on morphometric and phytochemical data analysis.

Plant *chemophenetics* [24] is a term that was recently proposed for exploring characteristic arrangements of specialized plant taxon metabolites; this analysis contributes to the phenetic description of the taxa – similar to anatomical, morphological, and karyological approaches – and represents an opportunity to describe organisms classified with molecular methods. Thus, the specialized metabolism products could be treated as phenotypic characters that can be used as arguments, e. g., the existence of botanical varieties in the same way as, e. g., traditional morphological characters [25].

Hence, the present study aims to apply a chemophenetic [24] approach to three *Senecio* (*S. hercynicus, S. ovatus* and *S. rupestris*) and two *Jacobaea* species (*J. pancicii* and *J. maritima*). Morphometric data and phytochemical profiling of phenolic specialized metabolites are combined to give insight into the similarity of species in the *Senecio* taxa.

2. Results and Discussion.

2.1. Morphometric analysis

Samples for each of three Senecio species (S. hercynicus, S. ovatus and S. rupestris) and two Jacobaea species (J. pancicii and J. maritima) were characterized by 12 independent variables (X1-12). These variables were ordinarily applied to differentiate the studied taxa [20, 37-39]. The raw morphometric data, together with descriptive statistics, are presented in Table S1 and Table S2. A combination of parametric (MANOVA) and non-parametric (Kruskal-Wallis) tests, together with post-hoc Bonferroni tests, were used to derive relationships between the tested variables and the samples' taxonomic membership. An $\boldsymbol{\alpha}$ level of 0.05 was set as significant. Some notable relationships will be drawn. Root diameter (X1) was the only parameter by which a discrimination was evident between samples belonging to the different genera, however, without differentiation within the species of the same genus, i.e., J. pancicii was not differentiated from J. maritia. Similarly, the three Senecio species showed a relative homogeneity on the X4 parameter, and were distinguished from the two Jacoboea species; X4 also differentiated J. pancicii from J. maritima. On the other hand, parameters X2, X5-7 showed similarity between S. rupestris and J. maritima, and between S.ovatus, S.hercynicus, and J. pancicii. The other parameters showed quite different relationship to the species, and it is evident from the LDA analysis shown below that a combination of parameters is needed for confident differentiation of the discussed species.

2.1.1 Correlation and Linear Discriminant Analysis (LDA)

The correlation matrix (Figure 1) revealed that stem height (X_2) was positively correlated to leaf length (X_3) and negatively correlated to variables X_6, X_8, X_{10}. The number of ray flowers (X_8) was positively associated to involucral bracts number (X_6) and number of disc flowers (X_{10}). Additionally, variables X_6, X_8, X_{10} show high positive correlation between each other.

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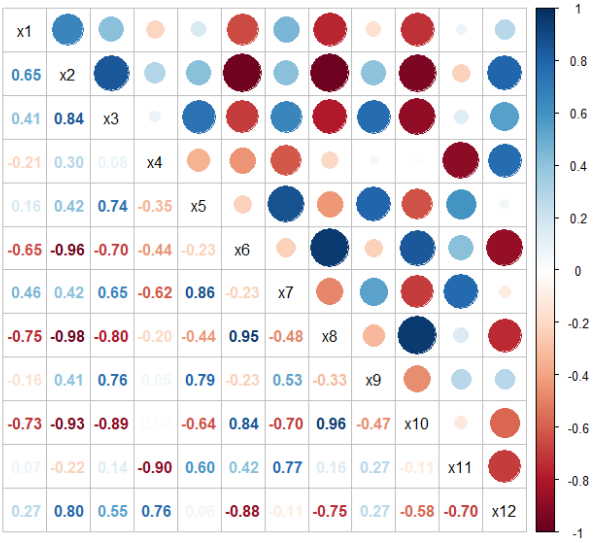
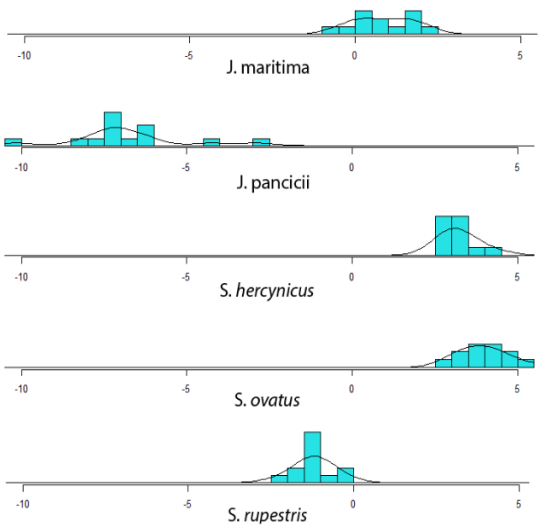


Figure 1. Correlogram of the 12 morphometric characters.

Next, a Linear Discriminant Analysis (LDA) was performed on the $X_{1\text{-}12}$ variables [30, 40]. Based on the residual sum of squares (RSS), adjusted R2, Mallow's Cp, and Bayesian information criterion (BIC), a six variable model was selected, including variables X_1, X_4, X_7, X_8, X_9, and X_{11} (Figure S1 and Table S3). Then, 80% of the data was used as a training set (n = 60) and 20% - as a test set (n = 15), on a random principle. The training set was used to derive a linear model for predicting the species of a plant, based on the selected set of 6 parameters. The linear model was able to correctly predict the species on the test set (n = 15), but one (Table S4). On the one-dimensional plot derived from the linear model (Figure 2A), *S. ovatus* and *S. hercynicus* were not well distinguished, while on the two-dimensional plot, all species were separated, except *S. ovatus* and *S. hercynicus* with a partial overlap (Figure 2B).

A



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B

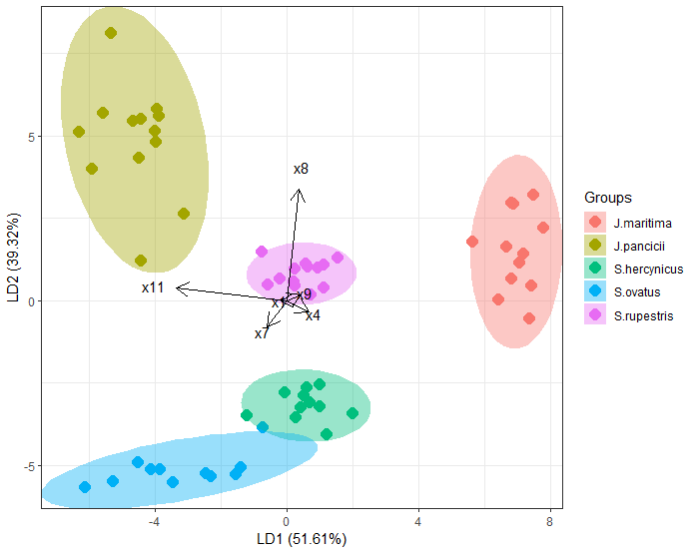

Figure 2. Discriminatory power of LD1 and LD2 functions. A – one-dimensional (1D) and B – two-dimensional (2D) discrimination.

Given these results, the morphological variability of the *Senecio* and *Jacobaea* species is not random and it is long-established for the tribe Senecioneae taxa as prominent [41]. Although the *Jacobaea* is distinguished from *Senecio sensu stricto*, a clear morphological synapomorphies for *Jacobaea* have not been recognized yet [1]. The received data by the morphometrical study unequivocally confirm the taxonomical relationship of *S. hercynicus* and *S. ovatus* belonging to *S. nemorensis* group and the transfer of the last-mentioned species to genus *Senecio*. Moreover, the results favor the delimitation of *J. maritima* and *J. pancicii* from genus *Senecio* and distinguishing of the other studied taxa [20].

2.2. *UHPLC-HRMS identification and tentative annotation of specialized natural products*

In order to establish a phenolic metabolite profiling, combined hydromethanolic plant extracts were prepared, as described in Section 3.4., and analyzed by UHPLC-HRMS. Based on chromatographic retention times, MS and MS/MS accurate measurements, fragmentation patterns, and comparison with reference standards and literature data, 46 hydroxybenzoic, hydroxycinnamic, acylquinic acids and their derivatives, 1 coumarin, and 21 flavonoids were annotated in the tested extracts. LC-MS and MS/MS data of all 68 identified phenolic compounds are presented in Table 1 along with their distribution in the studied extracts.

Table 1. Specialized metabolites in studied Senecio and Jacoboea extracts assayed by UHPLC-HRMS

№	Annotated compounds	Molecular formula	Exact mass $[M-H]^-$	MS^2	t_R (min)	Distribution
Hydroxybenzoic, hydroxycinnamic and acylquinic acids, their derivatives and coumarin						
1	protocatechuic acid-O-hexoside	$C_{13}H_{16}O_9$	315.0722	315.0726 (100), 153.0182 (29.5), 152.0104 (60.9), 109.0284 (10.1)	1.74	A, B, C, D, E
2	vanillic acid 4-O-hexoside	$C_{14}H_{18}O_9$	329.0878	329.0885 (1.8), 167.034 (100), 152.0103 (23), 123.0438 (14.3), 108.0202 (37.8)	1.81	A, B, C, E

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3	syringic acid*	$C_9H_{10}O_5$	197.0456	197.0449 (16.5), 182.0211 (3.2), 153.0549 (8.9), 138.0314 (3.3), 123.0437 (58.3)	1.76	C
4	vanillic acid*	$C_8H_8O_4$	167.0350	167.0339 (31.1), 152.0103 (100), 123.0438 (32.1), 108.0202 (52.9), 95.0486 (8)	1.82	A, B, C, D, E
5	protocatechuic acid*	$C_7H_6O_4$	153.0193	153.0182 (15.2), 109.0281 (100), 91.0173 (1.2), 81.033 (1.4)	2.04	A, B, C, D, E
6	p- hydroxyphenylacetic acid-O-hexoside	$C_{14}H_{18}O_8$	313.0929	313.0923 (13), 151.0389 (100), 133.0284 (0.2), 123.0075 (0.8), 109.0281 (4.2)	3.00	A, B, C
7	gluconic acid-O- hexoside	$C_{15}H_{18}O_{10}$	357.0827	357.083 (100), 195.0293 (10.8), 177.0183 (8), 151.039 (71.8)	2.25	C
8	syringic acid 4-O-hexoside	$C_{15}H_{20}O_{10}$	359.0984	359.0985 (8), 197.0448 (100), 182.0212 (21.7), 153.0546 (16.1), 138.031 (29.3), 123.0074 (33.1)	2.27	A, B, C, D, E
9	neochlorogenic acid*	$C_{16}H_{18}O_9$	353.0878	353.0882 (46.1), 191.0553 (100), 179.0341 (68.1), 173.0444 (4.1), 161.0235 (5.9), 135.0439 (54.4), 127.0385 (0.9), 111.0438 (0.7), 93.0329 (3.7), 85.0279 (8.5)	2.37	A, B, C, D, E
10	caffeic acid- O-hexoside	$C_{15}H_{18}O_9$	341.0878	341.0884 (2.2), 179.034 (2.9), 135.0438 (100), 107.0488 (0.7)	2.54	A, B, C, D, E
11	4-hydroxybenzoic acid-O-hexoside	$C_{13}H_{16}O_8$	299.0773	299.0775 (13.7), 137.0231 (100), 93.033 (0.2)	2.46	A, B, C, E
12	esculetin-O- hexoside	$C_{15}H_{16}O_9$	339.0722	339.0721 (10.6), 177.0184 (100), 149.0233 (0.9), 133.0282 (8), 105.0331 (3.3), 89.0381 (2.4)	2.72	A, B, C, D, E
13	4- hydroxybenzoic acid	$C_7H_6O_3$	137.0244	137.0232 (100), 108.0203 (11.2), 93.0333 (3.3)	2.84	A, B, C, D, E
14	ferulic acid*	$C_{10}H_{10}O_4$	193.0506	193.05 (100), 178.0264 (74.8), 163.0391 (34.4), 149.0598 (38), 134.036 (82.5)	2.96	A, B, C
15	ferulic acid-O- hexoside	$C_{16}H_{20}O_9$	355.1035	355.1048 (1), 193.0499 (100), 178.0263 (10.9), 149.0596 (21.4), 134.036 (62.1)	2.96	A, B, C
16	gentisic acid*	$C_7H_6O_4$	153.0193	153.0182 (46.7), 123.0074 (20.8), 109.0283 (40.6), 81.0331 (5.4)	2.98	A, B, C, D
17	4-hydroxybenzoic acid-O-hexoside isomer	$C_{13}H_{16}O_8$	299.0773	299.0783 (1.3), 137.0231 (100), 93.033 (50.8)	3.00	A, B, C
18	3-p-coumaroylquinic acid	$C_{16}H_{18}O_8$	337.1500	191.0554 (19.6), 163.039 (100), 161.0443 (4.2), 119.0488 (23.7)	3.04	C
19	caffeic acid-O-hexoside	$C_{15}H_{18}O_9$	341.0878	341.088 (26.8), 179.034 (100), 135.0438 (77), 107.0486 (0.8)	3.07	A, B, C, D, E
20	quinic acid	$C_7H_{12}O_6$	191.0561	191.0553 (100), 173.0446 (2), 155.0338 (0.2), 127.0388 (4.3), 111.0437 (1.9), 93.0331 (6.4), 85.0279 (18.1)	3.19	A, B, C, D, E
21	chlorogenic acida	$C_{16}H_{18}O_9$	353.0878	353.0881 (3.9), 191.0553 (100), 179.0343 (1.1), 173.0449 (0.4), 161.0232 (1.6), 135.0439 (0.5), 127.0386 (1.3), 111.0433 (0.3), 93.033 (2.2), 85.0279 (7.2)	3.19	A, B, C, D, E

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22	caffeic acid-O-hexoside isomer I	$C_{15}H_{18}O_9$	341.0878	341.0881 (9.5), 179.034 (100), 135.0438 (60.8), 107.049 (0.6)	3.27	A, B, C, D, E
23	4-caffeoylquinic acid	$C_{16}H_{18}O_9$	353.0878	353.0882 (32.1), 191.0554 (97.5), 179.0341 (72.6), 173.0446 (100), 135.0439 (56.3), 127.0387 (1.8), 111.0435 (3.3), 93.0331 (22), 85.028 (11.3)	3.37	A, B, C, D, E
24	p-hydroxyphenylacetic acid	$C_8H_8O_3$	151.0401	151.0389 (100), 136.0154 (2), 123.0074 (4.2), 109.028 (15)	3.47	C, E
25	coumaric acid-O-hexoside	$C_{15}H_{18}O_8$	325.0929	325.0923 (1.7), 163.039 (100), 145.0284 (3.5), 119.0488 (92.1), 93.0333 (0.8)	3.33	A, B, C, D, E
26	p-coumaric acid*	$C_9H_8O_3$	163.0401	163.0389 (6.7), 135.0438 (0.7), 119.0488 (100)	3.33	A, B, C, D, E
27	caffeic acid*	$C_9H_8O_4$	179.0350	179.0341 (20.5), 135.0438 (100), 117.0332 (0.7), 107.0489 (1.4)	3.53	A, B, C, D, E
28	caffeic acid-O-hexoside isomer II	$C_{15}H_{18}O_9$	341.0878	341.088 (24.5), 179.0341 (100), 135.0439 (85.6), 107.0489 (0.5)	3.79	B, C, D, E
29	5-p-coumaroylquinic acid	$C_{16}H_{18}O_8$	337.0929	337.0933 (8.7), 191.0554 (100), 173.0449 (6), 163.0389 (5.7), 127.0391 (1), 119.0489 (4.8), 111.0437 (1.9), 93.033 (17.2), 85.028 (4.9)	3.95	A, B, C, D, E
30	3-hydroxy-dihydro-caffeoyl-5-caffeoylquinic acid	$C_{25}H_{26}O_{13}$	533.1301	533.1306 (100), 191.0554 (83.4), 173.0447 (10.1), 161.0596 (3.2), 127.0387 (3.3), 93.033 (17.8), 85.028 (11.8)	3.09	A, B, C, D, E
31	isoferulic acid	$C_{10}H_{10}O_4$	193.0506	193.0499 (100), 178.0265 (0.8), 163.0391 (41.6), 149.0597 (18.8), 135.0439 (38.8)	4.10	C
32	syiringaldehide	$C_9H_{10}O_4$	181.0506	181.0497 (15.2), 166.0261 (100), 151.0025 (58.4), 123.0074 (15.7)	4.22	C
33	acetoxy-hydroxyacetophe-none-O-hexoside	$C_{16}H_{20}O_9$	355.1035	355.1039 (13.7), 193.0494 (1.2), 151.0389 (100), 123.0076 (0.9), 109.0281 (4.4)	4.27	C
34	taraxafolin B-(caffeoyl)-hexoside	$C_{26}H_{28}O_{16}$	595.1305	595.1308 (100), 341.0883 (25.4), 253.0353 (23.5), 235.0245 (3.5), 209.0446 (1.4), 191.0341 (31.1), 179.0341 (93.7), 165.0545 (16.4), 135.0438 (56)	4.41	C
35	5-feruoylquinic acid	$C_{17}H_{20}O_9$	367.1035	367.1038 (15.4), 191.0554 (100), 173.0445 (10.5), 134.0359 (13.1), 111.0437 (3.8), 93.0331 (25.5), 85.028 (5.1)	4.42	A, B, C, E
36	m- coumaric acid *	$C_9H_8O_3$	163.0401	163.039 (7.6), 135.0439 (0.5), 119.0489 (100)	4.56	A, B, C, E
37	3,4- dicaffeoylquinic acid*	$C_{25}H_{24}O_{12}$	515.1195	515.1198 (100), 353.0881 (15), 335.0773 (5.3), 203.0344 (0.5), 191.0555 (29.1), 179.0341 (53), 173.0446 (58.7), 161.0233 (17), 135.0439 (53.1), 127.0386 (2.2), 111.0437 (3.6), 93.0331 (16.8), 85.028 (3.7)	5.69	A, B, C, D, E

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38	3,5- dicaffeoylquinic acid*	$C_{25}H_{24}O_{12}$	515.1195	515.1202 (13.5), 353.0881 (100), 191.0554 (91.3), 179.0341 (49.4), 173.0443 (3.7), 161.0234 (4.2), 135.0439 (55.8), 111.0437 (1.3), 93.0332 (4.2), 85.028 (9.8)	5.86	A, B, C, D, E
39	1,5- dicaffeoylquinic acid*	$C_{25}H_{24}O_{12}$	515.1195	515.1199 (25.5), 353.088 (92.4), 335.0777 (1.9), 191.0554 (100), 179.0341 (53.3), 173.0446 (8.7), 135.0439 (65), 127.0387 (4.2), 111.0437 (2.1), 93.0332 (6.5), 85.028 (10.2)	6.02	A, B, C, D, E
40	4,5- dicaffeoylquinic acid*	$C_{25}H_{24}O_{12}$	515.1195	515.1197 (100), 353.0883 (72.3), 203.0341 (1.5), 191.0553 (38.9), 179.0341 (66.6), 173.0446 (98.1), 135.0439 (69.5), 111.0435 (5.2), 93.033 (30.8), 85.0279 (8.3)	6.22	A, B, C, D, E
41	shikimic acid	$C_7H_{10}O_5$	173.0456	173.0444 (100), 111.0437 (10), 93.033 (68.4)	6.22	E
42	salicilic acid*	$C_7H_6O_3$	137.0244	137.023 (8.7), 93.0331 (100)	6.29	A, C
43	3-p-coumaroyl-5-caffeoylquinic acid	$C_{25}H_{24}O_{11}$	499.1246	499.1238 (16.4), 353.0901 (1.5), 337.0933 (73.9), 335.0797 (1.7), 191.0553 (12.4), 173.0449 (7.9), 163.039 (100), 135.0441 (4.2), 119.0489 (34.4), 93.0334 (4.4)	6.51	B, C, E
44	3-caffeoyl-5-p-couma-roylquinic acid	$C_{25}H_{24}O_{11}$	499.1246	499.125 (26.1), 353.0882 (64.8), 337.0938 (17.5), 191.0554 (100), 179.0341 (34.5), 173.0446 (6.9), 163.0389 (2.9), 161.0231 (5.5), 135.0439 (36.8), 119.0488 (2.8), 111.0436 (1.4), 93.0331 (10.5), 85.0279 (7.1)	6.57	B, E
45	3-feruoyl-5-caffeoylquinic acid	$C_{26}H_{26}O_{12}$	529.1352	529.1296 (2.3), 367.1036 (99.2), 335.078 (1.1), 193.0499 (100), 191.0557 (3), 173.0443 (6.9), 161.0235 (2), 134.036 (68.5), 93.0331 (3.2)	6.82	A, B, C, E
46	3-caffeoyl-5-feruoylquinic acid	$C_{26}H_{26}O_{12}$	529.1352	529.1353 (41.1), 367.1037 (0.7), 353.0882 (43.5), 335.0794 (0.8), 191.0555 (100), 179.0342 (40.7), 173.0446 (11.5), 161.0238 (5.4), 135.0439 (37.7), 134.0361 (12.9), 127.0383 (1.3), 111.0437 (1.3), 93.0331 (15.5), 85.028 (7.5)	6.89	A, B, C, E
47	3,4,5-tricaffeoylquinic acid	$C_{34}H_{30}O_{15}$	677.1512	677.1517 (100), 515.1202 (46.2), 353.0883 (47.1), 335.0783 (13.9), 191.0554 (45.2), 179.0342 (65.2), 173.0446 (90.2), 161.0234 (24.1), 135.0439 (72.1), 111.0436 (5.6), 93.0331 (21.7)	7.77	B, C, E
			Flavonoids			
48	6, 8-di-C-hexosyl-naringenin	$C_{27}H_{32}O_{15}$	595.1669	595.1677 (100), 475.1247 (3.8), 457.1138 (1.3), 427.1055 (1.2), 415.1037 (11.6), 385.0933 (36.1),	3.63	D, E

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				355.0822 (38.9), 343.0825 (3.9), 313.0722 (6.1), 119.0489 (15.5), 107.0123 (3.5),		
49	kaempferol-O-dihexoside	$C_{27}H_{30}O_{16}$	609.1461	609.1462 (100), 447.0931 (24.7), 285.0405 (50.3), 284.0325 (7.6), 255.0300 (33.4), 227.0347 (5.7), 211.0391 (2.2)	3.81	C
50	isorhamnetin-O-dihexoside	$C_{28}H_{32}O_{17}$	639.1567	639.1575 (100), 477.1039 (34.6), 315.0514 (56.7), 300.0275 (11.8), 314.0429 (12.6), 285.0408 (6.4), 270.0172 (20.8), 242.0218 (14.0), 227.0344 (0.7), 151.0027 (5.5), 107.0124 (1.3)	4.04	C
51	rutin*	$C_{27}H_{30}O_{16}$	609.1461	609.1469 (100), 301.0352 (39.6), 300.0279 (64.0), 271.0249 (29.6), 255.0298 (14.6), 243.0296 (6.4), 227.0345 (2.2), 178.9975 (3.4), 163.0015 (0.8), 151.0025 (5.6), 121.0286 (0.9), 107.0125 (1.0)	5.07	A, B, C, D, E
52	isorhamnetin-O-pentosylhexoside	$C_{27}H_{30}O_{16}$	609.1461	609.1464 (100), 315.0504 (19.9), 314.0436 (85.1), 299.0196 (18.4), 271.0252 (22.0), 243.0297 (20.1), 227.0350 (5.1), 178.9978 (1.3), 151.0023 (34.0)	5.16	A, B, C, D, E
53	isoquercitrin*	$C_{21}H_{20}O_{12}$	463.0882	463.0887 (100), 301.0352 (44.4), 300.0278 (69.8), 271.0250 (46.2), 255.0300 (20.0), 243.0298 (11.0), 227.0346 (4.6), 211.0389 (1.4), 178.9976 (4.4), 151.0026 (7.8), 121.0280 (1.5), 107.0123 (2.8)	5.27	A, B, C, D, E
54	quercetin 7-O-hexuronide	$C_{21}H_{18}O_{13}$	477.0675	477.0671 (47.4), 301.0356 (100), 283.0245 (2.1), 255.0302 (3.1), 227.0343 (2.0), 211.0396 (1.8), 178.9976 (9.9), 163.0028 (2.4), 151.0025 (20.2), 121.0281 (6.3), 107.0124 (8.9)	5.22	A, B, C, D
55	luteolin-O-hexuronide	$C_{21}H_{18}O_{12}$	461.0726	461.0730 (39.5), 285.0406 (100), 257.0457 (4.6), 229.0505 (6.0), 213.0544 (2.0), 175.0242 (5.8), 151.0023 (0.9), 107.0125 (2.5)	5.84	A, B
56	kaempferol 7-O-rutinoside*	$C_{27}H_{30}O_{15}$	593.1512	593.1516 (100), 285.0405 (90.5), 255.0298 (42.9), 227.0346 (31.3), 163.0025 (1.1)	5.62	A, B, D, E
57	quercetin 3-O-acetylhexoside	$C_{23}H_{22}O_{13}$	505.0988	505.0996 (100), 463.0891 (0.8), 301.0351 (34.1), 300.0278 (88.9), 271.0251 (42.8), 255.0299 (21.5), 243.0297 (11.7), 227.0343 (3.1), 178.9976 (2.5), 163.0027 (2.8), 151.0024 (7.8), 121.0283 (1.1), 107.0124 (2.4)	5.61	A, B, C, D

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58	kaempferol-3-O-gluco-side*	$C_{21}H_{20}O_{11}$	447.0933	447.0938 (100), 285.0401 (21.4), 284.0329 (51.3), 255.0300(38.4), 227.0347 (40.6), 151.0024 (2.7),	5.87	B, C
59	isorhamnetin 3-O-gluco-side*	$C_{22}H_{22}O_{12}$	477.1039	477.1041 (100), 315.0495 (9.7), 314.0437 (51.2), 299.0213 (3.2), 271.0251 (18.8), 257.0460 (3.9), 243.0299 (22.3), 227.0341 (2.9), 215.0340 (3.7), 178.9972 (0.6), 151.0021 (1.7)	6.02	A, B, C, D, E
60	isorhamnetin -O-hex-uronide	$C_{22}H_{20}O_{13}$	491.0831	491.0836 (48.3), 315.0515 (100), 300.0278 (29.1), 271.0251 24.7), 255.0299 (10.7), 227.0347 (1.8), 175.0238 (5.7), 151.0029 (2.6), 107.0122 (0.8)	6.09	A, B
61	quercetin-3-O-rhamnoside (quercitrin)*	$C_{21}H_{20}O_{11}$	447.0933	447.0936 (100), 301.0355 (81.9), 300.0278 (22.24), 271.0248 (1.7), 255.0298 (2.0), 227.0352 (1.6), 178.9974 (2.6), 151.0025 (40.6), 121.0281 (8.9), 107.0124 (15.2)	6.76	B
62	luteolin*	$C_{15}H_{10}O_6$	285.0405	285.0406 (100), 175.0392 (3.0), 151.0024 (4.7), 133.0282 (22.8), 107.0124 (3.7)	7.56	C
63	quercetin*	$C_{15}H_{10}O_7$	301.0354	301.0356 (100), 273.0411 (3.3), 257.0469 (1.8), 245.0444 (0.8), 229.0500 (0.6), 215.1699 (0.3), 178.9977 (21.3), 151.0024 (49.4), 121.0281 (14.2), 107.0123 (12.9)	7.61	B, C, D, E
64	apigenin*	$C_{15}H_{10}O_5$	269.0456	269.0458 (100), 225.0549(1.9), 201.0550 (0.9), 151.0025 (5.7), 121.0282 (1.3), 117.0332 (18.4), 107.0124 (5.3)	8.62	C
65	kaempferol*	$C_{15}H_{10}O_6$	285.0405	285.0406 (100), 257.0465 (0.8), 243.0298 (0.2), 227.0353 (0.9), 211.0397 (1.3), 151.0025 (1.3), 107.0123 (1.2)	8.85	C, E
66	chrysoeriol*	$C_{16}H_{12}O_6$	299.0561	299.0564 (63.8), 284.0329 (100), 255.0300 (46.5), 227.0344 (38.2), 211.0394 (1.5), 151.0024 (0.3)	9.32	C
67	cirsiliol	$C_{17}H_{14}O_7$	329.0667	329.0672 (100), 314.0441 (43.6), 299.0199(89.6), 271.0248 (55.9), 243.0296 (6.5), 227.0345 (3.4), 211.1333 (6.8)	9.60	C
68	cirsimaritin	$C_{17}H_{14}O_6$	313.0718	313.0721 (100), 298.0483 (65.2), 283.0251 (52.5), 255.0298 (64.1), 227.0341 (4.4), 211.0396 (5.7)	12.27	C

*identified by reference standards

A - *S. hercynicus*; B - *S. ovatus*; C - *S. rupestris*; D - *J. pancicii*, E - *J. maritima*

2.2.1.Hydroxybenzoic, hydroxycinnamic acids, and their glycosides

Five hydroxybenzoic acids (compounds **3-5**, **16** and **42**) and 4 hydoxycinnamic acids (compounds **14, 26, 27,** and **36**) along with *p*-hydroxyphenylacetic acid (compound **24**) were identified in the studied species by comparison with reference standards (Table 1).

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Compounds **1, 2, 6-8, 10, 11, 15, 17, 19, 22, 25**, and **28** presented similar fragmentation patterns indicating phenolic acid-hexosides. They gave indicative fragment ions at *m/z* 153.018 (compound **1**), *m/z* 167.034 (compound **2**), *m/z* 151.039 (compound **6**), *m/z* 197.045 (compound **8**), *m/z* 179.034 (compounds **10**, **19**, **22**, **28**), *m/z* 137.023 (compounds **11**, **17**), *m/z* 193.050 (compound **15**), *m/z* 163.039 (compound **26**) and fragmentation pathways consistent with protocatechuic, vanillic, *p*-hydroxyphenylacetic, syringic, caffeic, 4-hydroxybenzoic, ferulic and *p*-coumaric acid, respectively (Table 1).

Compound **33** afforded a base peak at *m/z* 151.039 [(M-H)-162-42]$^-$, and fragment ions at *m/z* 123.008 [(M-H)-162-60]$^-$ and 109.028 [(M-H)-162-2×42]$^-$, suggesting two acetyl groups and hexose unit. Thus, **33** was ascribed to acetoxy-hydroxyacetophenone-*O*-hexoside. MS/MS of **34** at *m/z* 595.131 [M-H]$^-$ was acquired; taraxafolin B residue was deduced from the abundant ions at *m/z* 341.0883 [M-H-$C_{11}H_{10}O_7$]$^-$ (25.4%) and 253.035 [taraxafolin (TF)-H]- (23.5%), supported by *m/z* 209.045 [TF-H-CO_2]$^-$, 191.034 [TF-H-H_2O-CO_2]- and 165.055 [TF-H-2CO_2]$^-$. Accordingly, **34** was tentatively identified as taraxafolin B-(caffeoyl)-hexoside (Table 1).

2.2.2.Acylquinic acids

Six mono-, nine di- and one triacylquinic acids (AQAs) were identified or annotated in the assayed species. Fragmentation behaviors were consistent with those reported [42, 43]. Thus, **23**, **29** and **35** were assigned to 4-caffeoyl-, 5-p-coumaroyl- and 5-feruloylquinic acid, respectively. diAQA belongs to four widely spread in Asteraceae subclasses: dicaffeoylquinic acids (diCQA) (compounds **37**-**40**), feruloyl-caffeoylquinic acids (FCQA) (compounds **45**, **46**), *p*-coumaroyl-caffeoylquinic acids (p-CoCQA) (compounds **43**, **44**) and 3-hydroxy-dihydroxy-5-caffeoylquinic acid (HC-CQA) (compound **30**).

Compounds **43** and **45** gave abundant ions at *m/z* 337.093 (74%) and 367.104 (99%), respectively, indicating a loss of caffeoyl residue before the *p*-coumaroyl (compound **43**) and feruloyl (compound **45**) moiety. Moreover, both compounds gave base peaks at *m/z* 163.039 and 193.050, as observed in 3AQA, accompanied by *m/z* 119.049 [*p*-coumaric acid-H-CO_2]$^-$ (34%) (compound **43**) and 134.036 [ferulic acid-H-CH_3-CO_2]$^-$ (69%) (compound **45**) (Table 1). Thus, **43** and **45** were assigned to 3-*p*-Co-5CQA and 3F-5CQA, respectively. In the same way, **44** and **46** were annotated as 3C-5-*p*-CoQA and 3C-5FQA, witnessed by the base peak at *m/z* 191.055 [quinic acid-H]$^-$ as was seen in 3CQA. Likewise, the base peak at *m/z* 191.055 together with the abundant ions at *m/z* 179.034 and 135.044 defined 3,5-diCQA, while 1,5-diCQA was deduced from the low abundant dehydrated ion at *m/z* 335.078. Vicinal *di*CQA 3,4-diCQA (compound **37**) and 4,5-diCQA (compound **40**) were witnessed by the distinctive dehydrated ion at *m/z* 173.045; this assumption is supported by the chromatographic behavior of both compounds on the reverse phase support being the most polar and lipophilic isomers among the *di*CQA [44]. 3,4,5-triCQA (compound **47**) was discernable by the prominent ions at *m/z* 179.034, 173.045 and 135.044 as was observed in 3,4-disubstituted quinic acid skeleton.

2.2.3.Flavonoids

The flavonoid annotation was based on the fragmentation patterns for different flavonoid classes previously reported in a few Asteraceae species [42, 43, 45], or by using flavonoid standards. Retro-Diels-Alder (RDA) fragmentation allowed for the differentiation of flavon and flavonol derivatives. Thus, quercetin (compounds **51, 53, 54, 57, 61**), kaempferol (compounds **49, 56, 58)** and isorhamnetin (compounds **50, 52, 59, 60**) flavonols were identified from RDA ions $^{1,3}B^-$, $^{1,3}A^-$, $^{0,4}A^-$, $^{1,2}A^-$ and $^{1,2}B^-$ (Table 1). Compounds **51, 53, 56, 58, 59, 61, 62-66** were unambiguously identified by comparison with reference standards. Compound **48** ([M-H]$^-$ at *m/z* 595.168) showed typical fragmentation of *C*-glycosylflavone yielding a series of fragment ions at *m/z* 475.125 [M-H-120]$^-$, 415.104 [M-H-120-60]$^-$, 385.093 [M-H-120-90]$^-$, 355.0822 [M-H-2×120]$^-$ (Zheleva-Dimitrova et al., 2018). The aglycone naringenin in **48** was evidenced by the RDA ions at *m/z* 119.049 ($^{1,3}B^-$) and 107.012 ($^{0,4}A^-$). Thus, **48** was annotated as 6,8-di*C*-hexosyl-naringenin. In **49** and **50** the consecutive loss of two hexose units (2×162 Da) is related to an *O*-dihexosyl linkage, while in **52** *O*-pentosylhexosyl linkage was suggested. Compounds **54**, **55** and **60** presented similar

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fragmentation patterns yielding base peaks at *m/z* 301.036 (compound **54**), 285.041 (compound **55**) and 315.052 (compound **60**) [(M-H)-HexA]$^-$, respectively, indicating flavonoid hexuronides. In the case of **57**, a loss of an acetyl moiety at *m/z* 463.089 allowed to annotate quercetin 3-*O*-acetylhexoside. Unlike the Asteraceae species, only two 6-methoxylated flavonoids **67** and **68** were suggested on the base of the transitions: 329.067→314.044→299.020 (**67**) and 313.072→298.048→283.025 (**68**). Accordingly, **67** and **68** were ascribed to cirsiliol and cirsimaritin, respectively (Table 1).

The identified phenolic compounds as chlorogenic acid, caffeic acid, *p*-coumaric acid, rutin, quercetin etc. are typical for the Senecio taxa [9, 11, 13].

2.3. *Chemophenetic significance*

The chemophenetic significance of phenolic metabolite profiling coupled to morphometric data of the studied Senecioneae species is presented. The raw LC-MS data of annotated specialized compounds were converted and further manipulated with the R programming language as detailed in Section 3.6. A similarity/dissimilarity clustering analysis was conducted based on their normalized area under the curve (AUC) values of those reference compounds found in at least two, out of all five, species. Table S5 presents the selected compounds (rows), and the five studied species (columns). The cells show the calculated AUC, normalized from 0 (in case the compound was not detected in the extract) to 100, by rows. Figure 3 depicts a heatmap of the AUC values from Table S5. The dendrograms separated the compounds (columns) into 5 clusters, and the species (rows) into 2 clusters (Figure 3).

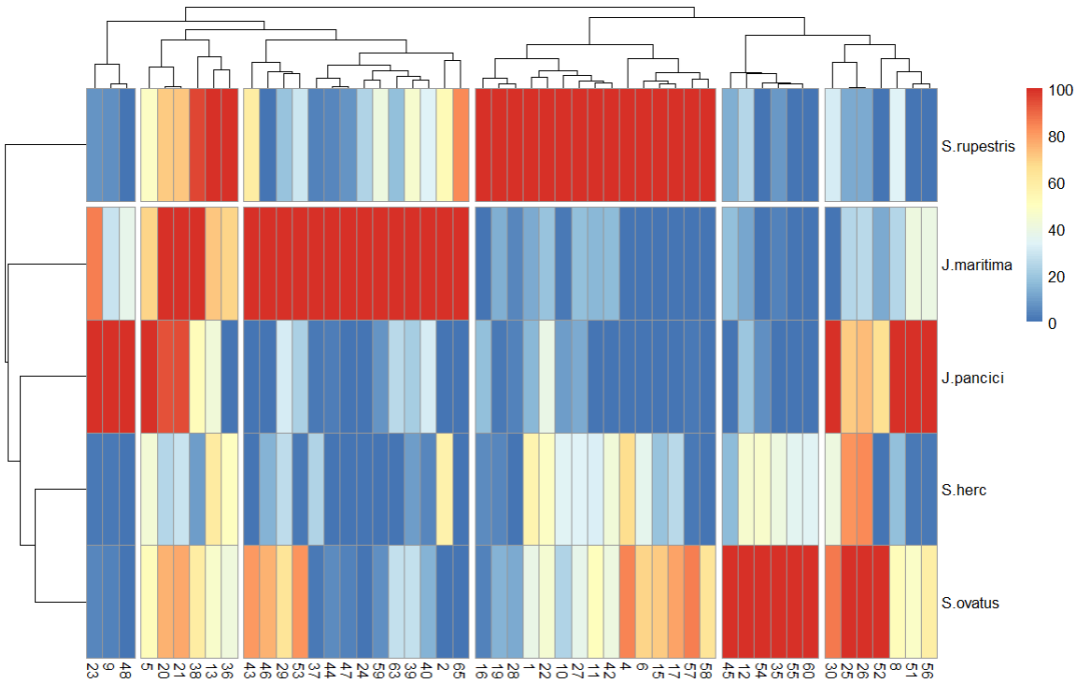

Figure 3. Heatmap of normalized (0-100) AUC values of identified compounds (columns) by species (rows).

The clustering, by rows, did not differentiate the two genera. The greatest resemblance was generated between *S. ovatus* and *S. hercynicus*; *J. pancicii* showed greater similarity to the last-mentioned two species compared to *J. maritima*; *S. rupestris* was cast as a separate node. Table S6 presents the grouped compounds from Figure 3, where it is notable which compounds were characteristic for a given species. Hence, the contribution of annotated phenolic compounds to the phenetic description of the selected taxa was determined. For example, hydroxybenzoic (**1**, **4**, **6**, **11**, **16**, **17**, **42**) and hydroxycinnamic (**10**, **15**, **19**, **22**, **27**, **28**), derivatives as well as the flavonol glucosides (**57** and **58**) were dominant in *S. rupestris*, while diAQAs (**37**, **38**, **39**, **40**, **43**, **44**, **46**), triAQA (**47**), and the flavonols (**53**, **59**,

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63, **65**), were in the highest amount in *J. maritima*. The coumarin **12**, acylquinic acids (**35** and **45),** and flavonoid hexuronides (**54**, **55**, and **60**) were characteristic for *S. ovatus*. *J. pancicii*, on the other hand, presented the highest amount of AQAs (**9**, **23**, **30**), hydroxycinnamic (**5**, **8**), and flavonol (**48**, **51**, **56**) derivatives.

A heatmap of the Euclidean distance, and a PCA plot (of the data in Table S5) are shown in Figure 4, where similar clustering is observed, compared to that in Figure 3.

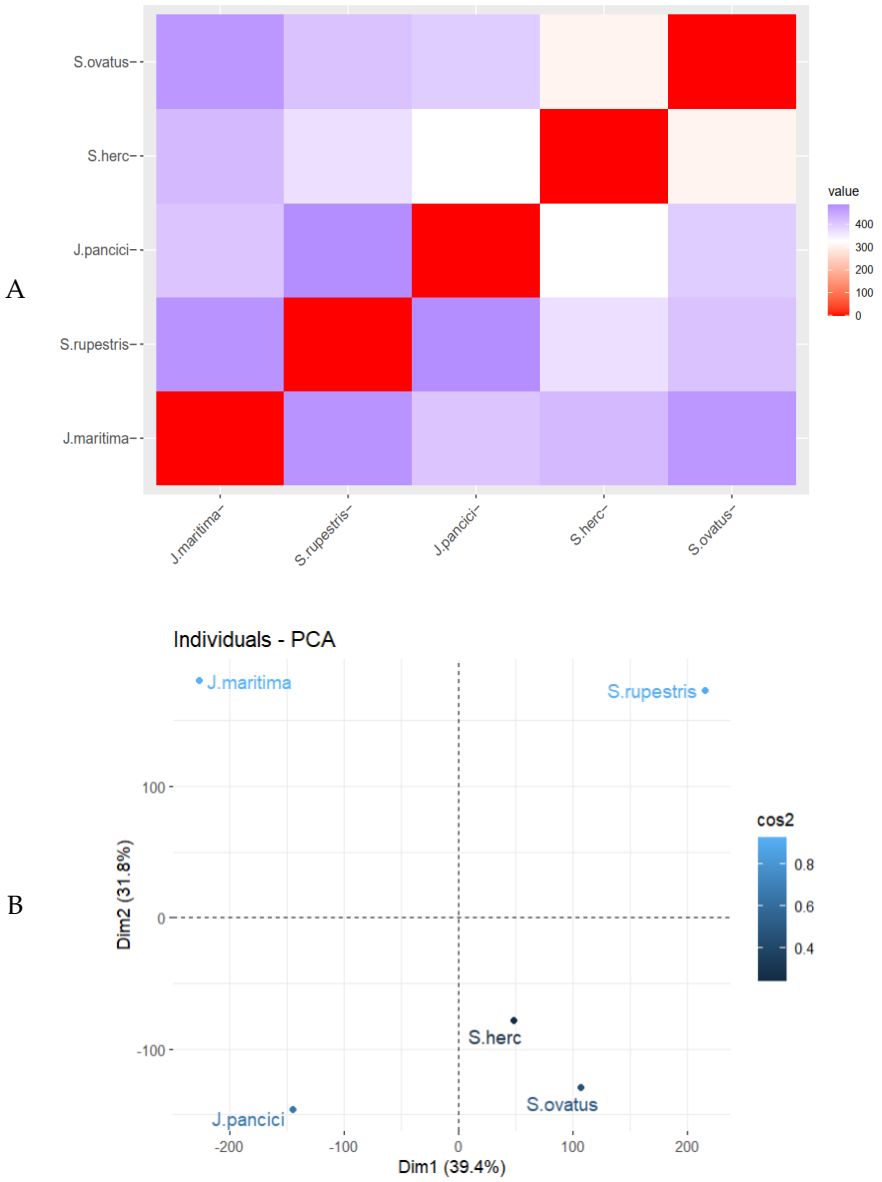

Figure 4. A: Heatmap of euclidean distance using normalized (0 to 100) AUC of identified compounds, in case a compound was not detected in an extract, the AUC for that compound was set to 0; B: Principal component analysis (PCA) (cos2 – quality of representation).

Overall, the morphometric data (Figure 2) corroborates the taxonomical relationship of *S. hercynicus* and *S. ovatus* to the *S. nemorensis* group. Also, a delimitation was observed between the two *Jacoboea* species (*J. maritima* and *J. pancicii*) from genus *Senecio* and distinguishing of the other studied taxa [20], and similar findings were detected by to the unsupervised clustering methods applied on the phytochemical data (Figure 3 and Figure 4). In both morphometric characteristics and phenolics content, *S. hercynicus* and *S. ovatus* showed the highest similarity.

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3. Materials and Methods

3.1. Plant material

The herbal drugs (aerial parts) were collected during the full flowering stage in July 2021 with location coordinates as follow: S. hercynicus at Vitosha Mt., "Zlatni mostove" locality at 1404 m a.s.l. (42.41°N 23.23°E); S. ovatus, S. rupestris, J. pancicii at Vitosha Mt., "Aleko hut" locality at 1855 m a.s.l. (42.58 N 23.29 E); J. maritima at the Black Sea coast, "Golden sand" resort at 24 m a.s.l. (43.28°N 28.04°E). The collected taxa at Vitosha Mt. inhabited one and the same plant community. The plant species were identified according to Vladimirov, 2012 [20]. Voucher specimen of S. hercynicus was deposited at Herbarium Academiae Scientiarum Bulgariae (SOM 177012). S. ovatus, S. rupestris, J. pancicii, J. maritima specimens were given at Herbarium Facultatis Pharmaceuticae Sophiensis, Medical University-Sofia, Bulgaria (Voucher specimen № 11 631 - 11 634).

3.2. Morphometric measurements

Morphometric measurements on the studied *Senecio* and *Jacobaea* species were performed on 15 randomly chosen plants, from each species, during the full flowering stage. The morphometric variability was determined using 12 quantitative characters (parameters) as follows: X_1 - root diameter [cm], X_2 - stem height [cm], X_3 - leaf length [cm], X_4 - leaf width [cm], X_5 - involucral bract length [cm], X_6 - involucral bracts number per capitula, X_7 - ray flower length [cm], X_8 - number of ray flowers per flower head, X_9 - disc flower length [cm], X_{10} - number of disc flowers per flower head, X_{11} - flower head diameter [cm], X_{12} - number of capitula per plant. The morphometric measurements are presented in Table S1. Descriptive statistics of the 12 characteristics was performed in the R programming language by the *arsenal* package [26] and presented in Table S2.

3.3. Chemicals and reagents

Acetonitrile and formic acid for LC-MS, and methanol of analytical grade, were purchased from Merck (Merck, Bulgaria). The reference standards used for compounds identification were bought from Phytolab (Vestenbergsgreuth, Bavaria, Germany).

3.4. Sample extraction and sample preparation

Air-dried powdered aerial parts (5 g, combined plant material belonging to the same species) were extracted with 80% MeOH (1:20 w/v) by sonication (100 kHz) for 15 min (×2) at room temperature. Then, the extracts were concentrated *in vacuo* and lyophilized to yield crude extracts: *S. hercynicus* – 0 .74 g, *S. ovatus* – 0.71 g, *S. rupestris* – 1.02 g, *J. pancicii* – 0.95 g, *J. maritima* – 0.96 g.

3.5. Ultra-High-Performance Liquid Chromatography – High Resolution Mass Spectrometry (UHPLC-HRMS)

Elution was achieved on a reversed phase column Kromasil EternityXT C18 (1.8 μm, 2.1 × 100 mm, AkzoNobel, Sweden) column maintained at 40°C. The binary mobile phase consisted of A: 0.1% formic acid in water and B: 0.1% formic acid in acetonitrile. The run time was 24.5 min. Prior to injection, the mobile phase was held at 50% B for 4.5 min and then gradually turned at 5% B in 0.5 min. After injection, the % B was gradually turned to 60% B over 15 min, and then held at 60% B for 3 min, increased gradually to 95% B over 3 min, held at 95% B over 2 min, then turned to 50% B in 0.5 min. The retention time of identified compounds ranged between 1.74 and 9.60 min. The flow rate and the injection volume were set to 300 μL/min and 1 μL, respectively. The effluents were connected online with a Q Exactive Plus Orbitrap mass spectrometer (ThermoFisher Scientific) where the compounds were detected. Data were processed with Xcalibur software 4.2 (ThermoFisher Scientific).

Mass analyses were carried out on a Q Exactive Plus mass spectrometer (ThermoFisher Scientific) equipped with a heated electrospray ionization (HESI-II) probe (ThermoFisher Scientific). The tune parameters were as follows: spray voltage 3.5 kV; sheath gas flow rate 38; auxiliary gas flow rate 12; spare gas flow rate 0; capillary temperature 320 °C; probe heater temperature 320 °C and S-lens RF level 50. Acquisition was acquired

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at Full-scan MS and Data Dependent-MS^2 modes. Full-scan spectra over the *m*/*z* range 100 to 1000 were acquired in negative ionization mode at a resolution of 70,000. Other instrument parameters for Full MS mode were set as follows: AGC target 1e6, maximum ion time 80 ms, number of scan ranges 1. For DD-MS^2 mode, instrument parameters were as follows: microscans 1, resolution 17,500, AGC target 1e5, maximum ion time 50ms, MSX count 1, isolation window 1.0 *m*/*z*, stepped collision energy (NCE) 10, 30, 60. Data acquisition and processing were carried out with Xcalibur 4.2 software (ThermoFisher Scientific).

3.6. File conversions and data analysis

After the .raw (ThermoFisher Scientific) mass spectrometric files were obtained, they were converted to .ms1 (MS1 data) and .mgf (MS2 data) files by MSConvertGUI 3.1 (ProteoWizard). Then the .ms1 and .mgf files were imported to RStudio (2021, Build 382) and further manipulated under the R programming language (version 4.2.1, 2022-06-23, "Funny-Looking Kid"). The MS2 spectra were screened for the presence of the available target (hydroxybenzoic acid derivatives and flavonoids) standard compounds. The screening was achieved by selecting spectra based on the following criteria: *m*/*z* error of the molecular ion < 15 ppm (minimum 0.0010 Da), retention time error < 2% (minimum 0.05 min, maximum 0.15 min), number of fragment ions match > 2/3, error of the percentage intensity of matched fragment ion < 15. Similar MS2 scans found in the same chromatographic peak were grouped, i.e., the spectra were summed, the *m*/*z* were adjusted by weight averaging

$$(m/z)_{avg} = \frac{\sum_{i=1}^{N} int_i \times (m/z)_i}{N}$$

where $(m/z)_{avg}$ is the recalculated *m*/*z* value, $(m/z)_i$ and int_i are the *m*/*z* and the intensity of the ith fragment ion, respectively. The areas under the curve (AUC) of the identified compounds were calculated and normalized from 0 to 100.

Data analysis were performed in the R programming language (R version 4.2.1., 2022-06-23, Funny-Looking Kid), operated under the RStudio environment (2022.07.2 Build 576). R packages used include: "MASS" [27], "klaR" [28], "caret" [29], "ISLR" [30], "leaps" [31], "factoextra" [32], "cluster" [33], "lpSolve" [34], "DescTools" [35], "pheatmap" [36], "arsenal" [26]. Distance matrices were generated using the "euclidean" method, and hierarchical clustering was performed using the "ward.D2" method. The complete R code used for morphometric analysis is presented in the Supplementary material.

4. Conclusions

Herein, a chemophenetic study of three *Senecio* (*S. hercynicus, S. ovatus* and *S. rupestris*) and two *Jacobaea* species (*J. pancicii* and *J. maritima*) is presented. From the collected morphometric data, describing 12 parameters, a distinguishment of species by genera was performed by a Linear Discriminant Analysis (LDA). Among the studied species, *S. hercynicus* and *S. ovatus* presented the greatest similarity, and hence, their formed clusters were the closest. Even though no overlap in the LDA analysis was observed between the Jacoboea and Senecio species, *J. pancicii* and *J. maritima* did not demonstrate likeness. A phytochemical analysis by UHPLC-Orbitrap-HRMS revealed 46 hydroxybenzoic, hydroxycinnamic, acylquinic acids and their derivatives, 1 coumarin, and 21 flavonoids. Hierarchical and PCA clustering applied to the phytochemical data corroborated the similarity of *S. hercynicus* and *S. ovatus,* established in the morphometric analysis. The study highlights the similarity/dissimilarity, both morphometric, and in manner of specialized metabolytes, of the selected species belonging to *Senecio* and *Jacobaea* genera (*Senecioneae*).

Supplementary Materials: The following supporting information can be downloaded online, Table S1: Scaled and unscaled raw morphometric data; Table S2: Descriptive statistics on the morphometric data; Table S3: Selection of a model with n variables; Table S4: Prediction of membership of the test set (n = 15); Table S5: Normalized (by rows) AUC values of identified specialized natural

compounds found in at least two out of all five studied species; Table S6: The compounds presented in Figure 3 grouped by the hierarchical clustering; Figure S1: Residual sum of squares (RSS), adjusted R2, Mallow's Cp, and Bayesian information criterion (BIC) for the standardized morphological data. R code: contains the code written in the R programming language for the analysis of the morphological data.

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