Turk J Pharm Sci 2023;20(6):380-389
DOI: 10.4274/tjps.galenos.2023.60562

ORIGINAL ARTICLE

Phenolic Compound Composition of *Sambucus nigra* L. Wild-Growing Plants from Kosovo

Bujar QAZIMI[1], Jasmina Petreska STANOEVA[2], Marinela CVETANOSKA, Nikola GESKOVSKI[3], Shpend DRAGUSHA[1], Hyrije KORAQI[1], Vesel QAZIMI[4], Valon EJUPI[1]*

[1]UBT-Higher Education Institution, Faculty of Pharmacy, Prishtina, Republic of Kosovo
[2]University Ss. Cyril and Methodius, Faculty of Natural Sciences and Mathematics, Institute of Chemistry, Skopje, North Macedonia
[3]University Ss. Cyril and Methodius, Faculty of Pharmacy, Institute of Pharmaceutical Technology, Skopje, North Macedonia
[4]Medical High School, "Nikola Shtejn", Tetovo, North Macedonia

ABSTRACT

Objectives: The aim of this study was to determine the phenolic components in the flowers and leaves of wild-growing *Sambucus nigra* L.

Materials and Methods: Plant materials were collected from eleven localities in Kosovo. Before LC-DAD-ESI-MS^n analysis, an ultrasonic-assisted method with 70% methanol for 30 min extraction was used.

Results: In total, 34 and 37 phenolic compounds were identified in flower and leaf extracts, respectively, with a total content of 61321.82-85961.64 mg/kg dry weight (DW) and 36136.62-93890.37 mg/kg DW. In all of the analyzed extracts, 15 phenolic acids, 20 flavonoids, one lignan, and one coumaroyl iridoid were detected. The major components were flavonoids, especially flavonols (quercetin-3-rutinoside, caffeoyl-kaempferol, and isorhamnetin-3-rutinoside), followed by phenolic acids (dicaffeoylquinic acid isomer, caffeic acid derivative, dicaffeoylquinic acid isomer, and dicaffeoylquinic acid isomer).

Conclusion: In general, the methanolic extracts of flowers have shown higher polyphenolic content than those found in leaves. The multivariate statistical analysis of the phenolic content of the samples resulted in PLS-DA models with appropriate correlation coefficients of 0.903 and 0.921 for flower and leaf extracts, respectively. The models revealed distinctive clustering patterns, and the loading scatter plots depicted the unique phenolic compounds specific to each sample group.

Key words: *Sambucus nigra* L., flower, leaf, phenolic compounds, LC-DAD-ESI-MS^n

INTRODUCTION

Sambucus nigra L. (known as European elder, elderberry, black elder, or elder)[1] is a European species with an oceanic to suboceanic, cool-temperate, and west Mediterranean range.[2] *S. nigra* tolerates poor soil conditions or disturbed soils known as eutrophic soils and grows in sunlight-exposed locations.[1] It can be found in forests, thickets, parks, balks, and in-home gardens.[3]

S. nigra leaves, as its principal biomass, are a valuable source of flavonoids.[4] Historically, the leaves were considered to relieve pain and promote healing when applied as a poultice. The flowers and fruits of *S. nigra* have been used successfully for centuries for medicinal purposes and to prepare tea, wines, and liquors. Traditional folk medicine uses the infusion from dried flowers because of its diuretic effects, which include reducing fever, promoting perspiration, and moderating cough.[5] Elder cultivars are used as beverages and food flavoring.[6] Moreover, both the fruits and the flowers are sources of flavonols, proanthocyanidins, and phenolic acids. Dried flowers of elder (*Sambuci flos*) are most often used in various tea compositions,[7] but they are also used in several cosmetics and medicinal products.[5,8]

The beneficial health-promoting effects of elderberries and elderflowers are well known, including beneficial effects against

*Correspondence: valon.ejupi@ubt-uni.net, Phone: +383 38 542 138, ORCID-ID: orcid.org/0000-0002-0367-9878
Received: 08.08.2022, Accepted: 16.01.2023

degenerative diseases (cardiovascular and inflammatory diseases), cancer, and diabetes, as well as antioxidant, anti-inflammatory, immunostimulant, chemopreventive,[1,2] and atheroprotective effects.[1] More recent pharmacological studies have shown it to have antibacterial,[8] antiviral, anti-inflammatory,[2] and immunomodulatory activities.[4] Recent experimental evidence also suggests that it is a powerful antioxidant.[1,2,9]

Polyphenols represent a large and diverse group of plant secondary metabolites abundantly found in the plant kingdom.[10,11] Apart from their use as food supplements or as additives in functional foods, natural phenolic compounds have also become increasingly attractive from a technological point of view because of their possible exploitation in materials science.[12]

Elderberry flowers possess a substantial supply of bioactive flavonoids, antioxidants, and phenolic compounds, which have been frequently used in traditional medicine and healing. Different solvents and infusion times in extraction may induce differences in phenolic composition and bioactive features.[9] Several extraction methodologies have been reported for the extraction of phenolic compounds from plant materials, mostly based on the use of organic solvents such as methanol, ethanol, acetone[12], ethyl acetate, and their combinations, often with different proportions of water.[11]

Ultrasonic-assisted extraction is suitable for the extraction of plant bioactive molecules such as polyphenols, providing extracts with higher concentrations of active compounds and enhanced biological activity.[13]

The aim of this work was to determine the phenolic compounds in the methanolic extracts of flowers and leaves of wild-grown *S. nigra* using LC/DAD/ESI-MS^n.

MATERIALS AND METHODS

Plant material: Plant materials were collected at the flowering stage from eleven localities in Kosovo (Table 1) during May-June, 2021. Plant identity was verified by Professor Shkelzim Ukaj and voucher specimens were deposited at the Herbarium at the Department of Pharmacognosy, Faculty of Pharmacy, UBT, Prishtina, Kosovo (PhGNFF/UBT-02). They were air-dried and stored in a paper box until analysis.

Extraction of phenolic compounds: The plant material (flowers and leaves) was collected separately, dried, and homogenized (Ika Laborteknik, Staufen, Germany) for 30 s. The obtained powders were used for phenolic extraction. A total of 0.5 g of powdered plant material was extracted with 20 mL of 70% methanol for 30 min using an ultrasonic bath at room temperature. The supernatant was filtered through a 0.45 µm pore-size polyethersulfone filter before analysis.

LC/DAD/ESI-MS^n analysis: For LC/DAD/ESI-MS^n analysis, an Agilent HPLC 1100 system coupled with an ion trap mass spectrometer was used. A C18 Eclipse SB column (Agilent) with dimensions of 150 mm x 4.6 mm, 5 µm for chromatographic separation of polyphenolic compounds was used. Water-formic acid (0.1%, *v/v*) (A) and acetonitrile (B) were used as a mobile phase starting with 20% B for 5 min and after it was installed to reach 30% B at 10 min (5 min linear), 55% B at 25 min (5 min linear), 65% B at 45 min, and 100% B at from 50 to 65 min. The flow rate was 0.4 mL min^{-1} and the injection volume was 20 µL.

Spectral data from all peaks were accumulated in the 190-600 nm range, and chromatograms were recorded at 280, 330, and 350 nm.

MS and MS^2 spectra were acquired in the negative ionization mode with an electrospray ionization (ESI) system using nitrogen as the nebulizing gas at a pressure and flow of 50 psi and 12 L min^{-1}, respectively. The heated capillary and voltage were maintained at 325 °C and 4 kV, respectively. The full scan covered the mass range at *m/z* 100-1200.

Identification and quantification of polyphenolic compounds

The identification of various classes of polyphenols was based on a comparison of their retention times, UV-Vis spectra, and MS/MS^n fragmentation patterns with those of the available standards and literature data. Quantification was performed using the area under the peaks in HPLC/DAD chromatograms and the corresponding regression curves ($R^2 \geq 0.999$) of authentic standards. Phenolic acid derivatives were quantified as caffeic acid equivalents at 330 nm, whereas flavonoids were quantified as quercetin equivalent at 350 nm.

Statistical analysis

Statistical analysis was performed using Simca 14.1 statistical software (Umetrics, Sartorius Group, Sweden). Principal component analysis (PCA) was employed to elucidate patterns of polyphenolic content that was specific to certain samples (flowers or leaves), and hierarchical cluster analysis (HCA). For that purpose, Ward's method was performed to group the samples based on their PCA scores. A subsequent partial least-square discriminatory analysis (PLS-DA) was conducted to build a correlation model among the sample's group affiliation (dependent - Y- variables) and their quantitative polyphenolic composition (independent - X- variables). Correlation coefficients were used to determine the model goodness of fit, whereas score scatter plots and loading plots were employed to depict the polyphenolic compounds that made an important contribution to sample classification.

RESULTS and DISCUSSION

Phenolic compounds in wild-grown *S. nigra* methanolic extracts were identified by their retention times, UV spectra, deprotonated molecular ions, and corresponding ion fragments using LC/DAD/ESI-MS^n (Table 2).

A total of 34 and 37 individual compounds was identified in the methanolic extracts of flower (SN-FL) and leaf (SN-LE) of *S. nigra,* representing 61321.82 (SN10-FL) - 85961.64 (SN3-FL) mg/kg DW (Table 3) and 36136.62 (SN4-LE) - 93090.37 (SN8-LE) mg/kg DW (Table 4) of the total content, respectively.

Phenolic compounds in the SN-FL and SN-LE samples were classified into three groups, *e.g.* phenolic acids (14 and 15), flavonoids (18 and 20), and lignans (1 and 1), as one compound of the coumaroyl iridoids group (1 and 1), respectively.

Table 1. Collection of data for plant material from eleven different natural populations of *Sambucus nigra* L. from Kosovo

No	Localities	*Sambucus nigra* Voucher specimen		Latitude	Longitude	Altitude (m)
		Flower	Leaf			
1.	Zllakuqan (Klinë)	SN1-FL	SN1-LE	42°39′59″ N	20°32′14″ E	401
2.	Istog	SN2-FL	SN2-LE	42°47′11″ N	20°29′09″ E	519
3.	Vitomiricë	SN3-FL	SN3-LE	42°41′30″ N	20°19′13″ E	525
4.	Pejë	SN4-FL	SN4-LE	42°39′41″ N	20°15′43″ E	565
5.	Skivjan	SN5-FL	SN5-LE	42°26′11″ N	20°22′21″ E	428
6.	Strellc (Deçan)	SN6-FL	SN6-LE	42°32′49″ N	20°17′26″ E	617
7.	Krushë e made (Gjakovë)	SN7-FL	SN7-LE	42°19′13″ N	20°37′58″ E	314
8.	Sopij (Suharekë)	SN8-FL	SN8-LE	42°20′09″ N	20°50′50″ E	443
9.	*Nerodime e epërme* (Ferizaj)	SN9-FL	SN9-LE	42°22′13″ N	21°04′35″ E	638
10	Graçanicë	SN10-FL	SN10-LE	42°36′29″ N	21°10′24″ E	588
11	Podujevë	SN11-FL	SN11-LE	42°53′46″ N	21°13′02″ E	595

Phenolic acids, a subclass of plant phenolics, possess phenol moiety and resonance stabilized structure, which causes H-atom donation and results in antioxidant properties through a radical scavenging mechanism.[14]

Total amount of phenolic acids in the SN-FL and SN-LE samples ranged from 15816.44 to 26985.19 mg/kg DW and 8632.18 to 28883.19 mg/kg DW, respectively. Four peaks with MS fragmentation ions at *m/z* 353 were attributed to the dicaffeoylquinic acid isomer (Table 2).

Dicaffeoylquinic acid isomer (peak no: 24) (2997.09-7675.36 mg/kg DW) and caffeic acid derivative (peak no: 4) (1018.73-7675.36 mg/kg DW) comprised 44.35-73.74% of the total phenolic acid content, followed by dicaffeoylquinic acid isomer (peak no: 23), dicaffeoylquinic acid isomer (peak no: 25), and *p*-coumaroyl-caffeoylquinic acid isomer (peak no: 33) were the dominant phenolic acids in flower (Table 3). In the leaves (Table 4), the major phenolic acids were caffeic acid derivative (peak no: 4) (2965.58-8571.72 mg/kg DW), dicaffeoylquinic acid isomer (peak no: 24) (201.23-4830.37 mg/kg DW) followed by 4-caffeoylquinic acid (peak no: 5), and dicaffeoylquinic acid isomer (peak no: 23).

Flavonoids, a class of polyphenol secondary metabolites, are believed to have various bioactive effects, including antiviral, anti-inflammatory, cardioprotective, antidiabetic, anticancer, and antiaging *etc.*[15] Flavonoids were the dominant group of polyphenols in the flower (Table 3) and leaves (Table 4) of *S. nigra* and represented 57-66.50% and 56.65-84.60% of the total analyzed polyphenolics, respectively. The total content of flavonoids in SN-FL and SN-LE samples ranged from 34997.17 to 57124.80 mg/kg DW and 20474.20 to 78757.38 mg/kg DW, respectively.

Flavonols were the main flavonoids in all samples of flowers and leaves with a total content of 34997.17-57124.80 mg/kg DW and 20474.20-78757.38 mg/kg DW, respectively. The prevailing compounds of flavonols in flowers were quercetin-3-rutinoside (peak no: 19) (6970.56-26685.21 mg/kg DW), caffeoyl-kaempferol (peak no: 6) (5734-12724.98 mg/kg DW), isorhamnetin-3-rutinoside (peak no: 22) (2583.7-11456.17 mg/kg DW), comprising 46-95% of the total flavonols, followed by quercetine malonyl diglucoside (peak no: 10), kaempferol-3-rutinoside (peak no: 21), and quercetin galloyl pentoside (peak no: 27). The component quercetin coumaroyl-rhamno-glucoside (peak no: 8) was found only in sample SN4-FL (3182.32 mg/kg DW). The dominant components of flavonols in leaves were caffeoyl-kaempferol (peak no: 6) (1157.34-27342.46 mg/kg DW), quercetin-3-rutinoside (peak no: 19) (1393.79-11917.56 mg/kg DW), and quercetin coumaroyl-rhamno-glucoside (peak no: 8) (2271.74-8958.14 mg/kg DW), comprising 25-62% of the total flavonols, followed by quercetin malonyl diglucoside (peak no: 10). Qualitatively and quantitatively, flavones and flavanones were the flavonoid representatives with the lowest content.

Lignan coumaroyl glucoside (peak no: 13) is a polyphenol of the non-flavonoid group with contents of 2224.9-4243.42 mg/kg DW and 1387.27-5071.77 mg/kg DW in SN-FL and SN-LE samples, respectively.

p-Coumaroyl dihydromonotropein (peak no: 34) belongs to the group of coumaroyl iridoids and was present in all analyzed samples of flowers (989.70-3276.52 mg/kg DW), whereas it was found only in some analyzed leaf samples (77.91-166.5 mg/kg DW).

Generally, the polyphenol content is higher in methanolic extracts of the flowers than in the leaves, except for the SN8-LE sample, where the polyphenol content is higher (93090.37 mg/kg DW). The SN6 sample was characterized by an approximate polyphenolic content in the flowers and leaves of 65296.79 and 69548.62 mg/kg DW, respectively. In other samples, a difference was observed between the polyphenolic content in the flowers and leaves of *S. nigra.*

Moreover, in agreement with previous studies, flavonoid amounts are greater in flowers than in leaves of *S. nigra.*[18]

Uzlasir et al.[9] analyzed the phenolic compositions of methanol, ethanol, and aqueous extracts of elderberry flowers using LC-

Table 2. Spectral data and retention time of polyphenolic compounds identified in methanol extracts of *Sambucus nigra* L. flowers and leaves

	Components				
Peak no.	Phenolic acids and their derivatives	t_R/min	λ_{max}/nm	[M-H]$^-$	MS^2
1	Quinic acid	3.927	235, 255	191	127, 173
2	3-Caffeoylquinic acid	4.116	288, 328	353	191, 173
3	5-Caffeoylquinic acid	4.557	288, 298 sh, 328	353	191
4	Caffeic acid derivative	5.221	290, 326	709	621, 534, 463, 353, 324
5	4-Caffeoylquinic acid	5.986	218, 300 sh, 326	353	191, 179, 173, 161, 135, 127
7	Caftaric acid	7.069	234, 294, sh, 324	311	179, 135
14	Coumaroylquinic acid	10.03	244, 294, sh, 324	337	191
23	Dicaffeoylquinic acid isomer	17.153	266, 346	515	447, 353, 299, 203, 173
24	Dicaffeoylquinic acid isomer	17.462	246, 298, sh, 328	515	353, 191, 179
25	Dicaffeoylquinic acid isomer	18.002	232, 300, sh, 314	515	471, 353, 299, 203, 173
29	*p*-Coumaroyl-caffeoylquinic acid isomer	20.106	234, 300, sh, 316	499	337, 163
30	*p*-Coumaroyl-caffeoylquinic acid isomer	20.338	234, 300, sh, 316	499	353, 191
31	Dicaffeoylquinic acid isomer	20.619	262, 298, 328	515	353
32	*p*-Coumaroyl-caffeoylquinic acid isomer	21.37	316	499	353, 337, 163
33	*p*-Coumaroyl-caffeoylquinic acid isomer	21.636	318	499	353, 337, 191
36	Caffeic acid derivative	26.817	234, 296, sh, 320	709	671, 353
Flavonols					
6	Caffeoyl-kaempherol	6.235	242, 298, sh, 328	447	439, 401, 285
8	Quercetin coumaroyl-rhamno-glucoside	7.574	256, 354	755	591, 489, 343, 300, 271
9	Quercetin coumaroyl-rhamno-glucoside	7.995	232, 298, sh, 326	755	737, 609, 593, 573, 489, 343, 301, 271
10	Quercetin malonyl diglucoside	8.335	316	771	667, 625, 505, 487, 365, 301
11	Quercetin malonyl diglucoside	8.696	332	771	667, 625, 505, 487, 365, 301
12	Quercetin 3-*O*-diglucoside	8.732	312	623	505, 445, 343, 301, 271
15	Kaempferol coumaroyl-rhamno glucoside	10.964	266, 348	739	593, 575, 473, 327, 285, 255
16	Quercetin caffeoyl pentoside	11.438	246, 296, sh, 328	595	475, 445, 301
17	Isorhamnetin diglucoside	12.871	234, 254, 343	639	607, 459, 315
18	Kaempferol coumaroyl -glucoside	13.219	226, 292, sh, 312	593	521, 359, 329, 285
19	Quercetin-3-rutinoside	14.211	256, 354	609	343, 301, 271
20	Quercetin-3-glucoside	15.887	254, 330	463	301
21	Kaempferol-3-rutinoside	16.17	266, 348	593	357, 327, 285, 229, 211
22	Isorhamnetin-3-rutinoside	16.489	256, 356	623	357, 315, 301, 271, 255
26	Kaempherol-3-malonylglucosede	18.422	266, 346	533	489, 285
27	Quercetin galloyl pentoside	18.909	254, 354	585	541, 459, 315, 301
28	Isorhamnetin octylglucoside	19.233	254, 354	519	477, 357, 315, 271
Flavones					
36	Acetyl-isoorientin	29.34	312	312	327, 291
37	Hydroxy trimethoxy flavonoid	27.484	363	368	291, 271, 229, 211, 171
Flavanones					
38	Naringenin	28.156	288	271	151
Lignans					
13	Lignan coumaroyl glucoside	9.428	228, 312		
Iridoids					
34	*p*-Coumaroyl dihydromonotropein	22.536	236, 292, sh, 320	537	389, 373, 331, 313, 193, 163

sh: Shoulder

Table 3. Phenolic content (mg/kg DW) in the methanolic extract of *Sambucus nigra* L. flower (SN-FL) (peak no: Compound according to Table 2)

Peak no.	SN1-FL	SN2-FL	SN3-FL	SN4-FL	SN5-FL	SN6-FL	SN7-FL	SN8-FL	SN9-FL	SN10-FL	SN11-FL
Phenolic acids and their derivatives											
1	355.58	76.04	95.71	87.46	72.17	60.68	62.56	97.89	72.58	100.99	46.99
2	828.82	412.51	608.42	437.26	550.8	471.46	561.44	462.37	496.71	510.37	553.61
3	387.86	593.52	791.81	257.58	490.16	496.66	496.27	800.25	592.07	701.32	429.53
4	5193.94	5090.66	4846	5252.27	5840.06	6087.05	5977.34	5313.38	5453.59	4018.73	7675.36
5	40.9	122.19	125.64	126.31	110.99	73.01	77.59	109.25	116.18	84.36	114.02
7	328.1	188.28	288.32	291.67	195.78	240.84	306.36	203.54	323.21	128.19	303.15
14	90.99	90.35	93.61	133.27	116.45	85.54	182.12	107.21	176.58	92.36	119.98
23	1817.29	1459.72	2008.99	1471.75	1281.27	1371.71	1485.39	1291.26	1359.09	1275.94	1198.03
24	8965.25	7420.57	12223.06	4197.8	8930.07	6615.19	8458.61	7702.74	9760.46	9462.73	2997.09
25	-	1907.36	1803.57	1504.67	1580	1355.74	1361.32	1518.79	917.84	772.2	1625.98
29	777.6	590.94	621.91	315.59	575.82	467.08	817.33	580.04	598.69	878.16	274.05
30	979.59	779.07	709.34	517.36	777	795.76	1231.32	902.48	857.56	955.84	365.23
31	-	-	-	-	-	-	-	-	-	-	-
32	1442.15	1010.47	1244.48	1431.38	1278.06	1191.25	1436.6	1189.02	1289.73	1064.62	1108.88
33	1178.45	989.7	1244.48	3276.52	1278.06	1191.25	1436.6	1189.02	1289.73	1528.8	-
36	115.01	133.62	279.84	171.61	212.73	512.22	191.14	137.16	145.31	97.9	202.57
Flavonols											
6	8886.34	10182.37	8738.78	5734	8088.94	9385	9387.17	12724.98	8536.82	9731.51	9384.68
8	-	-	-	3182.32	-	-	-	-	-	-	-
9	-	-	-	-	-	-	-	-	-	-	-
10	1617.89	2630.86	3611.13	1981.46	1462.29	2214.63	1544.1	1593.8	1552.91	1684.56	1912.21
11	1651.32	2659.23	-	1334.75	2077.66	1333.66	1675.55	1358.87	1339.24	1650.73	1310.2
12	-	-	-	-	-	-	-	-	-	-	-
15	-	-	-	-	-	-	-	-	-	-	-
16	310.83	618.72	286.19	473.69	385.74	367.54	529.81	455.2	594.13	476.42	381.38
17	1543.47	1558.02	1706.34	1762.07	1943.74	1610.53	1910.31	1889.47	1657.88	1493.58	-
18	-	276.02	381.87	271.04	105.66	313.13	332.55	296.57	185.86	81.88	74.44
19	12746.03	21829.87	26685.21	11660.99	16427.71	10881.45	12006.64	12411.14	22686.34	6970.56	11374.09
20	1052.34	1085.05	1049.48	1239.46	1124.08	1060.88	1164.5	1271.33	-	-	1075.97
21	2398.14	1767.28	2441.29	1390.08	1378.72	1471.7	1689.34	1556.49	1683.33	1173.2	1272.23
22	8536.84	2583.7	4159.93	8113.13	6611.06	3650.84	9515.6	7870.72	11456.17	7431.44	9416.35
26	1819.36	1083.51	1256.33	1229.4	1274.39	1103.44	1317.37	1259.92	1268.08	1144.56	1125.02
27	2560.25	1154.33	1321.74	1929.12	1500.48	1389.63	3184	2842.77	2487.37	1461.26	2793.77
28	1080.71	1061.51	1194.27	1324.17	1292.18	1421.12	1200.25	1297.17	-	-	1315.88
Flavones											
35	1097.03	931.57	1000.52	956.56	1017.08	1017.51	1091.02	1012.75	1046.32	1062.99	902.87

Table 3. Continued											
37	895.22	764.72	1007.57	1044.04	884.89	1240.76	803.32	809.13	747.66	967.18	1302.3
Flavanones											
38	1.18	4.79	5.6	5.7	6.41	11.86	6.74	4.15	5.42	5.79	19.39
Lignans											
13	2879.48	3419.24	3164.58	2224.91	3377.39	2810.71	3562.25	4010.78	3187.96	4243.42	3070.25
Iridoids											
34	424.32	658.19	865.63	2097.35	530.93	2996.96	922.26	754.42	664.25	70.23	938.01

(-): Not detected

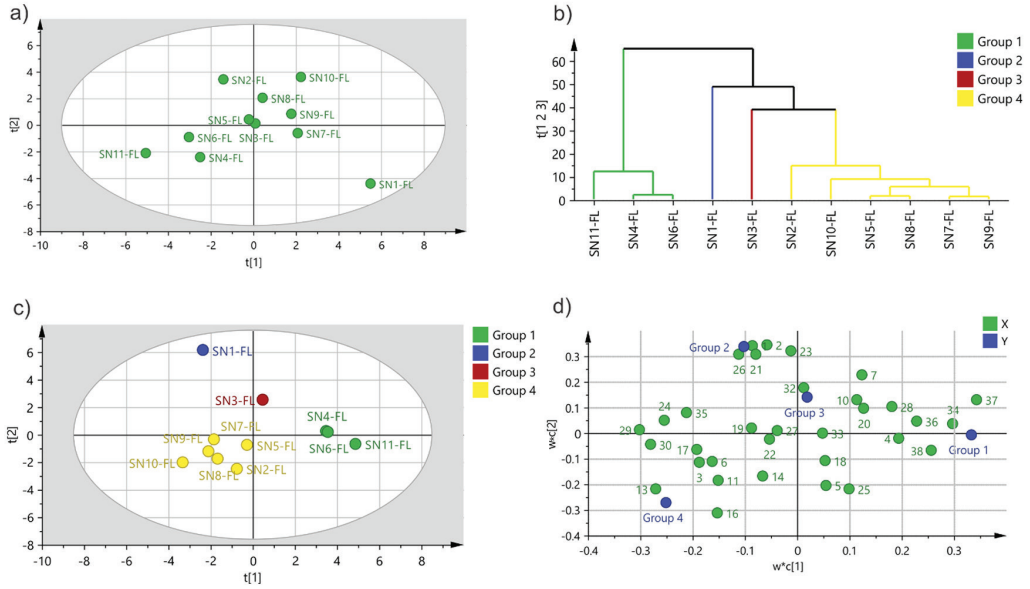

Figure 1. Multivariate analysis of the phenolic content of the flower extracts: a) score scatter plot from the first two components of the PCA model; b) HCA dendrogram with colored group clusters; c) score scatter plot from the first two components of the PLS-DA model colored according to the clustered sample groups; d) loading scatterplot for the first two components of the PLS-DA model

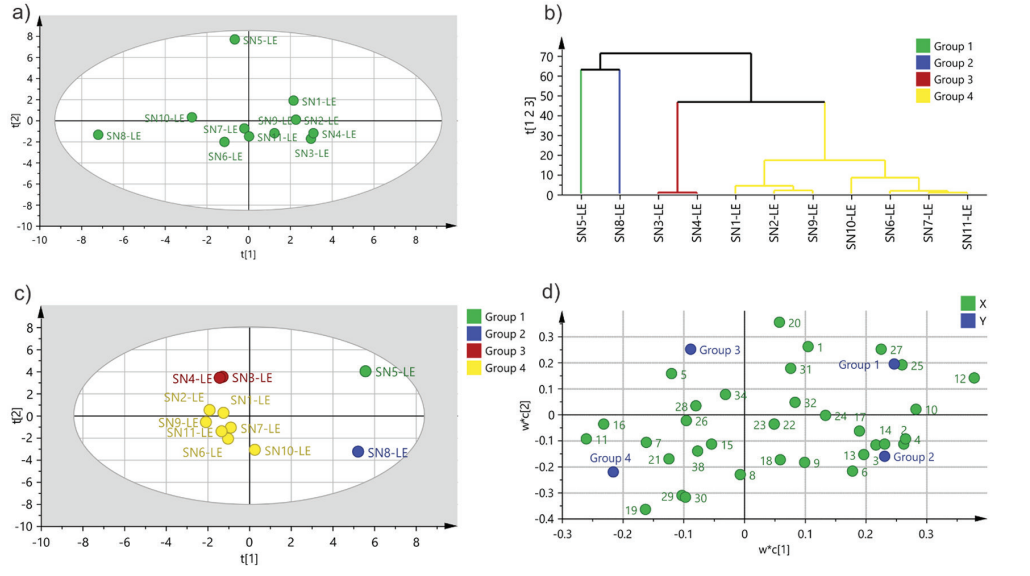

Figure 2. Multivariate analysis of the phenolic content of leaf extracts: a) score scatter plot from the first two components of the PCA model; b) HCA dendrogram with colored group clusters; c) score scatter plot from the first two components of the PLS-DA model colored according to the clustered sample groups; d) loading scatterplot for the first two components of the PLS-DA model

Table 4. Phenolic content (mg/kg DW) in the methanolic extract of *Sambucus nigra* L. leaf (SN-LE) (peak no: Compound according to Table 2)

Peak no.	SN1-LE	SN2-LE	SN3-LE	SN4-LE	SN5-LE	SN6-LE	SN7-LE	SN8-LE	SN9-LE	SN10-LE	SN11-LE
Phenolic acids and their derivatives											
1	44.76	60.84	67.05	65.19	62.33	31.65	61.06	47.05	24.33	51.04	19.45
2	508.77	570.68	516.34	520.83	941.46	980.2	676.83	1272.66	634.89	729.53	768.26
3	512.9	940.11	765.74	681.43	1711.3	2012.96	1291.38	3201.23	138.1	2214.58	1250.49
4	2965.59	3585.7	3309.91	3370.72	7109.55	6857.9	5011.2	8571.72	4984.25	5062.84	4826.13
5	974.03	855.74	3326.92	3353.26	252.65	1071.23	1141.95	943.27	3955.87	1397.89	908.63
7	-	116.81	99.92	40.82	-	70.43	106.46	59.97	77.32	55.22	64.51
14	-	-	-	-	137.74	48.81	-	485.96	-	326.23	57.65
23	2048.53	1074.78	1511.94	1098.61	1262.14	1083.56	1431.71	1550.62	1150.31	1076.47	1267.19
24	2954.96	281.28	342.56	322.54	5858.62	4351.7	1311.58	1711.67	663.17	4830.37	431.7
25	584.91	415.94	517.23	-	4654.29	631.87	372.82	530.55	187.94	695.26	135.62
29	123.05	106.61	-	-	-	128.36	82.08	102.3	60.33	198.62	-
30	118.82	99.95	-	-	-	122.3	87.66	106	63.56	190.54	-
31	1078.71	1035.75	1032.45	-	1145.54	-	-	-	-	-	-
32	1054.89	998.34	-	-	1066.49	-	-	-	-	-	-
33	-	-	-	-	-	-	-	-	-	1036.63	1003.52
36	-	-	-	-	-	-	-	-	-	-	-
Flavonols											
6	3659.09	4799.82	1157.34	1172.72	15375.53	19682.63	13326.51	27342.46	14526.94	15186.14	13465.24
8	4403.38	3314.95	4121.89	3312.19	-	5596.39	5752.36	8958.14	2982.94	7126.36	2271.74
9	1067.2	1520.37	1287.61	968.54	-	1104.19	777.96	3603.82	1176.64	957.84	1638.58
10	1531.06	1759.99	2659.38	2625.23	2653.66	2418.92	1989.14	5112.39	2494.34	2262.43	1874.89
11	1409.15	1542.27	2029.89	1637.58	-	1626.97	1489.62	1352.16	1467.83	1405.38	1532.11
12	1519.1	1279.68	1612.73	1751.45	3072.88	1558.27	1503.39	2991.26	1404.39	1349.35	1454.99
15	2516.14	-	-	-	1742.2	-	1834.4	-	1687.95	1601.79	1516.69
16	137.51	413.22	395.08	397.18	-	538.38	210.97	192.29	375.05	284.6	276.06
17	-	-	-	-	1451.5	-	1220.4	1262.7	-	1265.34	1290.86
18	260.44	446.65	431.45	433.75	121.85	120.89	1140.32	1295.5	615.82	921.33	295.33
19	6369.16	7412.85	4314.14	4337.13	1393.29	9901.72	8975.85	9288.86	7076.68	9231.39	11917.56
20	1043.53	1007.82	1433.5	1425.61	1091.36	1034.22	1021.5	1116.92	1048.71	963.82	1024.2
21	2617.2	1356.44	1361.96	1862.86	1415	1942.63	2527.97	1736.97	1600.06	1629.56	1886.53
22	1474.25	1476.64	1954.64	1346.72	1409.05	1366.45	1604.54	1859.85	1451.37	1773.68	1740.3
26	1086.3	-	1178.34	1065.49	-	1275.99	1011.54	1009.99	1076.47	-	1154.72
27	1237.56	-	1197.61	1226.6	1521.36	-	1168.29	1219.42	-	-	-
28	1053.47	1010.98	1258.45	1265.16	-	1007.53	977.86	1008.49	-	-	1063.67
Flavones											
35	-	-	-	-	991.71	-	-	-	-	-	-
37	-	-	-	-	-	634.11	-	-	-	-	-

Table 4. Continued											
Flavanones											
38	6.15	-	4.11	4.23	4.47	9.13	4.54	5.87	11.25	5.55	14.68
Lignans											
13	1844.59	1382.27	1821.43	1742.06	1513.8	2172.73	1773.02	5071.77	1970.62	2489.88	1993.38
Iridoids											
34	-	-	111.13	111.72	-	166.5	84.48	78.51	-	-	77.91

(-): Not detected

DAD-ESI-MS/MS. Chlorogenic acid, 5-*p*-coumaroylquinic acid, dicaffeoylquinic acid, and quercetin-3-rutinoside were the most abundant phenolic compounds in all the extracts. The results revealed significant differences among aqueous, methanol, and ethanol extracts of elderberry flowers with respect to the detected phenolics.[9] These components were also present and dominant in our samples of methanolic extracts of *S. nigra* flowers and leaves.

The aqueous extract of elderflower obtained at 90 °C was analyzed by GC-MS and HPLC-MS, which allowed the identification of 46 compounds, including quercetin and chlorogenic acid derivatives, representing 86% of the total phenolic compounds identified in the hydrophilic fraction of the aqueous extract. Naringenin (27.2%) was the major compound present in the lipophilic fraction.[8] In our examined samples of methanolic extracts from SN-FL and SN-LE, naringenin was the component with the lowest content. The reason for this difference might derive from the dichloromethane used in the extraction solution, which is selective for the isolation of lipophilic components from plant materials.[8]

Mikulic-Petkovsek et al.[17] reported phenolics in three elderflower extracts (one methanolic extraction and two water extracts prepared as fresh drinks according to local recipes). Hydroxycinnamic acids and flavonol glycosides are the major phenolic constituents in elderflowers.[17] These results are similar to our results for the methanolic extracts of flowers and leaves.

The methanolic extract contained higher levels of all phenolic groups than the aqueous extracts. The outcome of elderflower extracts is reliable on the solution used for extraction and time of extraction.[16]

A literature search also revealed that the efficiency of phenolic extraction depends on the extraction method, solvent type, and drying process used for the plant material.[6] We used an ultrasonic-assisted method for extraction of the plant air dried material and methanol as the solvent.

According to Tundis et al.,[18] the flowers and leaves of *S. nigra* were extracted by maceration using methanol and ethanol as solvents. Selected phenolic acid and flavonoid contents of extracts were analyzed by HPLC-DAD. Overall, the obtained data showed that target compounds exhibited higher content in methanol extracts than in ethanol extracts. However, the extraction yield depends not only on the extraction method but also on the solvent used for the extraction process. Generally, methanol has been found to be more efficient in the extraction of polyphenols with lower molecular weight.[18] Phenolic compounds are more soluble in polar organic solvents because of the presence of a hydroxyl group; therefore, methanol was selected as the extracting solvent.[10]

Elderberry growth phases represent an irreversible process involving a series of biochemical changes that have an extremely important impact on nutritional characteristics. The green buds and flowers had a high quercetin 3-rutinoside.[18] This compound was also dominant in the samples of *S. nigra* flowers and leaves collected during the flowering stage of the plant in the present study.

Total phenolic and flavonoid compounds are considered to be important antioxidant components. They are responsible for deactivating free radicals based on their ability to donate hydrogen atoms to free radicals based on their structural characteristics.[19]

Considering the multitude of phenolic compounds present in the flowers and leaves, a multivariate data analysis was performed to elucidate possible patterns in the phenolic content of the samples and further develop a correlation model. The initial PCA of the polyphenolic content of the flower extracts was performed using three main components that explain 60.6% of the variations in the X matrix (chemical composition of the flowers). The model score scatter plot (Figure 1a) reveals some grouping patterns of the samples that were further analyzed using HCA. The HCA dendrogram (Figure 1b) differentiated four samples groups based on the PCA scores. Group 4 contained 6 samples (SN2-FL, SN5-FL, SN7-FL, SN8-FL, SN9-FL, SN10-FL), Group 1 had 3 samples (SN4-FL, SN6-FL, SN11-FL), while groups 2 and 3 consisted of only one sample (SN1-FL and SN3-FL, respectively.

To analyze the relationship between the sample grouping and the polyphenolic content of the samples, further PLS-DA modeling was applied. Three main components were used to build the model that delivered a high correlation coefficient (R2Y= 0.903) and a distinctive grouping of the samples in the score scatter plot (Figure 1c). The variance important for projection (VIP) values revealed that nearly 20 phenolic components were assigned VIP coefficients larger than 1, indicating that more than half of the analyzed polyphenols were important and contributed to the sample classification. The VIP values are calculated by summing the squares of each loading weight, thus summarizing

the contribution of the variables to explain X and correlate to Y, where VIP values larger than one indicate important X variables (phenolic compounds).[20] The loading scatter plot (Figure 1d) reveals the relationships among the phenolic compounds and their corresponding groups, where the relative distance between each X (phenolic compound) and Y point (sample group) showcases the specificity of the phenolic chemical contents of each group. Considering the aforementioned, it can be observed that the Group 4 samples exhibit specific phenolic content that is richer in 5-caffeoylquinic acid, caffeoyl-kaempferol, quercetin malonyl diglucoside, lignan coumaroyl glucoside, and quercetin caffeoyl pentoside, whereas the Group 1 samples could be differentiated by higher contents of caffeic acid derivatives, *p*-coumaroyl dihydromonotropein, hydroxy trimethoxy flavonoid, and naringenin. The Group 2 sample presented specific phenolic content with high concentrations of quinic acid, 3-caffeoylquinic acid, kaempferol-3-rutinoside, and kaempferol-3-malonylglucoside, and the Group 3 sample was distinctive by its high *p*-coumaroyl-caffeoylquinic acid isomer content.

Statistical Analysis

Statistical analysis of the chemical composition of the leaf extracts was performed as described for the flower samples. The PCA model was built using three main components explaining 63.4% of the variability in the X-matrix, and the score scatter plot (Figure 2a) revealed two outlying samples (SN8-LE and SN5-LE) with distinctive scores in the first and second components, respectively. The subsequent HCA produced four distinctive sample clusters (groups) (Figure 2b), two of which were represented by the aforementioned samples (Group 1 - SN5-LE and Group 2 - SN8-LE), while Group 3 was composed of two samples (SN3-LE and SN4-LE) and Group 4 of seven samples (SN1-LE, SN2-LE, SN6-LE, SN7-LE, SN9-LE, SN10-LE, and SN11-LE).

The PLS-DA modeling of the data using group clustering as a Y-variable and phenolic content as an X-variable produced a model using three main components with R2Y= 0.921. Seventeen phenolic compounds were considered important in the regression model (VIP > 1), while the loading scatter plot (Figure 2d) revealed the distinctive phenolic compounds whose content was specific to each group. Therefore, high contents of dicaffeoylquinic acid isomer and quercetin galloyl pentoside were characteristic of SN5-LE (Group 1), whereas 3-caffeoylquinic acid, 5-caffeoylquinic acid, caffeic acid derivative, caffeoyl-kaempferol, lignan coumaroyl glucoside, coymaroylquinic acid, and isorhamnetin diglucoside were present in high concentrations in SN8-LE (Group 2). On the other hand, Group 3 samples were rich in 4-caffeoylquinic acid, and the phenolic content of the Group 4 samples was distinctive by its high concentrations of caftaric acid, quercetin malonyl diglucoside, quercetin caffeoyl pentoside, quercetin-3-rutinoside, and kaempferol-3-rutinoside.

CONCLUSION

A few dissimilarities in the qualitative composition of phenolic compounds between the methanolic extracts of flowers and leaves of *S. nigra* collected in the flowering stage from eleven different localities in Kosovo were revealed, nevertheless the quantitative differences were more evident. The multivariate statistical analysis revealed distinctive clustering patterns for the flower and leaf extracts, and several statistical indicators depicted the phenolic compounds that were present in higher concentrations and were specific for each group of samples.

Ethics

Ethics Committee Approval: Not necessary.

Informed Consent: Not necessary.

Peer-review: Externally peer reviewed.

Authorship Contributions

Concept: B.Q., V.E., Design: H.K., S.D., Data Collection or Processing: V.Q., Analysis or Interpretation: J.P.S., M.C., Literature Search: B.Q., V.E., N.G., Writing: B.Q., V.E., N.G.

Conflict of Interest: No conflict of interest was declared by the authors.

Financial Disclosure: The authors declared that this study received no financial support.

REFERENCES

1. Ferreira SS, Silva AM, Nunes FM. *Sambucus nigra* L. fruits and flowers: chemical composition and related bioactivities. Food Rev Int. 2020;38:1237-1265.
2. Schmitzer V, Veberic VR, Stampar F. European elderberry (S*ambucus nigra* L.) and American Elderberry (*Sambucus canadensis* L.): botanical, chemical and health properties of flowers, berries and their products. Berries: Properties, Consumption and Nutrition, Ljubljana; 2012:1-20.
3. Wojciechowicz-Żytko E, Jankowska B. *Sambucus nigra* L. as a reservoir of beneficial insects (Diptera, Syrphidae). Folia Hortic. 2016;28:209-216.
4. Petrut GS, Muste S, Muresan C, Paucean A, Muresan AE, Nagy M. Chemical profiles and antioxidant activity of black elder (*Sambucus nigra* L.) - a review. Bull.UASVM Food Sci. Techn. 2017;74:9-16.
5. Bajera T, Bajerováa P, Ventura K. Effect of harvest and drying on composition of volatile profile of elderflowers (*Sambucus nigra*) from Wild. Nat Prod Com. 2017;12:1937-1942.
6. Viapiana A, Wesolowski M. The phenolic contents and antioxidant activities of infusions of *Sambucus nigra* L. Plant Foods Hum Nutr. 2017;72:82-87.
7. Małgorzata D, Aleksandra P, Monika T, Ireneusz K. A new black elderberry dye enriched in antioxidants designed for healthy sweets production. Antioxidants (Basel). 2019;8:257.
8. Ferreira-Santos P, Badim H, Salvador ÂC, Silvestre AJD, Santos SAO, Rocha SM, Sousa AM, Pereira MO, Wilson CP, Rocha CMR, Teixeira JA, Botelho CM. Chemical characterization of *Sambucus nigra* L. flowers aqueous extract and its biological implications. Biomolecules. 2021;11:1222.
9. Uzlasir T, Kadiroglu P, Selli S, Kelebek H. LC-DAD-ESI-MS/MS characterization of elderberry flower (*Sambucus nigra*) phenolic compounds in ethanol, methanol, and aqueous extracts. J Food Process Preserv. 2020;45:e14478.
10. Brglez Mojzer E, Knez Hrnčič M, Škerget M, Knez Ž, Bren U. Polyphenols: extraction methods, antioxidative action, bioavailability and anticarcinogenic effects. Molecules. 2016;21:901.

11. Dai J, Mumper RJ. Plant phenolics: extraction, analysis and their antioxidant and anticancer properties. Molecules. 2010;15:7313-7352.
12. Panzella L, Moccia F, Nasti R, Marzorati S, Verotta L, Napolitano A. Bioactive phenolic compounds from agri-food wastes: an update on green and sustainable extraction methodologies. Front Nutr. 2020;7:60
13. Vujanović M, Majkić T, Zengin G, Beara I, Cvetanović A, Mahomoodally FM, Radojković M. Advantages of contemporary extraction techniques for the extraction of bioactive constituents from black elderberry (*Sambucus nigra* L.) flowers. Ind Crop Prod. 2019;136:93-101.
14. Kumar N, Goel N. Phenolic acids: natural versatile molecules with promising therapeutic applications. Biotechnol Rep (Amst). 2019;24:e00370.
15. Wang TY, Li Q, Bi KS. Bioactive flavonoids in medicinal plants: structure, activity and biological fate. Asian J Pharm Sci. 2018;13:12-23.
16. Marțiș Petruț GS, Mureșan V, Marc Vlaic RM, Mureșan CC, Pop CR, Buzgău G, Mureșan AE, Ungur RA, Muste S. The physicochemical and antioxidant properties of *Sambucus nigra* L. and *Sambucus nigra* Haschberg during growth phases: from buds to ripening. Antioxidants (Basel). 2021;10:1093..
17. Mikulic-Petkovsek M, Samoticha J, Eler K, Stampar F, Veberic R. Traditional elderflower beverages: a rich source of phenolic compounds with high antioxidant activity. J Agric Food Chem. 2015;63:1477-1487.
18. Tundis R, Ursino C, Bonesi M, Loizzo MR, Sicari V, Pellicanò T, Manfredi IL, Figoli A, Cassano A. Flower and leaf extracts of *Sambucus nigra* L.: application of membrane processes to obtain fractions with antioxidant and antityrosinase properties. Membranes (Basel). 2019;9:127.
19. Petkova DT, Mihaylova DS, Deseva IN, Denev PN, Krastanov AI. Green approach to obtain extracts of seven edible flowers. Mater Sci Eng. 2021;1031:012129.
20. Makraduli L, Makreski P, Goracinova K, Stefov S, Anevska M, Geskovski N. A comparative approach to screen the capability of raman and infrared (mid- and near-) spectroscopy for quantification of low-active pharmaceutical ingredient content solid dosage forms: the case of alprazolam. Appl Spectrosc. 2020;74:661-673.