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Phytochemical Profiles and Antioxidant Activity of *Salvia* species from Southern Italy

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Abstract: The purpose of this research was to investigate phytochemical profiles and antioxidant activity in four Salvia species growing in Salento (Southern Italy). The hydrodistillation products obtained from the aerial parts of *Salvia clandestina*, *Salvia fruticosa*, *Salvia officinalis* and *Salvia sclarea* were characterized by GC–MS and 50 compounds were detected. With the exception of *S. clandestina*, that did not produce essential oils, the other species shared different amounts of monoterpenes oxygenated (camphor 2.13%-9.16%) and sesquiterpenes hydrocarbons (caryophyllene 4.65%-18.33%; humulene 1.87%-12.39%). The phenolic profiling, analyzed by HPLC ESI/MS-TOF, highlights that *S. clandestina* is a rich source of danshensu (4.76 mg/g DW) while *S. sclarea* of rosmarinic acid (15.57 mg/g DW). Mutivariate statistical analysis (PCA) of hitherto studied *Salvia* phenols have shown similarities in profiles between *S. fruticosa* and *S. officinalis*, while *S. clandestina* and *S. sclarea* showed distinctive profiles. Otherwise, essential oil profiles analysed by PCA are clearly different among the three productive species. The extracts from collected plants were found to be effective antioxidant in three different in vitro assays (DPPH, ABTS, FRAP and Superoxide anion scavenging activity). Thus, they can be proposed as natural ingredients in functional foods, herbal medicines or as sources of bioactive molecules.

Keywords: *Salvia clandestina* L.; *Salvia fruticosa* Mill.; *Salvia officinalis* L.; *Salvia sclarea* L.; essential oils; phenolic compounds.

1. Introduction

The aromatic herbs belonging to the Lamiaceae family are a precious source of secondary metabolites because of their pharmacological properties and promising ingredients to develop products in cosmetic, food and pesticide industries [1]. Among this family, the *Salvia* genus is the most important aromatic species to be used as food spices, flavouring agents in cosmetics and in medicine to treat infections, malaria, cancer and inflammations [2,3,4]. A high number of useful plant secondary metabolites have been isolated from the genus such as: essential oils compounds, phenolic derivatives compounds and terpenoid [5]. The properties and the applications of essential oils (EOs) obtained from various species of *Salvia*, are numerous: antimicrobial [6,7], antioxidant [8,9], anti-inflammatory, cholinesterase inhibition, anxiolytic and sedative [10]. Furthermore, phenolic compounds are a class of plant secondary metabolites commonly widespread in the *Salvia* genus;

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soluble in water and usually immobilized in vacuole, with capacities of reducing agents, hydrogen donors and singlet oxygen quenchers and also metal chelation [11]. Some studies have shown that EOs and phenolic composition are greatly influenced by environmental and growing conditions, harvesting, genetic and other factors [12]. The environment might affect the chemical composition, the yield and quality of products and also probably the biological activity of their extracts [13]. The increased market demand of secondary metabolites in plants has brought to investigate some *Salvia* species collected from different areas of the world. In particular, *Salvia officinalis* L., *Salvia fruticosa* Mill., *Salvia sclarea* L. and recently *Salvia clandestina* L. deserve special attention for the complexity of the investigated compounds and the biological activity of their extracts.

Although there are some reports on the secondary metabolites of the *Salvia* genus, the present study was performed to characterize the chemical compositions and to evaluate the antioxidant activity of polar and aqueosus extracts of *S. clandestina*, *S. fruticosa*, *S. officinalis* and *S. sclarea*, grown in Salento (South Apulia, Southern Italy). The four *Salvia* species were grown in similar climatic state to avoid the environmental and growing conditions influence the production of secondary metabolites. The principal component analysis (PCA) were realized to characterize the species based on their main essential oil components and phenolic compounds. The aim of this work was to provide additional scientific evidence leading to a reconsideration of *Salvia* species for industrial uses, novel products as functional foods, herbal medicines and sources of natural antioxidants. This also could lead to effective conservation, protection and valorization strategies of these species.

2. Materials and Methods

2.1. Plants Material and Harvesting Season

All plant materials (five replicates for each species) were collected in 2017 during the floral budding and were gathered from different spots of the Botanical Garden of University of Salento. After collection, aerial parts from *S. clandestina* **(*Sc*)**, *S. fruticosa* **(*Sf*),** *S. officinalis* **(*So*)** and *S. sclarea* **(*Ss*)** were dried at 35°C until reaching a constant weight. The plants were authenticated by Systematic Botanical Laboratory (Drs. R.A. Accogli) and voucher specimen has been registered in the *Herbarium Lupiense* (LEC) of the Department of Biological and Environmental Sciences and Technologies, University of Salento, Italy (no. voucher ***Sc*:** 24978, ***Sf*:** 24979, ***So*:** 279780, ***Ss*:** 279781).

2.2. Hydrodistillation, GC-FID and GC–MS Analysis

According to European Pharmacopoeia method, dried aerial parts of four *Salvia* species were subjected to hydrodistillation for 2 h using a modified Clevenger-type apparatus [14]. The characterization of hydrodistillation products (EOs and hydrolate) was carried out by GC–MS with an Agilent 7890B using a mass selective detector Agilent 5977A, equipped with fused silica capillary column Agilent HP-5MS (30m x 0.25mm, 0.25 μm film thickness) (Agilent Technologies, Palo Alto, CA, USA). Identification of individual oil components was performed on the basis of retention index (RI), determined with reference to the homologous series of *n*-alkanes under identical experimental condition, injection with standard, an MS library (NIST 14, 2014) and/or literature data [15]. While, the quantification of EOs compounds was carried out by GC-FID analysis according to Negro *et al.* [16] using a PerkinElmer GC-FID system equipped with DB-5 column (30 m X 0.53 mm i.d., film thickness 1.5 μm, J&W, USA).

2.3. Extraction, HPLC ESI/MS-TOF Analysis and Total Quantification of Phenolic Compounds

The extraction of phenolic compounds was performed according to Zhou and co-authors [17] at a ratio of 1:20 (w/v). Water extraction was preferred because it has no negative impact on health and environment. After centrifugation, the resulting solutions were filtered using a 0.2 μm PFTE membrane and analyzed as described below.

Agilent 1200 Liquid Chromatography system (Agilent Technologies, Palo Alto, CA, USA) equipped with a standard autosampler was used for phenolic characterization, as reported by Nicolì *et al.* [18] and Sabella *et al.* [19]. The HPLC system was coupled to an Agilent diode-array detector. The detection wavelength was 280 nm and an Agilent 6320 TOF mass spectrometer equipped with a dual ESI interface (Agilent Technologies) operating in negative ion mode. Detection was carried out within a mass range of 50–1700 m/z. Accurate mass measurements of each peak from the total ion chromatograms (TICs) were obtained by means of an ISO Pump (Agilent G1310B) using a dual nebulizer ESI source that introduces a low flow (20 μL·min^{-1}) of a calibration solution which contains the internal reference masses at m/z 112.9856, 301.9981, 601.9790, 1033.9881, in negative ion mode. The accurate mass data of the molecular ions were processed through the software Mass Hunter (Agilent Technologies). Five standard calibration graphs for the quantification of the phenolic compounds were prepared using authentic standards (danshensu, caffeic acid, rosmarinic acid, salvianolic acid B and luteolin) (Tab. 1).

Table 1. Regression data, precision, LODs and LOQs for five phenolic compounds obtained with the optimized HPLC ESI–TOF–MS method

Compound	Equation[a]	R^2	LOD[b] μg/mL	LOQ[c] μg/mL	Linearity range μg/mL
danshensu	y = 214185x + 240522	0.999	0.011	0.039	0.5-100
caffeic acid	y = 1651849x + 122380	0.992	0.004	0.015	0.5-20
rosmarinic acid	y = 480627x + 111347	0.996	0.006	0.021	0.5-50
salvianolic acid B	y = 98350x + 236608	0.990	0.023	0.089	1-50
luteolin	y = 15921337x + 3231931	0.992	0.005	0.020	0.5-20

[a] *y* is the peak area in TIC chromatograms monitored, *x* is the concentration injected.
[b] LOD: limit of detection (S/N 3:1).
[c] LOQ: limit of quantification (S/N 10:1).

The total phenolic content (TPC) of plant aqueous extract was determined according to Folin-Ciocalteu's method [20]. The absorbance was read at 765 nm with a JASCO V-550 UV/VIS spectrophotometer (JASCO Corporation 2967-5, Ishikawa-machi, Hachioji-shi Tokyo, Japan). The calculation of TPC of each extract was made using caffeic acid standard curve (equation: y = 4.3039x). The results were expressed as mg caffeic acid equivalent per g of dry weight (CAE mg/g DW) and all measurements were performed in triplicate for each sample analyzed.

2.4. Antioxidant Activity of the Extracts

DPPH Assay. Antioxidant activity of the EOs was determined *in vitro* by evaluation of the free radical scavenging activity using 2,2-diphenyl-1-picrylhydrazyl (DPPH·) (DPPH assay) [21]. Inhibition of free radical DPPH in percent (AA%) was calculated as follow: %AA=(1−ODTF/ODT0) × 100, where ODTF was the absorbance at the final point (30 minutes) and ODT0 the absorbance at the initial point.

Radical Cation Decolorization Assay (ABTS). Scavenging activity of the extract was determined using ABTS (7mM) as described by Dudonné *et al.* [20]. The absorbance at 734 nm was measured in time using a PerkinElmer 2030 Multilabel reader Victor X5 (PerkinElmer, Waltham, Massachusetts, USA). All samples were measured in triplicate and scavenging capacities were expressed as Trolox equivalents antioxidant capacity (TEAC, μM Trolox equivalents per mg of dry plant).

Ferric Reducing Antioxidant Power (FRAP). The ferric reducing ability was determined by the FRAP method [22]. The absorption of the reaction mixture was measured at 593 nm using Perkin Elmer 2030 Multilabel reader Victor X5 after 3 min of incubation at 37°C. The samples were measured in triplicate and the Trolox equivalent antioxidant capacity (TEAC).

Superoxide anion scavenging activity assay. Superoxide radicals were done according to Beauchamp and Fridovich [23]. The photo-induced reactions were performed using fluorescent lamps (200W). All samples were measured in triplicate and the superoxide anion scavenging activity was expressed in IC_{50}.

2.5. Statistical Analysis

All data were analyzed in five independent biological replicates. The phenolic compounds quantification, total phenols content and antioxidant activity values were expressed as mean values ± standard deviation and subjected to one-way ANOVA analysis, followed by Tukey-HSD (Honestly Significant Difference) post hoc test ($p < 0.05$). Analyses were achieved using GraphPad software, version 6.01. Data from quantitative analysis on the six phenol compounds and on the main EOs constituents (content ≥ 1%) of samples, were also analyzed using a principal component analysis (PCA), to examine the interrelationships between metabolite profiles. The XLSTAT software (version 18.07.01.) was used for all data analyses.

3. Results and Discussion

3.1. Essential Oil Analysis and Evaluation of Antioxidant Activity

The hydrodistillation of *Salvia* species aerial part yielded the following yields reported as % dry weight (DW) in EOs: ***Sf*** 0.18, ***So*** 0.70, ***Ss*** 0.17, respectively. The hydrodistillation product yield of ***Sc*** was very low (≤ 0.05) and for this reason, it was decided not to carry out a qualitative analysis its EOs, but analized with GC/MS its hydrolate. Hydrolate is herbal water or aromatic water is obtained with essential oils in the process of isolation from plant materials by hydrodistillation. For plant materials with a low content of essential oils, hydrolates are the only valuable products of steam distillation [24].

The hydrolate from ***Sc*** was characterized by a prevalence of hydrocarbons monoterpenes, in particular: limonene, β-pinene, γ-terpinene and 2-carene (data no show). The EOs analyses with GC/MS of ***Sf***, ***So*** and ***Ss*** showed the presence of 50 components (Tab. 2). In particular: 29, 33 and 23 compounds were identified from the EOs of ***Sf***, ***So***, ***Ss*** respectively, which represented about 98, 98, and 82 % of the oils. The constituents from *Salvia* EOs, together with their retention index calculated, retention index from literature data and percentage composition were listed in Table 2.

In the qualitative and quantitative diversity composition of EOs compounds, among the **So**, **Sf** and **Ss** samples was observed. To evaluate the relationships between species, 27 identified oil components showing a content ≥ 1% in at least one of the EOs extracted from the three species were analyzed using principal component analyses. The biplot constructed by the two first principal components and showing the distribution of species and e EOs constituents was presented in Figure 1. Principal component 1 (PC1) and principal component 2 (PC2) accounted for 99.89% of the total variance in the data and showed a clear separation between ***Ss*** samples and ***So*** and ***Sf*** samples. The main constituents of the EOs of ***Ss*** were hydrocarbons sesquiterpenes (56.95%), in particular, germacrene D (35.73%) and oxygenated sesquiterpenes (20.48%) that contributed significantly to the distinction of this species when compared to EOs composition of ***Sf*** and ***So***. In fact, these last two species were characterized by oxygenated monoterpenes, 38.61% and 45.23%, and hydrocarbons sesquiterpenes, 34.96% and 37.45%, respectively. These compounds, according to Longaray Delamare *et al*. [4] Topçu *et al.* [25], include some most present in the two species: 1,8-cineole, caryophyllene and humulene. Previous studies found similar chemical compositions but in different proportions [26, 27]. The changes in the EOs compositions might arise from several environmental and genetic differences [28].

Table 2. Chemical composition and Antioxidant Activity of the essential oils (EOs) of *Salvia* species.

No.	RI[a]	RI[b]	Compound[c]	Composition[d] %		
				S. fruticosa	***S. officinalis***	***S. sclarea***
1	922	924	β-thujene	0.62	0.55	-
2	934	932	α-pinene	3.72	1.27	-
4	987	988	β-myrcene	5.01	0.94	-
5	1000	1001	2-carene	0.33	0.30	-
6	1012	1014	α-terpinene	0.72	0.63	-
7	1021	1020	*p*-cymene	-	0.58	
8	1024	1022	*o*-cymene	0.73	0.51	-
11	1029	1030	limonene	8.76	4.66	-
12	1051	1054	γ-terpinene	1.62	1.28	-
Hydrocarbons monoterpenes				**21.51**	**10.72**	**0.00**
3	943	946	camphene	0.61	0.58	-
9	1025	1025	β-phellandrene	-	0.54	-
10	1028	1026	1,8-cineole	27.57	13.93	-
13	1103	1101	α-thujone	2.24	14.41	-
14	1110	1112	β-thujone	1.37	3.25	-
15	1130	1132	o-cimenol	0.65	0.24	-
16	1139	1141	camphor	2.69	9.16	2.13
17	1164	1165	iso-borneol	-	0.52	-
18	1176	1174	terpinen-4-ol	1.00	1.09	-
19	1185	1186	α-terpineol	2.48	0.30	-
20	1287	1289	thymol	-	1.21	-
Oxygenated monoterpenes				**38.61**	**45.23**	**2.13**
21	1377	1374	α-copaene	-	-	1.42
22	1387	1389	β-elemene	-	-	0.72
23	1405	1407	longifolene	0.35	16.09	-
24	1409	1408	caryophyllene	18.33	5.93	4.65
25	1435	1437	α-guaiene	-	-	1.25
26	1440	1442	α-maaliene	-	0.18	-
27	1446	1445	calarene	-	0.20	-
28	1450	1452	humulene	7.64	12.39	1.87
29	1456	1458	allo-aromadendrene	-	0.33	
30	1471	1475	γ-gurjunene	3.69	-	-
31	1479	1478	γ-muurolene	-	0.28	-
32	1480	1481	γ-himachalene	-	-	0.52
33	1487	1484	germacrene D	-	-	35.73
34	1490	1491	valencene	1.74	1.56	0.56
35	1492	1494	bicyclogermacrene	-	-	4.96
37	1498	1500	α-muurolene	-	-	0.39
38	1502	1501	epizonarene	0.41	-	-
39	1507	1508	germacrene A	0.41	-	0.60
40	1510	1513	γ-cadinene	0.41	-	0.71
41	1524	1522	δ-cadinene	0.74	-	-
42	1525	1528	cis-calamenene	1.24	0.49	3.57
Hydrocarbons sesquiterpenes				**34.96**	**37.45**	**56.95**
36	1495	1496	viridiflorene	-	-	3.38
43	1579	1577	spathulenol	0.59	0.36	5.86
44	1605	1608	humulene 1-2 epoxide	0.84	1.49	0.48
45	1637	1639	*allo*-aromadendrene oxide	1.80	0.44	2.76
47	2015	2017	manoil oxide	-	-	0.66
48	2058	2057	manool	-	2.73	2.47
49	2220	2222	sclareol	-	-	4.87
Oxygenated sesquiterpenes				**3.23**	**5.02**	**20.48**
46	1732	1730	longifolene aldehyde	-	-	0.54
50	2301	2303	1-heptatriacotanol	-	-	0.80
Others compounds				**-**	**-**	**2.20**
Total compounds identified %				**98.31**	**98.42**	**81.76**
Unidentified compounds				**0.68**	**0.62**	**10.67**

[a] RI, retention indices relative to C7 -C30 n-alkanes on the HP-5 column. [b] RI, linear retention index taken from Adams [15] and/or NIST 14 (2014). [c] Constituents listed in order of elution from a DB-5 column. [d] Peak areas relative to total peak area (means of three samples). Sample codes: ***Sf*** (*S. fruticosa* L.), ***So*** (*S. officinalis* L.), ***Ss*** (*S. sclarea* L.)

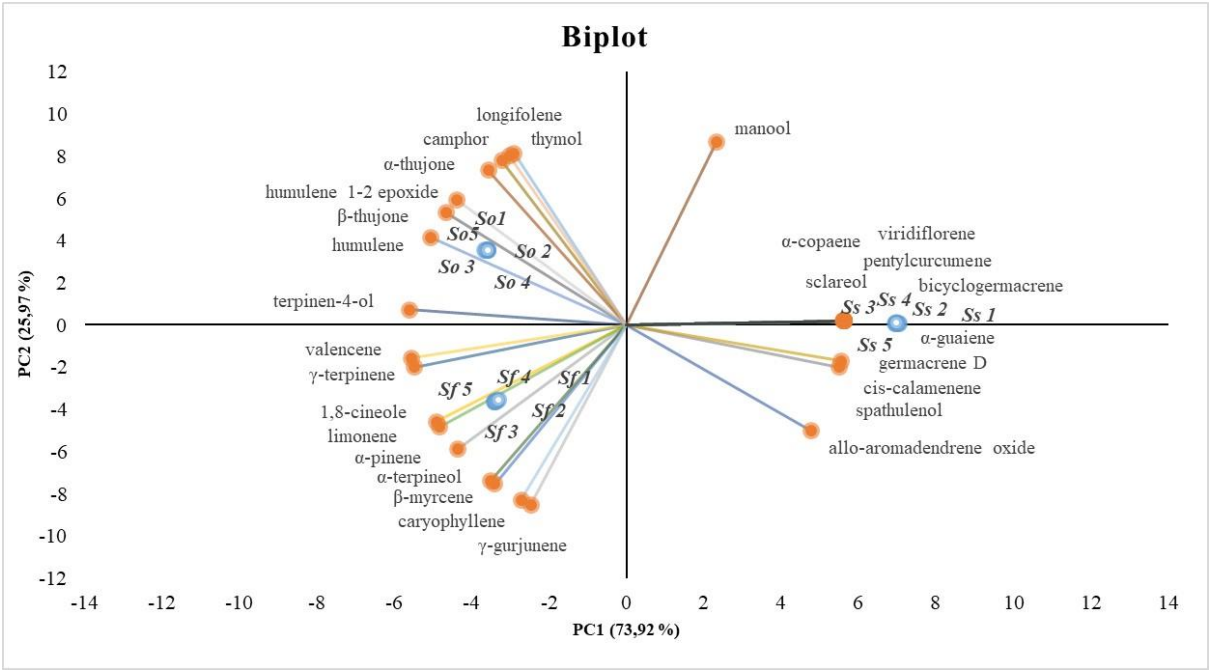

Figure 1. Graph based on 27 essential oil compounds with contents 1.0 % in at least one of the species. The length of the vectors is matched to their significance within each specie. Between vectors and between a vector and an axis, the correlation is positive if the angle is <90° and negative if the angle reaches 180°. The is no linear dependence if the angle is 90°. Sample codes: ***Sc*** (*S. clandestina* L.), ***Sf*** (*S. fruticosa* L.), ***So*** (*S. officinalis* L.), ***Ss*** (*S. sclarea* L.). The number after acronyms represent the five independent biological replicates analysed for each species.

The antioxidant properties of the isolated EOs, measured by the DPPH assay and the results showed that AA for ***So*** (AA =18.81%) was significantly higher than that of ***Sf*** (AA=4.27%) and ***Ss*** (AA=5.32%). The antioxidant activity was evaluated also for hydrolate product of ***Sc*** and the value of DPPH assay is very low (AA=0.04%); this result is in agreement with the poor performance given by the oils with similar patterns and high presence of monoterpenes hydrocarbons [21, 29].

3.2. Phenolic Compound Analysis and evaluation of Antioxidant Activity

Qualitative and quantitative analysis of phenolic components in aqueous extracts, carried out with HPLC ESI/MS-TOF, allowed the identification of 30 different compounds and their distribution in the four *Salvia* species examined is showed in Table 3. In particular, 29 compounds have been identified in ***Sc***, 20 in ***Sf***, 16 in ***So*** and 21 in ***Ss***. The identification of many compounds was confirmed by comparing with an analytical authentic standard and data reported in the literature. Danshensu, caffeic acid, chlorogenic acid, luteolin, rosmarinic acid and salvianolic acid B have also been identified comparing literature data with the exact mass obtained (Tab. 3). The compounds identified to belong to five groups: hydroxybenzoic acid derivatives, hydroxybenzaldehyde, hydroxycinnamic acid derivatives, flavonoids and organic acids. 12 compounds were present in all species and among these, the following ones are previously described for other *Salvia* species: rosmarinic acid, salvianolic acid B, A, K and yunnaneic acid E [30, 31].

Table 3. HPLC/ESI-TOF-MS accurate masses of $[M–H]^-$ ions of constituents of four *Salvia* species extract.

No	Compound	RT (min)	Molecular formula [M–H]- proposed	*m/z* exp.	*m/z* calcd.	error (ppm)	Species				Reference
							Sc	***Sf***	***So***	***Ss***	
1	vanillic acid	1.071	$C_8H_7O_4$	167.0358	167.0350	-4.99	+	n.d.	+	n.d.	[31,35]
2	danshensu*	1.705	$C_9H_9O_5$	197.0465	197.0455	-5.07	+	+	n. d.	n.d.	[31,35]
3	protocatechuic acid	2.200	$C_8H_9O_3$	153.0566	153.0557	-5.76	+	+	+	+	[31,35]
4	protocatechuic aldehyde	2.950	$C_7H_5O_3$	137.0249	137.0244	-3.80	+	n.d.	n. d.	+	[31,35]
5	Unknow	4.638	$C_{16}H_{21}O_{11}$	389.1111	389.1089	-5.76	+	n.d.	n. d.	n.d.	
6	caffeic acid hexoside	4.818	$C_{15}H_{17}O_9$	341.0896	341.0878	-5.29	+	+	n. d.	n.d.	[35]
7	caffeic acid*	5.400	$C_9H_7O_4$	179.0357	179.0350	-3.91	+	n.d.	+	+	[31,35,36]
8	chlorogenic acid*	5.786	$C_{16}H_{17}O_9$	353.0887	353.0878	-2.34	+	+	n. d.	+	[35,36]
9	Unknow	6.880	$C_{17}H_{29}O_{21}$	569.1194	5691207	+2.32	+	n.d.	n. d.	n.d.	
10	Unknow	7.146	$C_{17}H_{29}O_{21}$	569.1191	5691207	+2.76	+	n.d.	n. d.	n.d.	
11	caffeoylmalic acid	7.500	$C_{13}H_{11}O_8$	295.0446	295.0459	4.92	+	n.d.	n. d.	n.d.	[37]
12	tuberonic acid glucoside	7.520	$C_{18}H_{27}O_9$	387.1694	387.1661	-8.61	n. d.	n.d.	n. d.	+	[38]
13	salvianic acid C	7.620	$C_{18}H_{17}O_9$	377.0857	377.0848	-2.38	+	+	n. d.	+	[39]
14	prolithospermic acid	7.670	$C_{18}H_{13}O_8$	357.0631	357.0616	-4.20	+	+	n. d.	+	[38]
15	luteolin diglucoronide	8.270	$C_{27}H_{25}O_{18}$	637.1061	637.1046	-2.35	+	n.d.	n. d.	+	[32]
16	lithospermic acid	9.510	$C_{27}H_{21}O_{12}$	537.0970	537.1038	2.63	+	+	+	+	[31]
17	salvianolic acid K	9.530	$C_{27}H_{23}O_{13}$	555.1124	555.1148	4.07	+	+	+	+	[32]
18	yunnaneic acid F	9.810	$C_{29}H_{25}O_{14}$	597.1296	597.1250	-4.60	+	+	n. d.	+	[40]
19	luteolin 7-O-glucoside	10.329	$C_{21}H_{19}O_{11}$	447.0952	447.0933	-4.38	+	+	+	n.d.	[32,35]
20	yunnaneic acid E	10.579	$C_{27}H_{23}O_{14}$	571.1129	571.1152	4.05	+	+	+	+	[30,35]
21	Unknow	10.887	$C_{17}H_{14}O_7$	331.0843	331.0823	-5.97	+	+	n. d.	+	
22	Salvianolic acid K	10.977	$C_{27}H_{23}O_{13}$	555.1162	555.1144	-3.24	+	+	+	+	[32]
23	rosmarinic acid*	11.143	$C_{18}H_{15}O_8$	359.0764	359.0772	2.22	+	+	+	+	[30,31,36]
24	salvianolic acid A	11.574	$C_{26}H_{21}O_{10}$	493.1152	493.1140	-2.43	+	+	+	+	[31,36]
25	unknown	11.723	$C_{29}H_{34}O_{22}$	736.1627	736.1625	-0.14	+	n.d.	+	n.d.	
26	unknown	12.200	$C_{18}H_{34}O_{18}$	539.1815	539.1829	2.6	+	+	+	+	
27	salvianolic acid B*	12.552	$C_{36}H_{29}O_{16}$	717.1547	717.1461	-1.19	+	+	+	+	[31,36]
28	luteolin*	12.867	$C_{15}H_9O_6$	285.0429	285.0405	-1.40	+	+	+	+	[32,35]
29	apigenin*	14.393	$C_{15}H_9O_5$	269.0465	269.0455	-3.71	+	+	+	+	[32,35]
30	salvianolic acid F	15.388	$C_{17}H_{13}O_6$	313.0735	313.0718	-5.43	+	+	+	+	[31,32]

*Positively identified via comparison with authentic standard. n.d.: not determined. Sample codes: **Sc** (*S. clandestina* L.), **Sf** (*S. fruticosa* L.), **So** (*S. officinalis* L.), **Ss** (*S. sclarea* L.)

The total phenol concentrations (TPC) detected in the selected *Salvia* species are shown in Table 4 followed by multiple comparison procedure (Tukey post hoc test). TPC values ranging between 36.70 and 59.10 mg/g dry weight has been observed and four different classes: “a”, “b”, “c”, and “d” (different letters correspond to statistically different means) has been identified. According to Cvetkovikj *et al*. [32], the most abundant phenolic compound was determined to provide an overview of the content and offer the possibility for a fast comparing the Sage chemodiversity. In particular, the amounts of rosmarinic acid in four species was: 7.56 (b) in ***Sc***, 6.55 (c) in ***Sf***, 5.46 (d) in ***So*** and 15.57 (a) mg/g of dry weight ***Ss***. Thus, the quantity of rosmarinic acid is considerably higher in ***Ss*** when

compared to the amounts detected in the aqueous extracts of the other species examined. The presence and the amount of danshensu, (3-(3,4-dihydroxy-phenyl)-2-hydroxy-propinic acid), a stronger antioxidant, which has been recently determined in ***Sc*** as already reported by Nicolì *et al*. [18], is remarkable if compared with *Salvia miltiorrhiza* Bunge, the main source of this compound [17]. The other phenols concentration (caffeic acid, caffeoymalic acid, salvianolic acid B and luteolin) is analogue with other species belonging to the *Salvia* genus [33].

Table 4. Total phenolic content (TPC) (Folin-Ciocalteu spectrophotometric method), phenolic compounds quantification (HLC ESI–TOF–MS method) and antioxidant activity of four *Salvia* species aqueous extract.

Compound	***S. clandestina*** (mg/g DW)[1]	***S. fruticosa*** (mg/g DW)[1]	***S. officinalis*** (mg/g DW)[1]	***S. sclarea*** (mg/g DW)[1]
TPC[2]	36.70 ± 0.46 **d**	45.30 ± 0.44 **c**	59.10 ± 0.10 **a**	55.60 ± 0.17 **b**
danshensu	4.76 ± 0.21 **a**	0.26 ± 0.05 **b**	<LOQ **b**	<LOQ **b**
caffeic acid	0.90 ± 0.06 **a**	<LOQ **b**	0.05 ± 0.03 **b**	0.04 ± 0.02 **b**
caffeoymalic acid[2]	0.36 ± 0.03 **a**	<LOQ **b**	<LOQ **b**	<LOQ **b**
rosmarinic acid	7.56 ± 0.51 **b**	6.55 ± 0.18 **c**	5.46 ± 0.31 **d**	15.57 ± 0.84 **a**
salvianolic acid B	1.60 ± 0.20 **a**	0.87 ± 0.08 **b**	0.13 ± 0.03 **c**	0.15 ± 0.04 **c**
Luteolin	0.03 ± 0.01 **a**	0.06 ± 0.01 **a**	0.05 ± 0.03 **a**	0.06 ± 0.02 **a**
Antioxidant Assay				
ABTS[3]	542.97 ± 4.17 **d**	648.4 ± 5.18 **c**	945.01 ± 8.89 **a**	919.77 ± 4.37 **b**
FRAP[3]	348.83 ± 4.13 **b**	1428.74 ± 5.92 **a**	1443.37 ± 8.07 **a**	1439.86 ± 10.35 **a**
Superoxide anion scavenging activity[4]	0.05 ± 0.01 **c**	0.14 ± 0.03 **a**	0.09 ± 0.01 **b**	0.10 ± 0.01 **b**

[1] mean ± SD, n = 5. [2] Data expressed as milligrams equivalents of caffeic acid per mg/g of dry weight (DW).[3] Values expressed as µM Trolox equivalents per mg of dry weight (DW). [4] Values expressed as IC_{50}. In the column, values followed by the same small letter did not share significant differences at $p < 0.05$ (Tukey test).

To highlight the differences between *Salvia* species a principal component analysis was carried out on phenols quantification data. PCA data (Figure 2) showed that the first two dimensions account for 84.77% of the total variance. The first axis (F1) explains 68.41 % and the second axis (F2) 16.36 % of the total variance. The Biplot identified three well-defined clusters, ***So*** and ***Sf*** clustered together and are characterized by the lowest value of six phenols. To the second cluster belongs ***Ss*** characterized by an intermediate level of six phenols quantified and the third cluster characterized by ***Sc*** with the highest level of six phenols, in particular: danshensu, caffeic acid and salvianolic acid B.

Many studies demonstrated plant phenolic compounds present considerable free radicals scavenging activities as hydrogen- or electron-donating agents, and metal ion-chelating properties [2].

But because of the heterogeneity in the expression of results above all the tests of antioxidant activity carried out, it is difficult to compare them with similar works. Nevertheless, a stronger correlation between the TPC and the antioxidant activity was observed for the ABTS and FRAP assay compared to the Superoxide anion scavenging activity assay. In fact, the aqueous extract of ***So*** presents a greater quantity of phenolic compounds, as shown in the Table 4, and consequently a greater antioxidant activity (945.01 ABTS and 1443.37 FRAP µM TE mg/g DW), followed by ***Ss*** (919.37 ABTS and 1439.86 FRAP µM TE mg/g DW), ***Sf*** (646.40 ABTS and 1428.74 µM TE mg/g DW) and ***Sc*** (542.97 ABTS and 348.58 FRAP µM TE mg/g DW). Tukey post hoc test results reported four classes in ABTS test suggesting a continuum distribution of antioxidant activity and only two class in FRAP test suggesting a homogenous antioxidant activity for ***So***, ***Sf*** and ***Ss***. Different results are based on the superoxide anion scavenging activity assay, in which the main anti-oxidant capacity of ***Sc*** (0.05 (a) IC_{50}) has been highlighted. This can be determined by a higher concentration of salvianolic acid B and danshensu, scavenger of radicals and antioxidants, of which ***Sc*** is particularly abundant [34]. Considering all the results obtained, it is feasible to refer that aqueous extracts of *Salvia* species have a significant bioactivity, which seems to be positively correlated to their phenolic composition.

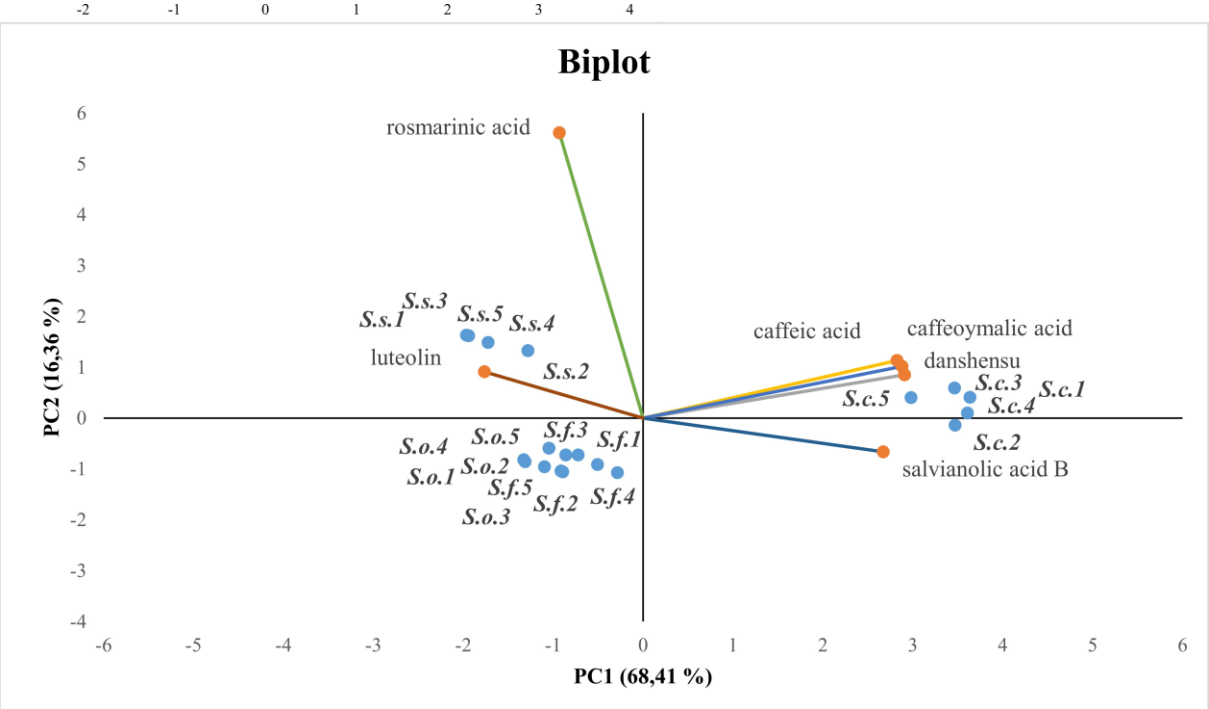

Figure 2. Principal component analysis (PCA) graph of phenols quantitative analysis. The PC1 and PC2 axes represent the two principal components with 68.41 % and 16.36 % of the total variance, respectively. The PCA identified three clusters, ***So*** **(***Salvia officianalis* L.**)** and ***Sf*** (*Salvia fruticosa* Mill.) clustered toghether and are charactererized by the lowest value of six phenols. To the second cluster belongs ***Ss*** (*Salvia sclarea* L.) characterized by an intermediate level of six phenols quantificated and the third cluster characterized by ***Sc*** (*Salvia clandestina* L.) with the highest level of six phenols. The number after acronyms represent the five independent biological replicates analysed for each species.

4. Conclusion

In the phytochemical profiles, *Salvia* species showed polymorphism in the chemical composition of EOs and phenolic compounds. In particular, EOs characterization of the three *Salvia* species (no EOs was recovered from the hydrodistillation of ***Sc*** aerial part) indicated a clear distinction of EOs profiles of ***Ss*** respect to ***Sf*** and ***So***. The collection of plants within the same pedoclimatic area, link this result to the genetic diversity of these *Salvia* species. Similar considerations can be made, for the four species, with regards to the phenolic compounds composition analyzed. In fact, the phenolic compounds present in ***Sf*** and ***So*** were very similar, while ***Sc*** and ***Ss*** showed distinctive profiles. In particular, ***Sc*** and ***Ss*** are rich sources of danshensu and rosmarinic acid, respectively. Regarding antioxidant activity, EOs of ***So*** has the highest antioxidant power, followed by the EOs of ***Ss*** and ***Sf***.

The assays carried out on the phenolic fraction confirm this different antioxidant capacity. The ***Sc***, instead, showed an excellent scavenging activity, probably for its higher concentration of danshensu, of which ***Sc*** is particularly rich. Moreover, the wide distribution, the easiness of cultivation and the widespread use of these species all over the world make them of particular interest in the future research to develop novel products as functional foods, herbal medicines and/or biopesticides. This preliminary investigation should be encouraged for the growing interest of consumers in natural substances as alternatives to synthetic antioxidants and this also could lead to the protection and the valorization of these species.

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