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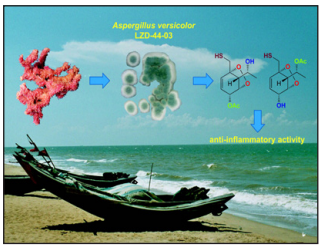

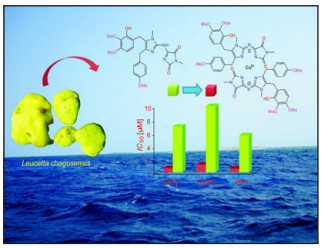

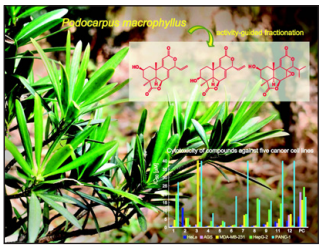

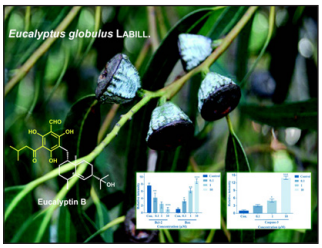

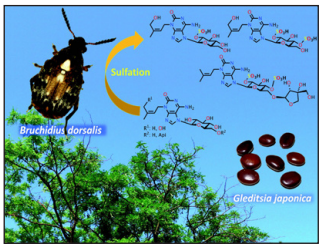

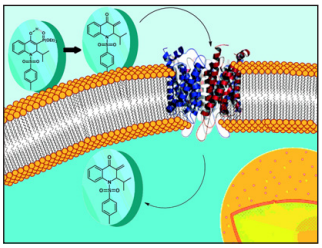

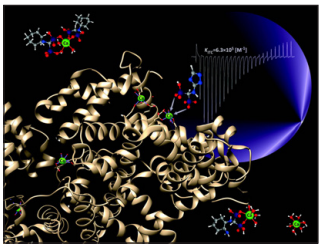

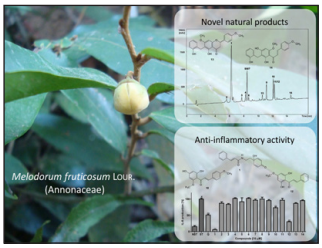

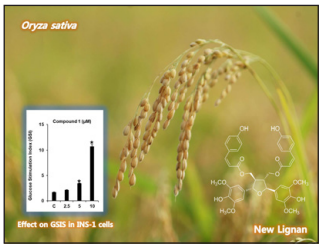

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FULL PAPER

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Phytochemical Profile and Antioxidant Activity of the Aerial Part Extracts from *Matthiola incana* subsp. *rupestris* and subsp. *pulchella* (*Brassicaceae*) Endemic to Sicily

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As part of a project aimed at investigating the *Matthiola* taxa endemic to Sicily (Italy), this study focused on *Matthiola incana*, an edible species used in the traditional medicine of various countries. Herein, the characterization of phenolic and volatile compounds, the antioxidant capacity *in vitro* (1,1-diphenyl-2-picrylhydrazil (DPPH), reducing power and Fe^{2+} chelating activity assays) and the toxicity test (*Artemia salina* lethality bioassay) of the hydroalcoholic extracts from the aerial parts of *M. incana* subsp. *rupestris* from Mt. Pellegrino (Palermo) and Mt. Erice (Trapani), and of *M. incana* subsp. *pulchella* are reported. The results are compared with those previously shown for *M. incana* subsp. *incana*, to achieve a comprehensive overview of the three subspecies. The HPLC-PDA/ESI-MS and SPME-GC/MS analyses led to the identification of 13 phenolics and 54 volatile compounds. Differences in the qualitative-quantitative profile of these phytochemicals have been highlighted between the *M. incana* subspecies. The antioxidant tests showed different activity for the extracts, which were found to possess better chelating properties. At last, none of the tested extracts displayed toxicity against brine shrimp larvae. These findings enrich the knowledge on the *Matthiola* taxa growing wild in Sicily, both from the strictly systematic point of view and for the possible applications as sources bioactive compounds that can be used in the nutraceutical field.

Keywords: Sicilian vascular flora, *Matthiola incana*, infraspecific taxa, chemical composition, biological activity.

Introduction

Several species belonging to the *Brassicaceae* family have been recognized as rich sources of phytochemicals with healthy nutritional effects and a promising wide range of biological activities.[1,2] Indeed, the majority of these vegetables, as also highlighted by phytochemical analyses on the Sicilian wild cabbages, contain precious molecules such as vitamins, carotenoids, phenolic compounds, terpenes and glucosinolates.[3]

For some years, our team has been carrying out research focused on taxa within the *Brassicaceae* family growing spontaneously in Sicily (Italy) that have been little or no investigated before, in order to highlight their potential as sources of bioactive compounds with promising applications in pharmaceutical, nutraceutical and cosmetic fields.[4–6] In this context, the taxa included in the genus *Matthiola* R. Br. endemic to Sicily have been selected for our current studies.

The genus *Matthiola* consists of about 50 annual and biennial herbaceous species or perennial shrubs, distributed throughout the Macaronesian islands, the Mediterranean basin, the Saharo-Sindian region and the north-eastern Africa and Asia.[7,8] The genus is represented by 10 taxa in Europe;[9] in Italy, the presence of 7 taxa is reported by Pignatti,[10] 4 of which occurring in Sicily, as also confirmed by Raimondo et al. in the 'Checklist of the vascular flora of Sicily',[11] namely *Matthiola incana* (L.) R. Br. and *M. fruticulosa* (L.) Maire (perennial), *M. tricuspidata* (L.) R. Br. and *M. sinuata* (L.) A. Br. (annual).

As part of a project aimed at investigating the four *Matthiola* taxa that grow wild in Sicily, at present our research is focused on *Matthiola incana*, a species not fully studied so far, despite its various utilizations.[12–14]

Matthiola incana is widely cultivated in several countries as an ornamental species, due to its colorful flowers; in fact, it has become an economically important floral crop.[15] Characteristic in Sicily is the use of the flowers to decorate horses during the popular festival of Saint Joseph which takes place in the village of Scicli.[16] *Matthiola incana* is also an edible species; in particular, the flowers are used in salads or as a garnish and are widely consumed as tea in China.[17–20] Moreover, the traditional use of this species against various ailments has been documented in many regions around the world. In the Islamic Iranian folk medicine, *M. incana* poultice has been reported to be used against the non-ulcerative cancers, being very effective in eliminating hard swellings, particularly those in the breast and testicles.[21] In India, the plant is utilized as an antidote to poisonous bites.[22] In Ecuador, *M. incana*, is used as a remedy for inflammation, nervousness, cough, stomach-ache, cardiac ailments.[23] In Bolivia, the aerial part decoction is utilized to treat eczemas.[24] In Italy (Sardinia) the infusion and decoction of the whole plant are used as emollient for the skin.[25] Furthermore, *M. incana* seeds contain an oil rich in omega-3-linolenic acid, representing a new and valuable potential source of oil for food supplements and industrial uses.[26]

In the latest edition of the 'Flora d'Italia' three infraspecific subdivisions are recognized under *M. incana*: the nominal subspecies, i.e., subsp. *incana*, the most widespread, the subsp. *rupestris* (Raf.) Nyman and the subsp. *pulchella* (Conti) Greuter & Burdet, confined to the island of Pantelleria. However, the 'Flora d'Italia' reports that the described morphological differences are not particularly indicative, and the three subspecies are not completely distinguished.[10] Indeed, in the 'Flora Europaea' only two subspecies are indicated under *M. incana*, i.e., subsp. *incana* and subsp. *rupestris*, and the consulted taxonomic database 'The Plant List' reports *M. incana* subsp. *pulchella* as a synonym of *M. incana* subsp. *rupestris*.[9,27] From the nomenclatural point of view, a very recent review recognizes to the endemic taxon of Pantelleria the subspecific rank but modifies its name in *Matthiola incana* subsp. *glandulifera* (Lojac.) C. Brullo & Brullo.[28]

Recently, we have reported the phytochemical characterization of the aerial parts of *M. incana* subsp. *incana*, also demonstrating the valuable *in vitro* antioxidant properties of this subspecies.[12] In this context, we believed it might be interesting to include in our studies also the other two subspecies under *M. incana* endemic to Sicily, namely *M. incana* subsp. *rupestris*, whose aerial parts were collected in two different localities (Mt. Pellegrino – Palermo and Mt. Erice – Trapani), and *M. incana* subsp. *pulchella* from Pantelleria. Hence, the qualitative-quantitative profile of phenolic and volatile compounds was characterized and compared to determine any differences that could be useful for the chemosystematic distinguishing of all subspecies under *M. incana*. Moreover, the antioxidant potential and the toxicity of these infraspecific taxa were evaluated.

Results and Discussion

Identification of Phenolic Compounds by HPLC-PDA/ESI-MS

Figure 1 shows the HPLC-PDA chromatograms, extracted at a 280 nm wavelength, of the phenolic compounds determined in the hydroalcoholic extracts of the aerial parts of *M. incana* subsp. *rupestris* from Mt. Pellegrino – Palermo (A) and Mt. Erice – Trapani (B), and of *M. incana* subsp. *pulchella* (C). Compound identification was carried out by combined data arising from retention times, PDA and MS spectra and literature.

As regards the *M. incana* subsp. *rupestris* extracts, the samples from different localities showed the same qualitative phenolic profile: 9 compounds, all belonging to the flavonoid class, were detected in both extracts. However, it should be noted that the total amount of identified flavonoids was quite different, resulting slightly higher in the samples from Mt. Erice (Trapani) respect to those collected from Mt. Pellegrino (Palermo) (152.37 mg/g extract and 120.19 mg/g extract, respectively). The peak n. 7 viz. naringin was the most abundant flavonoid found in both extracts and its content was superimposable (58.34 ± 11.42 mg/g extract and 60.23 ± 10.52 mg/g extract, respectively),

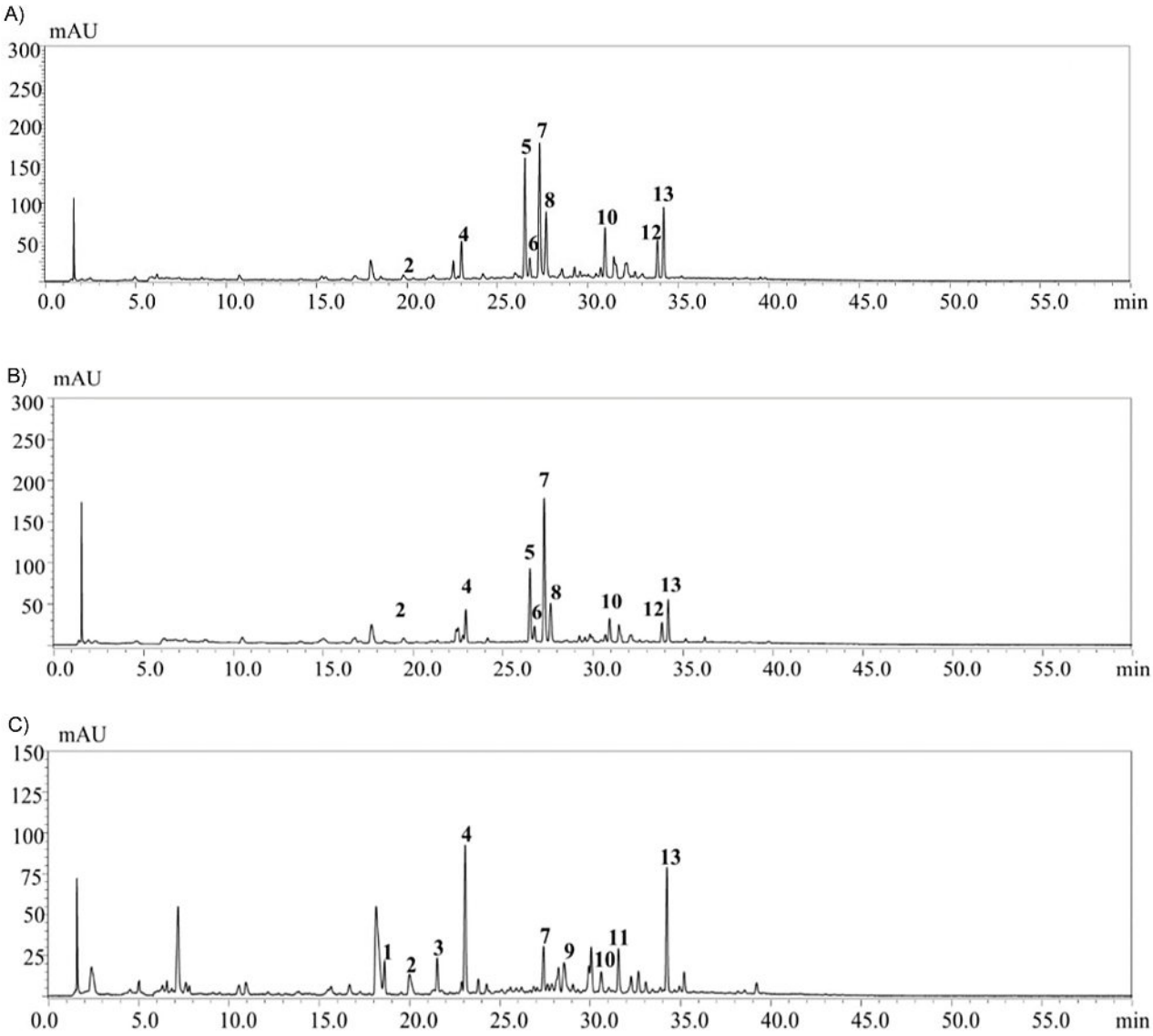

Figure 1. HPLC-PDA chromatograms of the phenolic compounds, extracted at 280 nm wavelength, of the hydroalcoholic extracts obtained from the aerial parts of *M. incana* subsp. *rupestris* from Mt Pellegrino – Palermo (**A**) and Mt. Erice – Trapani (**B**), and *M. incana* subsp. *pulchella* (**C**). For peak identification, see *Table 1*.

Table 1. HPLC-PDA/ESI-MS[a] (negative ionization mode) phenolic fingerprint of the hydroalcoholic extracts obtained from the aerial parts of *M. incana* subsp. *rupestris* from Mt. Pellegrino – Palermo (**A**) and Mt. Erice – Trapani (**B**), and *M. incana* subsp. *pulchella* (**C**).

No.	t_R (min)	Molecular Formula	$[M-H]^-$	UV/VIS (nm)	Compound	Class	mg/g extract (A)[b]	mg/g extract (B)[b]	mg/g extract (C)[b]
1	18.2	$C_{11}H_{12}O_5$	223	234, 321	Sinapic acid	Cinnamic acid-like	–	–	1.86 ± 0.78
2	19.5	$C_{15}H_{12}O_5$	271	283	Naringenin	Flavanone-like	6.13 ± 3.75	5.67 ± 1.58	11.61 ± 1.16
3	21.3	$C_{21}H_{22}O_{10}$	433, 271	254, 354	Naringenin-hexoside	Flavanone-glycoside-like	–	–	9.24 ± 3.94
4	22.9	$C_{21}H_{20}O_{11}$	447, 285	265, 365	Luteolin-hexoside	Flavone- glycoside-like	5.59 ± 2.87	6.41 ± 1.24	12.18 ± 1.21
5	26.5	$C_{27}H_{30}O_{16}$	609, 301	254, 354	Rutin	Flavonol-glycoside-like	29.24 ± 7.08	47.92 ± 6.55	–
6	26.7	–	725, 269	266, 335	Apigenin derivate	Flavone-glycoside-like	2.37 ± 0.57	3.24 ± 1.41	–
7	27.3	$C_{27}H_{32}O_{14}$	579, 271	254, 354	Naringin	Flavanone-glycoside-like	60.23 ± 10.52	58.34 ± 11.42	10.10 ± 0.15
8	27.7	$C_{26}H_{28}O_{14}$	583, 269	266, 348	Apigenin-dihexoside	Flavone-glycoside-like	6.22 ± 1.03	10.68 ± 1.99	–
9	28.5	–	739, 515	255, 351	Sinapoylhydroxyferuloyl-dihexoside	Cinnamic acid-like	–	–	2.76 ± 1.49
10	30.8	$C_{21}H_{20}O_{10}$	431, 269	270, 330	Vitexin	Flavone-glycoside-like	3.84 ± 0.11	7.96 ± 2.30	1.78 ± 0.97
11	31.6	$C_{21}H_{22}O_{11}$	449, 287	256, 346	Dihydrokaempferol-hexoside	Flavonol-glycoside-like	–	–	1.84 ± 1.70
12	33.4	$C_{21}H_{20}O_{11}$	447, 285	256, 346	Kaempferol-hexoside	Flavonol-glycoside-like	2.74 ± 1.25	5.52 ± 0.26	–
13	34.1	$C_{15}H_{12}O_6$	287	256, 346	Dihydrokaempferol	Flavonol-like	3.83 ± 0.65	6.63 ± 1.45	5.21 ± 0.22

[a] Column: Ascentis Express C_{18}, 15 cm × 4.6 mm, 2.7 µm d.p. (ESI, negative ionization mode; when observed, secondary fragment ions are reported). [b] Values are expressed as the mean ± S.D. ($n = 3$).

followed by peak n. 5 viz. rutin, whose amount resulted higher in the samples collected from Mt. Erice than those from Mt. Pellegrino (47.92 ± 6.55 mg/g extract and 29.24 ± 7.08 mg/g extract).

These small quantitative differences in the phenolic profile of the extracts could be related to the different ecological conditions of the two localities of origin of *M. incana* subsp. *rupestris* samples: in particular, to the more mesophilic conditions of Mt. Erice and to the more thermoxerophilic ones of Mt. Pellegrino.

Matthiola incana subsp. *pulchella* showed a phenolic profile quite different, both qualitatively and quantitatively, from that of subsp. *rupestris*. In the extract obtained from the aerial parts of this species, which grows exclusively on the island of Pantelleria, 9 phenolics were detected: 2 cinnamic acid-like compounds, namely sinapic acid (peak n. 1) and sinapoylhydroxyferuloyl dihexoside (peak n. 9), and 7 flavonoids. A much lower content of phenolics was highlighted (56.58 mg/g extract), being the total amount of phenolic acids 4.62 mg/g extract and the flavonoids 51.96 mg/g extract. Among the phenolics identified, peak n. 4 viz. luteolin-hexoside was the most abundant one (12.18 ± 1.21 mg/g extract), followed by naringenin (11.61 ± 1.16 mg/g extract).

From the comparison of the phenolic profile of the two subspecies included in this study, some qualitative differences were highlighted: the phenolic acids were detected only in the samples obtained from the subsp. *pulchella*, as well as the flavonoids naringenin-glucoside and dihydrokaempferol-glucoside. Instead, the flavonoids peaks n. 6 and 8, viz. two apigenin derivates, and peaks n. 5 and 15, namely rutin and kaempferol-hexoside were present exclusively in the extracts of the subsp. *rupestris*.

The phenolic profile of the two subspecies here analyzed differs also from that of the *M. incana* subsp. *incana* previously investigated.[12] Indeed, in the extract obtained from the nominal species a greater number of phenolic compounds was identified, 2 phenolic acids and 10 flavonoids.

Quantitatively, the total amount of phenolics detected in *M. incana* subsp. *incana* was similar to that of the subsp. *rupestris* and about 3 times higher than that of the subsp. *pulchella*.

Comparing the qualitative profile of the subsp. *rupestris* with that of the nominal species, it was observed that the latter contains phenolic acids, hexosides of quercetin and dihydrokaempferol, whereas the apigenin derivatives were not detected

As compared to the subsp. *pulchella*, some difference regarding the phenolic acid composition of *M.*

incana was found; in fact, the vanillic acid was identified in the nominal subspecies only. Concerning the flavonoids, *M. incana* subsp. *incana* contained rutin and kaempferol-hexoside, which were not detected in the subspecies endemic to Pantelleria.

Identification of Volatile Compounds by SPME-GC/MS

The volatile compounds identified in the hydroalcoholic extracts of the aerial parts of *M. incana* subsp. *rupestris* from Mt. Pellegrino – Palermo (A) and Mt. Erice – Trapani (B), and of *M. incana* subsp. *pulchella* (C) are reported in *Table 2*. Several volatile components belonging to the chemical classes of esters (n. 6), alcohols (n. 6), acids (n. 10), ketones (n. 10), aldehydes (n. 11), sulfur compounds (n. 5), hydrocarbons (n. 4) and nitriles (n. 2) were detected in the headspace of the analyzed extracts.

As regards the *M. incana* subsp. *rupestris* extracts, the samples from different localities (Trapani and Palermo) have similar volatile profiles with just little quantitative differences; their volatile fractions were constituted mainly of sulfur compounds and nitriles which accounted for much more than 50% of all volatiles. The main constituents were dimethyl trisulfide, dimethyl disulfide, benzyl nitrile and dimethyl tetrasulfide. Isothiocyanates were present at very low levels, with 3-butenyl isothiocyanate as the only one here detected. Among the other chemical classes, ketones were the most represented with 3-hexanone and hexahydrofarnesyl acetone as the main compounds.

Matthiola incana subsp. *pulchella* showed a volatile profile quite different, both qualitatively and quantitatively, from that of subsp. *rupestris*. In the case of *M. incana* subsp. *pulchella* the volatile profile was composed mostly of ketones, sulfides, and esters. Contrary to *M. incana* subsp. *rupestris* samples, neither isothiocyanates nor nitriles have been detected. Dimethyl trisulfide, 3-hexanone, hexahydrofarnesyl acetone, ethyl decanoate, dimethyl disulfide, dimethyl tetrasulfide and tetradecanol were the compounds quantitatively most represented.

The volatile composition of the two subspecies here analyzed differs also from that of *M. incana* subsp. *incana* previously investigated.[12] The *M. incana* subsp. *incana* aerial part hydroalcoholic extract was characterized by high levels of sulfides and, in decreasing order, of ketones, aldehydes and acids, whereas nitriles and isothiocyanate were present in very low amounts.

These differences are closely related to the glucosinolate hydrolysis process. Glucosinolates are secondary metabolites present in numerous plant species, mainly included in the order Brassicales, and from their hydrolysis sulfides, isothiocyanates and nitriles may arise. In fact, the glucosinolate cleavage, by the action of the myrosinase enzyme, can lead to different products, being influenced by the structure of the glucosinolate itself and by the presence of many factors which modify the enzyme action.[29]

Antioxidant Activity

Antioxidants have been traditionally classified into two main types, based on their mechanism of action: the chain-breaking antioxidants, i.e., the primary antioxidants, react with free radicals producing less reactive species or interrupting the radical chain reaction. The secondary antioxidants, the so-called preventive, act through indirect pathways and delay the oxidation process.[30]

The primary antioxidant abilities of the extracts of *M. incana* subsp. *rupestris* from Mt. Pellegrino – Palermo and Mt. Erice – Trapani, and *M. incana* subsp. *pulchella* were evaluated through the DPPH test and the evaluation of the reducing power, the secondary antioxidant efficacy was assessed using the ferrous ion chelating assay.

Figure 2a shows the results of the DPPH test, utilized to establish the free scavenging properties of the extracts. From the comparison of the extracts from the different subspecies of *M. incana* and the reference standard BHT, it is evident that all of them display a moderate activity, dose-dependent, in the range of concentrations assayed. Among the extracts, *M. incana* subsp. *rupestris* from Mt. Pellegrino showed the greatest radical scavenging ability, causing about 56% of inhibition at the greatest concentration tested. Comparing the results obtained from the samples of *M. incana* subsp. *rupestris* collected from two different localities, the radical scavenging activity differed significantly ($P < 0.0001$); actually, the extract of *M. incana* subsp. *rupestris* from Mt. Pellegrino, with an IC_{50} value of 1.73 ± 0.02 mg/mL, resulted much more active than the samples collected from Mt. Erice ($IC_{50} = 2.60 \pm 0.01$ mg/mL). According to the calculated IC_{50} values, the radical scavenging activity of the extracts and the standard decreases in the following order: BHT > *M. incana* subsp. *rupestris* (Mt. Pellegrino) -> *M. incana* subsp. *rupestris* (Mt. Erice) > *M. incana* subsp. *pulchella* (*Table 3*).

The radical scavenging activity of the subspecies selected for the present study differs also from that of *M. incana* subsp. *incana* previously investigated, result-

Table 2. Composition as volatile constituents and classes of substances of the hydroalcoholic extracts obtained from the aerial parts of *M. incana* subsp. *rupestris* from Mt. Pellegrino – Palermo (**A**) and Mt. Erice – Trapani (**B**), and *M. incana* subsp. *pulchella* (**C**).

Compounds	LRI[a] on DB-5 ms	LRI[a] on VF-WAXms	Amount[b]		
			A	**B**	**C**
Esters					
Isopentyl propanoate	972	1183	181	170	–[c]
Ethyl octanoate	1196	1430	138	153	235
Methyl decanoate	1323	1594	–	–	41
Ethyl 9-decenoate	1386	1703	295	319	899
Ethyl decanoate	1394	1639	1032	1165	3138
Methyl hexadecanoate	1926	2199	265	309	171
All			**1911**	**2115**	**4483**
Alcohols					
1-Octen-3-ol	980	1452	609	549	–
2-Ethyl-1-hexanol	1029	1028	178	194	280
Benzyl alcohol	1035	1860	67	75	–
(E)-2-Octen-1-ol	1068	1617	156	201	–
Phenylethanol	1114	1920	30	44	–
Tetradecanol	1592	2175	–	–	1032
All			**1040**	**1063**	**1312**
Acids					
3-Methylbutanoic acid (Isovaleric acid)	835	1681	–	–	179
2-Methylbutanoic acid	842	1687	119	133	–
Pentanoic acid	877	1758	171	183	–
Hexanoic acid	976	1860	55	43	–
Heptanoic acid	1075	1966	190	225	–
2-Ethylhexanoic acid	1117	1129	136	154	–
Octanoic acid	1174	2069	926	881	830
Phenylacetic acid	1245	2572	–	–	175
Nonanoic acid	1269	2171	525	583	333
Decanoic acid	1366	2273	233	269	424
All			**2355**	**2471**	**1940**
Ketones					
4-Methylpentan-2-one	736	1010	193	152	–
Acetylacetone	780	1196	482	387	–
2-Hexanone	790	1083	181	144	–
3-Hexanone	801	1046	3831	3404	4608
2-Heptanone	885	1185	145	175	–
4-Methylhentan-2-one	936	1210	306	479	460
1-Octen-3-one	977	1308	–	–	305
6-Methy-5-hepten-2-one	985	1340	50	62	–
2-Octanone	988	1286	168	200	–
6,10,14-Trimethylpentadecan-2-one (Hexahydrofarnesyl acetone)	1844	2119	2952	3953	3301
All			**8308**	**8956**	**8673**
Aldehydes					
3-Methylbutanal	668	949	–	–	360
Heptanal	903	1187	–	–	153
(E)-2-Heptenal	957	1331	–	–	315
Benzaldehyde	962	1529	354	482	–
Octanal	1004	1288	37	91	231
Phenylacetaldehyde	1043	1640	136	283	53
(E)-2-Octenal	1059	1432	–	–	397

Table 2. (cont.)

Compounds	LRI[a] on DB-5 ms	LRI[a] on VF-WAXms	Amount[b]		
			A	**B**	**C**
Nonanal	1105	1389	489	596	555
Safranal	1200	1651	201	199	–
Decanal	1206	1498	232	200	366
(E)-2-Decenal	1263	1647	–	–	171
All			**1449**	**1851**	**2602**
Nitriles					
Heptanenitrile	978	1408	197	276	–
Benzyl nitrile	1137	1893	6975	8512	–
All			**7172**	**8788**	
Sulfur Compounds					
Diethyl sulfide	680	902	980	1294	–
Dimethyl disulfide	743	1082	6790	8495	1300
Dimethyl trisulfide	968	1391	14548	22067	5642
3-Butenyl isothiocyanate	975	1464	77	59	–
Dimethyl tetrasulfide	1215	1750	4224	7692	1315
All			**26619**	**39607**	**8257**
Hydrocarbons					
Ethylbenzene	858	1129	–	382	–
1,3-Dimethylbenzene	868	1148	–	1675	–
1,4-Dimethylbenzene	893	1139	–	552	–
1-Octadecene	1792	1851	–	–	571
All				**2610**	**571**

[a] Linear retention indexes calculated according to the Van Den Dool and Kratz equation. [b] Peak area arbitrary scale; [c] not detected.

Table 3. Free radical scavenging activity (DPPH test), Reducing power, and Ferrous ion chelating activity of the hydroalcoholic extracts obtained from the aerial parts of *Matthiola incana* subsp. *rupestris* from Mt. Pellegrino – Palermo and Mt. Erice – Trapani, and *M. incana* subsp. *pulchella*.[a]

Matthiola incana subspecies	DPPH IC_{50} (mg/mL)[b]	Reducing power ASE/mL[b]	Fe^{2+} chelating activity IC_{50} (mg/mL)[b]
M. incana subsp. *rupestris* (Mt. Pellegrino)	1.73 ± 0.02^{a}	13.28 ± 1.20^{a}	1.54 ± 0.01^{a}
M. incana subsp. *rupestris* (Mt. Erice)	2.60 ± 0.01^{b}	13.45 ± 1.17^{a}	1.78 ± 0.03^{b}
M. incana subsp. *pulchella*	3.69 ± 0.14^{c}	21.15 ± 0.98^{b}	1.57 ± 0.02^{a}
Reference standard	BHT 0.07 ± 0.01^{d}	BHT 1.44 ± 0.02^{c}	EDTA 0.01 ± 3.55 E-05^{c}

[a] (a–d) Different superscript letters within the same column indicate significant differences between mean values ($P < 0.05$). [b] Values are expressed as the mean ± SD ($n = 3$).

ing the latter less active than the subsp. *rupestris* from Mt. Pellegrino but more active than the others (IC_{50} = 2.32 ± 0.24 mg/mL)[12]

The results of the reducing power assay of the extracts are shown in *Figure 2b*. All the extracts from the subspecies of *M. incana* exhibit mild reducing power as compared to BHT, which increases with increasing concentrations. As concerns the extracts of *M. incana* subsp. *rupestris* from the two different geographic locations, a similar effect was observed, slightly higher for that from Mt. Pellegrino considering the absorbance values, and superimposable according to the calculated ASE/mL (*Table 3*). Based on the ASE/mL values, the reducing power of the extracts and the standard decreases as follows: BHT > *M. incana* subsp.

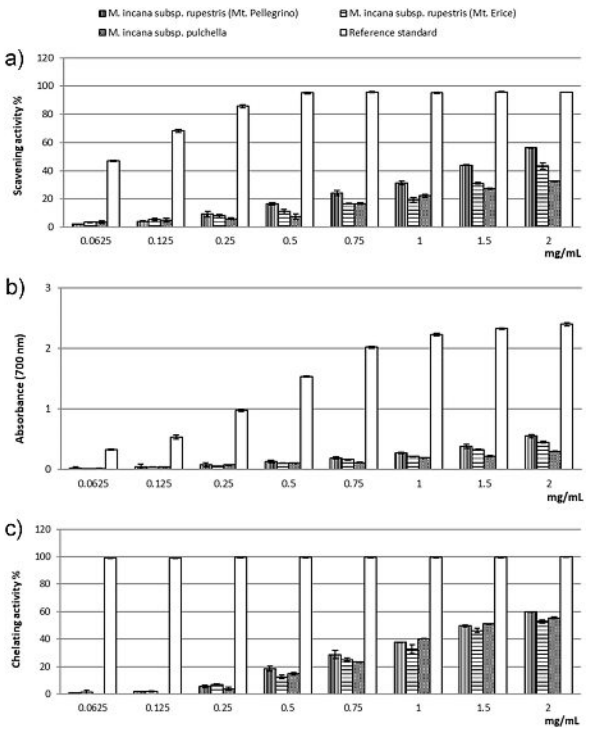

Figure 2. Free radical scavenging activity (DPPH test) a), Reducing power b), and Ferrous ion chelating activity c) of the hydroalcoholic extracts obtained from the aerial parts of *Matthiola incana* subsp. *rupestris* from Mt. Pellegrino – Palermo and Mt. Erice – Trapani, and *M. incana* subsp. *pulchella*. Reference standard: BHT a) and b), EDTA c). Values are expressed as the mean ± SD ($n = 3$).

rupestris (Mt. Pellegrino) = *M. incana* subsp. *rupestris* (Mt. Erice) > *M. incana* subsp. *pulchella*.

Anyway, all the tested extracts are less effective than that of *M. incana* subsp. *incana* previously investigated (ASE/mL = 12.28 ± 0.42 mg/mL).[12]

Figure 2c shows the results of the Fe^{2+} chelating activity of the tested extracts. It is clear that all the samples of the subspecies of *M. incana* display chelating ability. Comparing the results obtained from the samples of *M. incana* subsp. *rupestris* collected from two different localities, a significant difference ($P < 0.001$) of chelating activity was highlighted; the extract of *M. incana* subsp. *rupestris* from Mt. Pellegrino, with an IC_{50} of 1.54 ± 0.01 mg/mL, resulted more active than the samples collected from Mt. Erice. Interestingly, the activity of the former was superimposable to that of the subsp. *pulchella* (*Table 3*). On the basis of the IC_{50} values shown in *Table 3*, the chelating activity of the extracts and the standard was in the following decreasing order: EDTA > *M. incana* subsp. *rupestris* (Mt. Pellegrino) = *M. incana* subsp. *pulchella* > *M. incana* subsp. *rupestris* (Mt. Erice).

However, none of the extracts investigated in this study is able to overcome the effect previously observed for the *M. incana* subsp. *incana* extract, which turned out to be much higher, showing about 90% activity at the maximum concentration assayed, and with an IC_{50} value of 0.53 ± 0.02 mg/mL.[12]

Taken together, the results obtained demonstrate that all the tested extracts displayed higher secondary antioxidant properties than the primary ones. The extracts from *M. incana* subsp. *rupestris* collected in the two different locations displayed significantly different activity in both the DPPH and the chelating activity assays, although they showed only slight differences from a phytochemical point of view. The antioxidant activity of flavonoids has been widely demonstrated; thus, the flavonoid constituents of the extracts, especially naringin and rutin, which are contained in high amounts, could reasonably contribute to the antioxidant effects highlighted.[31,32] Nonetheless, the greater activity of the samples collected on Mt. Pellegrino cannot be justified with flavonoid components only, since the two extracts have the same qualitative profile but the extract obtained from the samples collected on Mt. Erice contains some flavonoids even in larger amount. This difference might be explained with the involvement of other phytochemicals belonging to different chemical classes contained in the extracts.

The extract of *M. incana* subsp. *pulchella* showed the lowest activity in both the DPPH and the reducing power assays; on the other hand, the extract showed a chelating effect comparable to that of *M. incana* subsp. *rupestris* from Mt. Pellegrino, despite the total amount of detected phenolics being about half. This could be explained taking into consideration the different phenolic profile highlighted for this subspecies, in particular, the presence of higher amounts of luteolin derivatives, that are considered moderate chelators, but mainly of naringenin glucoside and sinapic acid, that are strong iron chelators; indeed, these compounds are absent in the extracts of *M. incana* subsp. *rupestris*.[33,34]

Artemia salina Leach Lethality Bioassay

Artemia salina Leach (brine shrimp), a small marine invertebrate, is a versatile and valuable organism used as a biological system for toxicity testing. The brine shrimp lethality bioassay is extensively utilized as an alternative model for toxicity evaluation because it

offers numerous advantages such as rapidity, cost-effectiveness, continuous availability of cysts (eggs), and ease of handling and maintenance under laboratory conditions.[35] This experimental model has been applied as an alternative method for preliminary estimation of the toxicity of a large number of plant extracts.[36]

Based on the Clarkson's toxicity criterion, all the tested extracts from the *M. incana* subspecies showed no toxicity against brine shrimp larvae.[37] Indeed, the median lethal concentration values were greater than 1000 μg/mL, thus indicating that the extracts are potentially safe.

Conclusions

In this work, the phenolic and volatile profiles, and the antioxidant properties of *M. incana* subsp. *rupestris* and *M. incana* subsp. *pulchella* growing wild in Sicily (Italy) are reported for the first time. From the results of phytochemical analysis of the aerial part extracts from the two *M. incana* subspecies, compared with those of the similar study carried out on *M. incana* subsp. *incana*, it appears that there are some differences between the three taxa, both in the qualitative and quantitative profile of the phenolic and volatile compounds identified and in the potential antioxidant activity. Anyway, all the tested extracts displayed higher secondary antioxidant properties than the primary ones, also showing the absence of toxicity against brine shrimp larvae.

These findings show that the aerial parts of all the three *M. incana* infraspecific taxa are a safe source of antioxidants, thus increasing the number of *Brassicaceae* plants already known for their beneficial properties for human health and they could be useful for a chemosystematic distinction of these subspecies. Indeed, the data reported here support and clarify the distinction between the *M. incana* infraspecific taxa, so far considered incomplete only on the basis of morphological characters, also suggesting a further taxonomic deepening extended to the other species of the same genus endemic to Sicily.

Experimental Section

Chemicals and Reagents

LC/MS grade water (H_2O), acetonitrile (ACN), catechin, chlorogenic acid, apigenin, luteolin, rutin and kaempferol were obtained from Merck Life Science (Merck KGaA, Darmstadt, Germany). LC/MS grade formic acid was purchased from Riedel-de Haën (Seelze, Germany). Methanol (MeOH) was purchased from Baker Analyzed Reagents. Ferrous chloride ($FeCl_2$) was obtained from Carlo Erba (Milan, Italy). Unless indicated otherwise, all chemicals were purchased from Sigma–Aldrich (Milan, Italy).

Plant Material and Extraction Procedure

The aerial parts of *Matthiola incana* subsp. *rupestris* (Raf.) Nyman were collected in May 2019 from two different localities: at Mt. Erice (Trapani) Sicily, Italy, on limestone cliffs, about 260 m (a.s.l.), and at Mt. Pellegrino (Palermo), Sicily, Italy, on limestone rocks, about 50 m (a.s.l.). The aerial parts of *Matthiola incana* subsp. *pulchella* (Conti) Greuter & Burdet were collected in Contrada Campobello (Pantelleria), Sicily, Italy, in June 2020, on volcanic lithosol near the coast, about 6 m (a.s.l.). Voucher specimens were identified by Prof. F.M. Raimondo, *PLANTA*/Center for Research, Documentation and Training (Palermo) and Prof. V. Spadaro, University of Palermo, and have been deposited to the Herbarium Mediterraneum of the University of Palermo, Italy (PAL-Gr) (voucher numbers: Raimondo & Spadaro n. 04/19; Raimondo & Schimmenti n. 01/19; Cambiano & Greco n. 03/20). The aerial parts of the selected species were frozen just after collection, then, the freeze-dried plant material underwent preventive maceration with 80% MeOH (1:10 w/v) for 150 min followed by extraction with 80% MeOH (1:10 w/v) in an ultrasonic bath at 50 °C for 15 min, for three times. After filtration, the solvent was removed by rotavapor. The yields of the extracts, referred to 100 g of lyophilized plant material, were 20.26% and 20.15% for *M. incana* subsp. *rupestris* from Mt. Pellegrino and Mt. Erice, respectively, and 7.50% for *M. incana* subsp. *pulchella*.

Phytochemical Investigations

Identification of Phenolic Compounds by HPLC-PDA/ESI-MS

Samples preparation: An amount of 20 mg of *M. incana* subsp. *rupestris* from Mt. Pellegrino (A) and Mt. Erice (B) and of *M. incana* subsp. *pulchella* (C) aerial part extracts was dissolved in 1 mL of MeOH/water (80/20 *v/v*).

Instrumentation: The analyses were carried out using a Shimadzu HPLC system (Kyoto, Japan) equipped with a CBM-20 A controller, LC-20AD pumps,

a DGU-20A3 degasser, a SIL-20AC autosampler, an SPD-M20 A photo diode array detector (PDA) and a quadrupolar mass analyzer (LCMS-8050, Shimadzu, Kyoto, Japan), equipped with an ESI interface, in negative ionization mode. Data acquisition was performed by Shimadzu LabSolution software ver. 5.91.

Chromatographic conditions: Analyses were carried out on an Ascentis Express C18, 15 cm × 4.6 mm i.d. with particle size of 2.7 μm (Merck Life Science, Merck KGaA, Darmstadt, Germany). The injection volume was 5 μL, mobile phase consisted of water/formic acid (99.9 : 0.1) (solvent A) and ACN/formic acid (99.9 : 0.1) (solvent B), the linear gradient profile was as follows: 0 min, 0% B, 5 min, 5% B, 15 min, 10% B, 30 min, 20% B, 60 min, 50% B, 70 min, 100% B, 71 min, 0% B. The flow-rate was 1 mL/min and it was split to 0.2 mL/min prior to MS detection.

PDA conditions: The wavelength range was 200–400 nm and the chromatograms were extracted at a wavelength of 280 nm. Time constant was 25 ms and sample frequency 40.

MS conditions: The MS acquisition was performed using ESI, in negative mode, under the following conditions: mass spectral range 100–600 *m/z*; interval: 0.5 sec; nebulizing gas (N_2) flow: 1.5 L/min; interface temperature: 350 °C Heat block: 300 °C, DL temperature: 300 °C; DL voltage −34 V; probe voltage 4.5 kV; Qarray voltage: 1.0 V, RF voltage: 90 V; detection gain 1.0 kV.

Quantitative determination was carried using calibration curves of seven standards, namely naringenin, chlorogenic acid, naringin, apigenin, luteolin, rutin and kaempferol. Standard calibration curves were prepared in a concentration range 0.1–50 mg/L with five different concentration levels. Triplicate injections were made for each level, and a linear regression was generated. The calibration curves with the external standards were obtained using concentration (mg/L) with respect to the area obtained from the integration of the PDA peaks at a wavelength of 283 nm for flavanone-like compounds, 325 nm for cinnamic acid-like compounds, 335 and 365 for flavone-like-glycoside compounds, 346 nm and 354 nm for flavonol-glycoside-like compounds and 370 nm for flavanol-like compounds. The amount of each compound was finally expressed in mg/g of extract.

Identification of Volatile Compounds by SPME-GC/MS

Extraction (HS-SPME): The aerial part extracts of *M. incana* subsp. *rupestris* from Mt. Pellegrino (A) and Mt. Erice (B) and of *M. incana* subsp. *pulchella* (C) were analyzed for their volatile composition by HS-SPME-GC/MS. The dried extracts were solubilized in saturated sodium chloride solution to a final concentration of 10 mg/mL; then, 3 ± 0.1 mL of each extract solution was transferred to a 7 mL vial closed with a 'mininert' valve (Supelco, Bellefonte, PA, USA) and extracted using a DVB/CAR/PDMS fiber, 50/30 μm film thickness (Supelco, Bellefonte, PA, USA) following the method previously reported.[12]

Analysis (GC/MS): The volatiles were analyzed by a Shimadzu GC 2010 Plus gas chromatograph coupled to a TQMS 8040 triple quadrupole mass spectrometer (Shimadzu, Milan, Italy). Two capillary columns of different polarity were used: 1) VF-WAXms, 60 m, 0.25 mm i.d., 0.25 μm film thickness polar column (Agilent Technologies Italia S.p.A., Milan, Italy); 2) DB-5 ms, 30 m, 0.25 mm i.d., 0.25 μm film thickness apolar column (Agilent Technologies Italia S.p.A., Milan, Italy).

The conditions were as follows. Injection mode: splitless. Oven temperature: 1) 45 °C held for 5 min, then, increased to 80 °C at a rate of 10 °C/min and to 240 °C at 2 °C/min, held at 240 °C for 5 min, for VF-WAXms column; 2) 45 °C increased to 160 °C at a rate of 3 °C/min and to 260 °C at 10 °C/min, held at 260 °C for 5 min, for DB-5 ms column. Carrier gas: helium at a constant flow of 1 mL/min. Transfer line temperature: 250 °C. Acquisition range: 40 to 360 *m/z*; scan speed of 1250. For the identification of the volatiles, mass spectral data, NIST' 14 (NIST/EPA/NIH Mass Spectra Library, version 2.0, USA) and FFNSC 3.0 database, linear retention indices (LRI), literature data and injection of the available standards were used.[38]

Antioxidant Activity

DPPH Assay

The 1,1-diphenyl-2-picrylhydrazyl (DPPH) assay was carried out to evaluate the free radical scavenging activity of the hydroalcoholic extracts of *M. incana* subsp. *rupestris* from Mt. Pellegrino and Mt. Erice and of *M. incana* subsp. *pulchella*, using the protocol reported in our previous work.[12] The extracts were tested in the range of 0.0625–2 mg/mL, and butylated hydroxytoluene (BHT) was utilized as positive control.

A volume of 0.5 mL of each sample was mixed with 3 mL of methanol DPPH solution (0.1 mM) and incubated for 20 min at room temperature in the dark. Then, the color change of the solutions was estimated by measuring absorbance with a spectrophotometer (UV-1601, Shimadzu) at the wavelength of 517 nm. Three independent experiments were carried out, and the results are reported as the mean radical scavenging activity percentage (%) ± standard deviation (SD) and mean 50% inhibitory concentration (IC_{50}) ± SD.

Reducing Power Assay

The reducing power of the hydroalcoholic extracts of *M. incana* subsp. *rupestris* from Mt. Pellegrino and Mt. Erice and of *M. incana* subsp. *pulchella* was estimated through the Fe^{3+}-Fe^{2+} transformation method, according to the protocol reported in our previous work.[12] The extracts were tested in the range of 0.0625–2 mg/mL, and butylated hydroxytoluene (BHT) and ascorbic acid were utilized as positive controls. After mixing 2.5 mL of phosphate buffer (0.2 M, pH 6.6) and 2.5 mL of 1% potassium ferrycyanide, 1 mL of each sample was added. Following incubation at 50 °C for 20 min and rapid cooling, 2.5 mL of 10% trichloroacetic acid was added, and the mixture was centrifuged (3000 rpm, 10 min). Then, 2.5 mL of the supernatant was transferred in another test tube and mixed with 2.5 mL of distilled water and 0.5 mL of 0.1% ferric chloride ($FeCl_3$). After incubation for 10 min of at room temperature in the dark, the color change of the samples was estimated by measuring absorbance at 700 nm. Three independent experiments were carried out, and the results are expressed as the mean absorbance values ± SD and ascorbic acid equivalent (ASE/mL) ± SD.

Ferrous Ion (Fe^{2+}) Chelating Activity Assay

The spectrophotometric measurement of the Fe^{2+}-ferrozine complex was used to determine the Fe^{2+} chelating activity of the hydroalcoholic extracts of *M. incana* subsp. *rupestris* from Mt. Pellegrino and Mt. Erice and of *M. incana* subsp. *pulchella*, following the protocol previously reported.[12] The extracts were tested in the range of 0.0625–2 mg/mL, and ethylenediaminetetraacetic acid (EDTA) was used as positive control. After mixing 1 mL of each sample with 0.5 mL of methanol and 0.05 mL of 2 mM $FeCl_2$, the reaction was initiated by adding of 0.1 mL of 5 mM ferrozine. The mixture was incubated at room temperature in the dark for 10 min and the color change of the solutions was estimated by measuring absorbance spectrophotometrically at 562 nm. Three independent experiments were carried out, and the results are reported as the mean inhibition of the ferrozine-(Fe^{2+}) complex formation (%) ± SD and IC_{50} ± SD.

Artemia Salina Leach Lethality Bioassay

The brine shrimp (*Artemia salina* Leach) lethality bioassay was used to establish the acute toxicity of the hydroalcoholic extracts of *M. incana* subsp. *rupestris* from Mt. Pellegrino and Mt. Erice and of *M. incana* subsp. *pulchella*, as previously reported.[12] Ten brine shrimp larvae, taken 48 h after hatching in artificial seawater, were incubated for 24 h at 25–28 °C in 5 mL of artificial seawater mixed with different amounts of the extracts (10–1000 μg/mL). Then, the surviving larvae were counted using a magnifying glass and median lethal concentration (LC_{50}) values were determined by the Litchfield and Wilcoxon's method. The assay was carried out in triplicate. The toxicity level of the extracts was assessed according to the toxicity scale reported by Clarkson et al.; LC_{50} values above 1000 μg/mL indicate absence of toxicity.[37]

Statistical Analysis

Statistical comparison of data was carried out by using one-way analysis of variance (ANOVA) followed by Tukey-Kramer multiple comparisons test (GraphPADPrism Software for Science). *P*-values lower than 0.05 were considered statistically significant.

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Author Contribution Statement

N. Miceli, F.M. Raimondo, V. Spadaro and M.F. Taviano, conceived and designed the study; F. Raimondo and V. Spadaro collected and identified the plant material; F. Cacciola, Y. O. El Majdoub, K. Arena, L. Mondello, C. Condurso, and F. Cincotta carried out the phytochemical studies; N. Miceli, M.F. Taviano, E. Cavò and S. Ragusa performed the antioxidant and toxicity experiments and analysed the data. N. Miceli, M.F. Taviano,

V. Spadaro, F. Cacciola and C. Condurso wrote the original draft of the manuscript. All the authors revised the article critically and gave approval of the final version.

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