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Bioactive Constituents of *Juniperus turbinata* Guss. from La Maddalena Archipelago

Alessandro Venditti,*[a] Filippo Maggi,[b] Luana Quassinti,[b] Massimo Bramucci,[b] Giulio Lupidi,[b] Luigi Ornano,[a] Mauro Ballero,[c] Cinzia Sanna,[d] Maurizio Bruno,[e] Sergio Rosselli,[e] and Armandodoriano Bianco[a]

[a]Dipartimento di Chimica, Università di Roma "La Sapienza", Piazzale Aldo Moro 5, 00185 Rome, Italy, e-mails: alessandro.venditti@gmail.com; alessandro.venditti@uniroma1.it

[b]Scuola di Scienze del Farmaco e dei Prodotti della Salute, Università di Camerino, Via Sant'Agostino 1, 62032, Camerino, Italy

[c]CoSMeSe, Consorzio per lo Studio dei Metaboliti Secondari, Via Sant'Ignazio 13, 09123, Cagliari, Italy

[d]Dipartimento di Scienze della Vita e dell'Ambiente, Università di Cagliari, Via Sant'Ignazio 13, 09123, Cagliari, Italy

[e]Dipartimento di Scienze e Tecnologie Biologiche Chimiche e Farmaceutiche, Università di Palermo, Viale delle Scienze – Parco d'Orleans II Ed. 17, 90128 Palermo, Italy

A comprehensive phytochemical study of *Juniperus turbinata* (Cupressaceae) collected from La Maddalena Archipelago (Sardinia, Italy) is reported. Both the essential oil and the ethanolic extract obtained from the aerial parts were analyzed. The essential oil appears to belong to a new chemotype compared to other Mediterranean juniper accessions, as it was favored by geographic isolation of the isles. It showed a low content of monoterpene hydrocarbons and α-terpineol, *ent*-manoyl oxide, 1,10-di-*epi*-cubenol as the major constituents. The ethanolic fraction contained mainly diterpenoids. Among these, 15-formyloxyimbricatolic acid (**7**) is a new natural product since it has hitherto been obtained only by synthetic route. The phenolic fraction contained biflavonoids: cupressuflavone (**9**), followed by minor amounts of amentoflavone (**10**) and hinokiflavone (**11**). The essential oil and six purified compounds (**1** – **4**, **8** and **9**) were assessed for biological activities, namely antioxidant (assessed by $DPPH^{\cdot}$, $ABTS^{\cdot+}$ and FRAP methods) and cytotoxic effects towards selected human tumor cell lines (MDA-MB 231, A375 and HCT116 cells). Compound **3** exhibited higher radical scavenging activity against $ABTS^{\cdot+}$ radical than the reference Trolox. Noteworthy, compound **8** showed powerful effects towards tumor cell lines, with IC_{50} values in the range of 0.060 – 0.201 μM, which make it a promising anticancer drug candidate.

Keywords: *Juniperus turbinata*, polar compounds, imbricataloic acid, essential oil, biological activity.

Introduction

Juniperus turbinata Guss. is a Mediterranean shrub or small tree, up to 6 – 8 m tall, belonging to Cupressaceae family. It has been regarded as *Juniperus phoenicea* ssp. *turbinata* (Guss.) Nyman[1][2] or *Juniperus phoenicea* var. *turbinata* (Guss.) Parl.,[3][4] and included in the *Juniperus phoenicea* L. complex, along with *Juniperus phoenicea* s.s. However, according to morphological[5][6] and genetic differences[7] between the two taxa, *Juniperus turbinata* has recently been accepted at species rank.

J. turbinata grows in coastal areas around the Mediterranean Sea and in the mountains of northwest of Africa, up to 800 m a.s.l.,[8] while *J. phoenicea* s.s. is present on mountains of Iberian Peninsula, southern France and north-western Italy.[5]

The *J. phoenicea* complex enjoys good reputation as an important medicinal plant as it has largely been used in traditional medicine to heal various illnesses such as dysentery, rheumatism, intestinal, urinary and respiratory problems,[9] diabetes[10][11] and infectious diseases.[12 – 14]

Two major classes of secondary metabolites are found in the *Juniperus* genus such as terpenoids, with diterpenes as the main compounds, and phenolics, in particular biflavonoids.[15 – 19]

Concerning *J. phoenicea*, a significant degree of morphological and genetic variability has been reported in literature.[20 – 24] This may undoubtedly affects the secondary metabolism expression of the species. It is worth of note that most of studies on *J. phoenicea* are related to its monoterpene-rich essential oil[14][25 – 29] and the monoterpene hydrocarbon α-pinene emerged as the principal characterizing volatile compound. Conversely, the non-volatile secondary metabolites were investigated to a minor extent, and

the following classes of natural products were the main groups of secondary metabolites characterizing the species *J. phoenicea*: abietane diterpenoids,[19][30] sesquiterpenes,[31] norterpenoid glucosides,[32] scutellarein derivatives,[33] furanones, phenylpropane glucosides[17][34][35] and lignans.[36] However, in this context, *J. turbinata* has hitherto been poorly explored and showed labdane diterpenes as the main constituents.[37]

The aim of this study was to perform a complete phytochemical analysis on both polar and volatile compounds of *J. turbinata* collected from a geographically isolated population living in the La Maddalena Archipelago and to assess their biological activity, namely the cytotoxicity on a panel of human tumor cells (MDA-MB 231, A375 and HCT116 cells) together with the radical scavenging and ferric reducing antioxidant capacity. For this purpose, the main secondary metabolites occurring in the *J. turbinata* ethanolic extract were isolated by column chromatography and structurally elucidated by MS and NMR techniques. The essential oil was hydrodistilled from the aerial parts and analyzed by GC-FID and GC/MS. Antioxidant activity and cytotoxic effects of essential oil and six isolated compounds were evaluated by $DPPH^{\cdot}$, $ABTS^{\cdot+}$, FRAP and MTT methods, respectively.

Results and Discussion

Analysis of the Ethanolic Extract

As already recognized from other Mediterranean populations of *J. turbinata*, the sample collected from La Maddalena Island showed a huge presence of diterpenoids, which were the major phytochemicals. Among these, imbricatolic acid (**1**), 13-*epi*-cupressic acid (**2**), *ent*-manoyl oxide (**3**), 7α-hydroxysandaracopimaric acid (**4**), 13-*epi*-torulosal (**5**), sandaracopimaric acid (**6**), 15-formyloxyimbricatolic acid (**7**) and imbricataloic acid (**8**) were identified (*Figure 1*). In this context, it is worthy to note that the isolation of 15-formyloxyimbricatolic acid (**7**), from a natural source, is reported here for the first time. In fact, compound (**7**) was previously obtained by a semisynthesis approach starting from imbricatolic acid (**1**).[38] From the chemical standpoint, compound **7** may be considered as the oxidation product of 15-formyloxyimbricatolal, recognized as one of the components of a Chilean resin sample obtained from *Araucaria araucana*.[39] The identification of compound **7** was performed by applying an extensive bidimensional NMR analysis (using both mono- and bidimensional experiments) and following a method reported in our recent works[40 – 42] which permit to identify the components of simple mixtures by unequivocal assignment of each resonance signal. In the present case, the assignment of resonances to the respective compounds was simplified because the components were already described in literature, while these methods have previously been applied in the structure elucidation of new natural products. As a new natural compound, the presence of **7** is also of high chemosystematic relevance because it was not previously recognized in this species. It might likely be a characteristic phytochemical trait owned by the population living in La Maddalena Island and probably derived by its geographic isolation. We already observed peculiar phytochemical

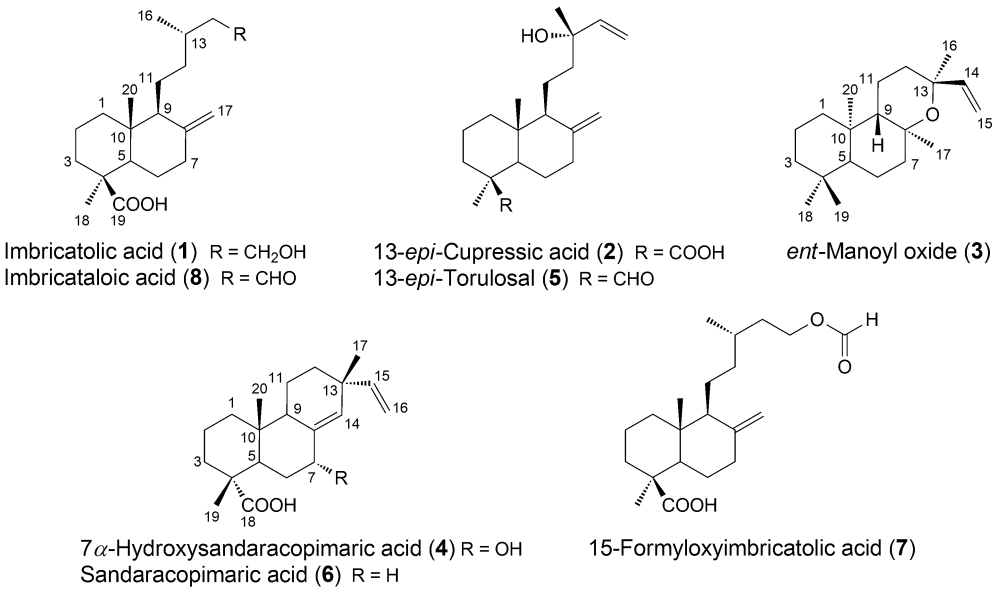

Figure 1. Diterpenoids isolated from *Juniperus turbinata* from La Maddalena Archipelago.

patterns in the polar fraction obtained from species living in isolated environments, restricted areas or by endemic entities, especially in species collected from Sardinia and La Maddalena islands,[43 – 51] therefore the results achieved in the present study are a further confirmation of these observations. Moreover, the majority of the diterpenes identified in this study have never been reported as constituents of *J. turbinata*, and this represent an additional evidence of the influence exerted by the isolated environment on the phytochemical pattern. Making a comparison, the observed diterpene composition was very similar to those observed in *J. rigida* from Korea[52] and partially to those reported for *J. phoenicea* s.s. from Saudi Arabia.[53]

The minor components of the ethanolic extract belong to the class of flavonoid and resulted to be three bi-apigenin derivatives with apigenin residues coupled in different ways: with the C–C linkage in cupressuflavone (**9**; C(8)–C(8″)) and amentoflavone (**10**; C(3′)–C(8″)), respectively, and with the C–O linkage in hinokiflavone (**11**; C(4′)–O–C(6″); *Figure 2*).

All these biflavonoids were already recognized in *Juniperus* spp. as well as in the related species of the Cupressaceae family,[18][53][54] while simple flavonoids, reported as constituents in accessions belonging to other populations of *J. turbinata*,[55][56] were not recognized in the present study.

Composition of the Essential Oil

The composition of the essential oil of *J. turbinata* is reported in *Table 1*. A total of 99 volatile components were identified, corresponding to 91.0% of the total composition. The major compounds were represented by α-terpineol (11.0%), *ent*-manoyl oxide (5.5%) and 1,10-di-*epi*-cubenol (5.1%). α-Terpineol is a safe compound because of its frequent use in fragrances and is being used as a scaffold for the synthesis of new drugs for the treatment of cancer, severe pains and inflammatory disorders.[57][58] The most abundant fraction in the oil was that of oxygenated monoterpenes (30.8%), followed by similar levels of sesquiterpene hydrocarbons (23.6%) and oxygenated sesquiterpenes (21.7%). Noteworthy was the presence of diterpenes (10.9%, ten identified compounds), mainly labdanes, abietanes and pimaranes, among which *ent*-manoyl oxide was the most abundant. Finally, scarce was the contribution of the monoterpene hydrocarbons with 1.3% of the total. Other components occurring in significant amounts (> 2%) in the oil were: *trans*-verbenol (2.4%) and piperitone (4.2%) among oxygenated monoterpenes; δ-cadinene (3.9%), germacrene D (2.6%), *epi*-bicyclosesquiphellandrene (2.4%), germacrene B (2.2%) and γ-cadinene (2.8%) for sesquiterpene hydrocarbons; elemol (2.3%) and shyobunol (3.4%) for oxygenated sesquiterpenes, and *trans*-totarol (2.1%) for diterpenes.

Some differences in volatiles were found with respect to other populations of *J. turbinata* studied previously. Rezzi and collaborators[59] analyzed a population growing in Corsica and found α-pinene, β-phellandrene, α-terpinyl acetate, δ-3-carene, myrcene and α-phellandrene as the main constituents. Similar composition was observed in populations of *J. phoenicea* var. *turbinata* (the previously accepted

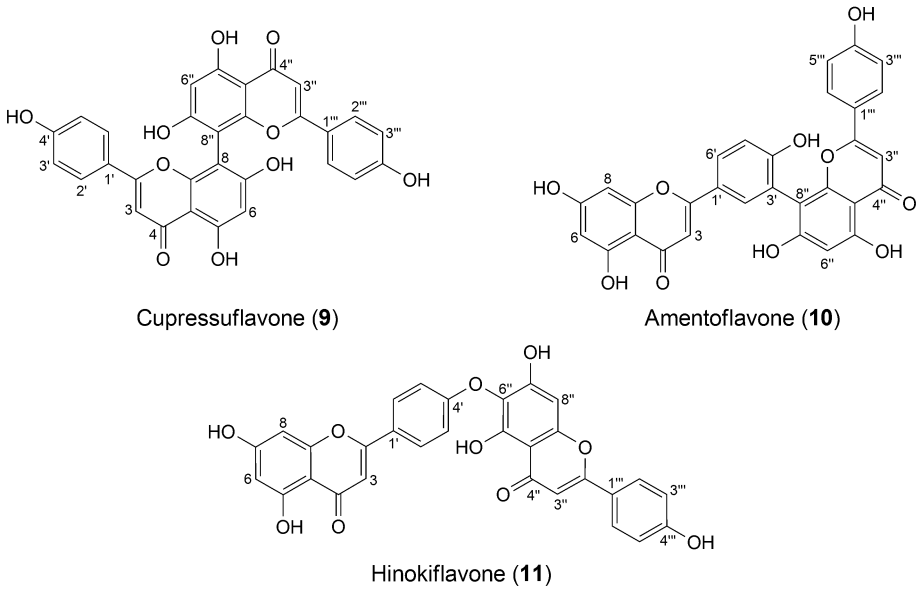

Figure 2. Biflavonoids isolated from *Juniperus turbinata* from La Maddalena Archipelago.

Table 1. Essential oil composition of *Juniperus turbinata*

No.	Component[a]	RI[b]	RI lit.[c]		[%][d]	ID[e]
			Adams	NIST08		
1	α-Pinene	932	939	932	0.2	Std
2	Myrcene	991	990	992	0.0	Std
3	α-Phellandrene	1003	1002	1002	0.1	Std
4	*p*-Cymene	1026	1024	1025	0.0	Std
5	β-Phellandrene	1029	1029	1030	0.9	Std
6	Linalool	1101	1096	1100	0.1	Std
7	*cis*-*p*-Menth-2-en-1-ol	1120	1121	1121	0.7	RI, MS
8	α-Campholenal	1125	1126	1123	0.7	RI, MS
9	*trans*-Pinocarveol	1136	1139	1137	1.3	Std
10	*trans*-*p*-Menth-2-en-1-ol	1140	1140	1140	0.4	RI, MS
11	Camphor	1141	1146	1139	0.6	Std
12	*trans*-Verbenol	1145	1144	1145	2.4	RI, MS
13	*trans*-Pinocamphone	1159	1162	1159	0.2	RI, MS
14	Pinocarvone	1162	1164	1162	0.2	RI, MS
15	Borneol	1164	1169	1164	0.1	Std
16	*p*-Mentha-1,5-dien-8-ol	1168	1170	1168	0.8	RI, MS
17	*cis*-Pinocamphone	1171	1175	1170	0.3	RI, MS
18	Terpinen-4-ol	1176	1177	1175	0.4	Std
19	Cryptone	1183	1184	1184	1.3	RI, MS
20	*p*-Cymen-8-ol	1186	1182	1186	0.5	RI, MS
21	α-Terpineol	1189	1188	1189	11.0	Std
22	Myrtenal	1193	1195	1193	0.4	RI, MS
23	Myrtenol	1193	1195	1193	0.6	Std
24	Verbenone	1204	1205	1204	1.1	Std
25	*trans*-Piperitol	1204	1208	1205	0.6	RI, MS
26	*trans*-Carveol	1218	1216	1219	0.7	RI, MS
27	Citronellol	1233	1225	1232	1.5	Std
28	Cuminaldehyde	1238	1241	1239	0.3	RI, MS
29	Piperitone	1252	1252	1252	4.2	Std
30	*trans*-Myrtanol	1258	1261		0.3	RI, MS
31	(4*E*)-Decen-1-ol	1261	1263		1.2	RI, MS
32	Phellandral	1271		1272	0.7	RI, MS
33	α-Terpinen-7-al	1281	1285		0.1	RI, MS
34	*p*-Cymen-7-ol	1290	1290	1289	0.1	RI, MS
35	δ-Elemene	1334	1338	1337	0.3	RI, MS
36	δ-Cubebene	1346	1348		0.1	RI, MS
37	δ-Copaene	1370	1376		0.1	RI, MS
38	β-Bourbonene	1377	1388	1377	0.1	RI, MS
39	β-Cubebene	1385	1388	1386	0.1	RI, MS
40	β-Elemene	1387	1390	1387	0.4	RI, MS
41	(*E*)-Caryophyllene	1409	1419	1409	1.0	Std
42	*cis*-Thujopsene	1420	1431		0.3	RI, MS
43	2,5-Dimethoxy-*p*-cymene	1425	1426		0.6	RI, MS
44	γ-Elemene	1429	1436		0.8	RI, MS
45	*cis*-Muurola-3,5-diene	1443	1450		0.7	RI, MS
46	α-Humulene	1445	1454	1446	1.1	Std
47	*trans*-Cadina-1(6),4-diene	1468	1476		1.3	RI, MS
48	Germacrene D	1473	1485	1472	2.6	RI, MS
49	β-Selinene	1477	1490	1478	0.2	RI, MS
50	*epi*-Bicyclosesquiphellandrene	1482		1471	2.4	RI, MS
51	Valencene	1485	1496	1485	0.3	RI, MS
52	*epi*-Cubebol	1487	1494		1.6	RI, MS
53	Butylated hydroxyanisole	1487	1489		0.8	RI, MS
54	α-Muurolene	1494	1500		1.0	RI, MS
55	γ-Cadinene	1508	1513	1509	2.8	RI, MS
56	*trans*-Calamenene	1518	1522	1517	1.0	RI, MS

Table 1. (cont.)

No.	Component[a]	RI[b]	RI lit.[c]		[%][d]	ID[e]
			Adams	NIST08		
57	δ-Cadinene	1518	1523	1518	3.9	RI, MS
58	*trans*-Cadina-1,4-diene	1526	1534		0.4	RI, MS
59	Methyl dodecanoate	1528	1525	1527	0.4	RI, MS
60	(*Z*)-Nerolidol	1533	1532		0.3	RI, MS
61	γ-Vetivenene	1533	1533		0.3	RI, MS
62	α-Calacorene	1536	1545	1547	0.2	RI, MS
63	Elemol	1545	1549	1545	2.3	RI, MS
64	Germacrene B	1547	1561	1548	2.2	RI, MS
65	*cis*-Muurol-5-en-4β-ol	1554	1551		0.1	RI, MS
66	β-Calacorene	1556	1564		0.1	RI, MS
67	*cis*-Muurol-5-en-4α-ol	1559	1561		0.1	RI, MS
68	(*E*)-Nerolidol	1564	1563	1564	0.2	Std
69	Germacrene D-4-ol	1568	1575	1568	0.7	RI, MS
70	Caryophyllene oxide	1573	1583	1573	1.5	Std
71	Dodecanoic acid	1575		1575	0.2	RI, MS
72	Salvial-4(14)-en-1-one	1584	1594		0.2	RI, MS
73	Widdrol	1588	1599	1587	0.3	RI, MS
74	Ethyl dodecanoate	1597	1598	1597	0.2	RI, MS
75	Humulene epoxide II	1597	1608		0.7	RI, MS
76	1,10-Di-*epi*-cubenol	1620	1619		5.1	RI, MS
77	*epi*-α-Muurolol	1635	1642		1.4	RI, MS
78	β-Eudesmol	1640	1650	1640	1.2	RI, MS
79	α-Eudesmol	1644	1653	1644	0.9	RI, MS
80	α-Cadinol	1647	1654	1646	1.2	RI, MS
81	Shyobunol	1680	1689		3.4	RI, MS
82	Amorpha-4,9-dien-2-ol	1699	1700		0.1	RI, MS
83	Pentadecanal	1714		1717	0.2	RI, MS
84	Methyl tetradecanoate	1728	1723	1728	0.0	RI, MS
85	Butyl dodecanoate	1787	1787	1786	tr[f]	RI, MS
86	Nootkatone	1792	1806	1794	0.3	RI, MS
87	Cyclohexadecanolide	1916	1934	1912	0.2	RI, MS
88	Methyl hexadecanoate	1929	1921	1928	0.0	RI, MS
89	Pimaradiene	1943	1949		0.1	RI, MS
90	*ent*-Manoyl oxide	1976	1987	1977	5.5	RI, MS
91	Abietatriene	2042	2056	2041	0.5	RI, MS
92	Abietadiene	2065	2087		0.1	RI, MS
93	unknown diterpene MW = 272	2094			1.8	MS
94	Sandaracopimarinal	2183	2184		0.1	RI, MS
95	Sempervirol	2282	2283		0.1	RI, MS
96	4-*epi*-Abietal	2295	2298		0.4	RI, MS
97	*trans*-Totarol	2299	2314	2299	2.1	RI, MS
98	*trans*-Ferruginol	2325	2331	2325	0.2	RI, MS
99	Nonacosane	2900	2900	2900	0.1	Std
	Total identified [%]				91.0	
	Monoterpene hydrocarbons				1.3	
	Oxygenated monoterpenes				30.8	
	Sesquiterpene hydrocarbons				23.6	
	Oxygenated sesquiterpenes				21.7	
	Diterpenes				10.9	
	Others				4.5	

[a] Compounds are listed in order of their elution from a HP-5MS column. [b] Linear retention index on HP-5MS column, experimentally determined using homologous series of $C_8 - C_{30}$ alkanes. [c] Linear retention index taken from Adams (2007) and NIST 08 (2008). [d] Percentage values are means of three determinations with a RSD% in all cases below 10%. [e] Identification methods: std, based on comparison with authentic compounds; MS, based on comparison with Wiley, Adams and NIST 08 MS databases; RI, based on comparison of LRI with those reported in Adams, FFNSC 2 and NIST 08. [f] tr [%] below 0.1%.

denomination of this taxon) from Portugal, Algeria and Morocco, where α-pinene, β-phellandrene and α-terpinyl acetate were detected as the major volatile compounds.[25][26][60] On the other hand, in populations from Tunisia and Morocco α-pinene was by far the most abundant volatile component.[14][28][29][61] Thus, one of the main features which differentiated the investigated population of *J. turbinata* from those previously studied appears to be the low content of monoterpene hydrocarbons along with a noteworthy presence of sesquiterpenes and diterpenes.

Overall, it seems that *J. phoenicea* complex exhibits an infraspecific chemical variability with more chemotypes depending on geographic and climatic factors. The geographic isolation of the La Maddalena Archipelago could have influenced the development of the observed chemical variability. In fact, we have already detected peculiar molecular patterns in EOs obtained from species collected from these Isles, especially in the case of endemic entities as *Artemisia caerulescens* subsp. *densiflora*[62] and *Helichrysum microphyllum* subsp. *tyrrhenicum*,[63] as well as in the case of more widespread species such as *Artemisia arborescens*.[64]

Cytotoxic Activity on Tumor Cells

The cytotoxic activity of essential oil and polar compounds **1** – **4**, **8** and **9** from *J. turbinata* was evaluated on a selection of human tumor cell lines such as colon carcinoma, breast adenocarcinoma and malignant melanoma cell lines by MTT. Increasing concentrations of essential oil and polar compounds isolated from the ethanolic extract were assayed on the three tumor cell lines for 72 h. The results showed that essential oil induced a concentration-dependent inhibitory effect on all cell lines tested in the dilutions range 1.56 – 400 μg/mL (*Table 2*, *Figure S1*). The IC_{50} values were 9.48, 25.10, and 33.69 μg/mL towards A375, HCT116 and MDA-MB 231 cell line, respectively. The essential oil resulted active mainly on human melanoma cell line. The chemical composition of the essential oil does not put in evidence the presence of a main compound that could be responsible for the cytotoxic activity. α-Terpineol (11.0%) has been reported moderately cytotoxic towards Madin–Darby canine kidney (MDCK) cell line with EC_{50} values of 0.025% (v/v).[65] Among other compounds present in the oil, minor components might also contribute to the cytotoxic activity being involved in some type of synergism with the major compounds.

Table 2. *In vitro* cytotoxic activity of *Juniperus turbinata* essential oil

	Cell line (IC_{50} [μg/mL])[a]		
	MDA-MB 231[b]	A375[c]	HCT116[d]
Essential oil			
J. turbinata	33.69	9.48	25.10
95% C.I.[e]	31.12 – 36.47	7.83 – 11.49	24.14 – 26.10
Reference			
Cisplatin	3.56	0.56	2.68
95% C.I.	2.90 – 3.75	0.32 – 0.68	2.17 – 2.96

[a] IC_{50} = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation). [b] Human breast adenocarcinoma cell line. [c] Human malignant melanoma cell line. [d] Human colon carcinoma cell line. [e] C.I. = Confidence Interval.

The cytotoxic activity of compounds isolated from the ethanolic extract is reported in *Table 3*. Data showed a strong cytotoxic activity of imbricataloic acid (**8**) against all cell lines tested, with IC_{50} values of 0.06, 0.114, and 0.201 μM against colon carcinoma, melanoma and breast adenocarcinoma cell lines, respectively (*Figure S2*). Noteworthy, its activity was higher than that of cisplatin used as positive control. Like in the case of essential oil, this compound showed the strongest effects on melanoma cells (nanomolar IC_{50} values). Significant cytotoxic activity was also shown by cupressuflavone (**9**), but with lower intensity (IC_{50} in the range of 12.71 – 19.25 μM; *Figure S3*) compared with that of imbricataloic acid (**8**), while the other purified compounds were inactive (IC_{50} > 100 μM).

The inhibitory properties of imbricatolic acid (**1**) on cell proliferation in several tumor cell lines have already been reported.[66] In particular, at a concentration of 10 μM, imbricatolic acid (**1**) was able to arrest the cell cycle in p53-null CaLu-6 human lung tumor cells. New derivatives of imbricatolic acid were evaluated for antiproliferative activity on MRC-5, AGS, SK-MES-1, J82 and HL-60 human tumor cell lines, with the most active compound showing an IC_{50} value of 17 μM on AGS cells.[67] In our assay, imbricatolic acid (**1**) resulted inactive at the concentrations tested on all cell lines. However, the substitution of the primary alcoholic function at C(15) (CH_2OH group) with an aldehydic function (CHO group) in imbricataloic acid (**8**) structure seems to be a crucial structural feature to strongly enhance the cytotoxic activity of the diterpenoidic base skeleton. Therefore, it will be interesting to perform further studies in order to determine the mode of action, as well as the cytotoxicity against non-tumor cells, and *in vivo* efficacy of this diterpene.

Antioxidant Activity

In *Table 4* is reported the antioxidant activity of *J. turbinata* essential oil against different radicals.

Table 3. *In vitro* cytotoxic activity of diterpenoids and biflavonoids isolated from *Juniperus turbinata*

Compound	Cell line (IC_{50} [μM])[a]		
	MDA-MB 231[b]	A375[c]	HCT116[d]
Diterpenoids			
Imbricatolic acid (**1**)	> 100	> 100	> 100
95% C.I.[e]			
13-*epi*-Cupressic acid (**2**)	> 100	> 100	> 100
95% C.I.			
ent-Manoyl oxide (**3**)	> 100	> 100	> 100
95% C.I.			
7α-Hydroxysandaracopimaric acid (**4**)	> 100	> 100	> 100
95% C.I.			
Imbricataloic acid (**8**)	0.201	0.114	0.060
95% C.I.	0.173 – 0.234	0.109 – 0.119	0.055 – 0.066
Biflavonoids			
Cupressuflavone (**9**)	16.06	12.72	19.25
95% C.I.	14.75 – 17.49	12.17 – 13.29	17.99 – 20.60
Reference			
Cisplatin	11.86	1.87	8.93
95% C.I.	9.66 – 12.49	1.07 – 2.26	7.23 – 9.86

[a] IC_{50} = The concentration of compound that affords a 50% reduction in cell growth (after 72 h of incubation). [b] Human breast adenocarcinoma cell line. [c] Human malignant melanoma cell line. [d] Human colon carcinoma cell line. [e] C.I. = Confidence Interval.

Table 4. *In vitro* radical scavenging activities of essential oil from *Juniperus turbinata*

	$DPPH^{\cdot}$		$ABTS^{\cdot+}$		FRAP
	TEAC[a] [μM TE/g]	IC_{50}[b] [μg/mL]	TEAC[a] [μM TE/g]	IC_{50}[b] [μg/mL]	TEAC[a] [μM TE/g]
Essential oil					
J. turbinata	383.2 ± 4.7	110 ± 1.5	160.4 ± 2.1	90.2 ± 4.2	139.0 ± 1.2
Reference					
Trolox		10.9 ± 0.2		3.7 ± 0.2	

[a] TEAC = Trolox equivalent (TE) antioxidant concentration. [b] IC_{50} = The concentration of compound that affords a 50% reduction in the assay. Values represent mean ± SD from triplicate experiments.

According to the $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ assays, the essential oil showed significant antioxidant activity with values of IC_{50} about 10- and 25-fold lower when compared with Trolox, respectively. The measured reducing capacity power (FRAP assay), revealed that the oil has a moderate capacity for iron binding that is related to its possible action as peroxidation protector. The reducing power may be attributed to hydrogen donation from compounds bearing hydroxylic functions as well as to the presence of reductant agent. The latter can reduce the oxidized intermediates in lipid peroxidation processes, convert them to more stable products and, consequently, terminate radical chain reactions. By comparing the results (*Figure S4*) obtained by the three different antioxidant activity methods and the relationships between the chemical composition and the antioxidant activity, it seems that *J. turbinata* essential oil possesses a good capacity to scavenge free radicals and to prevent lipid peroxidation, which can be ascribed to the presence of hydroxylated compounds occurring among oxygen-containing monoterpenes, sesquiterpenes and diterpenes.

Table 5 and *Figure S5* show the scavenging activity of compounds **1** – **4**, **8** and **9** isolated from *J. turbinata* ethanol extract against the radicals $DPPH^{\cdot}$ and $ABTS^{\cdot+}$ and the evaluation of their reducing capacity power (FRAP). All tested compounds showed good scavenging ability towards the $ABTS^{\cdot+}$ radical, with an effectiveness in the order: cupressuflavone (**9**) > *ent*-manoyl oxide (**3**) > imbricatolic acid (**1**) ≡ imbricataloic acid (**8**) > 7α-hydroxysandaracopimaric

Table 5. *In vitro* radical scavenging activities of isolated diterpenes and bioflavonoids from *Juniperus turbinata*

Compound	DPPH$^{\cdot}$		ABTS$^{\cdot+}$		FRAP
	TEAC[a] [μM TE/g]	IC_{50}[b] 10^{-5} M	TEAC[a] [μM TE/g]	IC_{50}[b] 10^{-5} M	TEAC[a] [μM TE/g]
Diterpenoids					
Imbricatolic acid (**1**)	–	> 1000	68 ± 0.4	79 ± 2.0	6 ± 0.10
13-*epi*-Cupressic acid (**2**)	–	> 1000	37 ± 0.04	146 ± 9.8	3 ± 0.06
ent-Manoyl oxide (**3**)	–	> 1000	146 ± 1.9	41 ± 2.1	8 ± 0.12
7α- Hydroxysandaracopimaric acid (**4**)	11 ± 0.10	358 ± 9.8	46 ± 0.3	118 ± 12	6 ± 0.09
Imbricataloic acid (**8**)	17 ± 0.1	227 ± 8.8	62 ± 0.3	87 ± 4.5	10 ± 0.17
Biflavonoids					
Cupressuflavone (**9**)	211 ± 0.1	11 ± 5.7	2391 ± 0.150	1.35 ± 0.18	26 ± 0.44
Reference					
Trolox	–	1.22 ± 0.07	–	1.74 ± 0.11	–

[a] TEAC = Trolox equivalent (TE) antioxidant concentration. [b] IC_{50} = The concentration of compound that affords a 50% reduction in the assay. Values represent mean ± SD from triplicate experiments.

acid (**4**) > 13-*epi*-cupressic acid (**2**; *Table 5*). In particular, the strongest activity was observed for cupressuflavone (**9**; IC_{50} 1.35 × 10^{-5} M) which was even higher than that of the reference Trolox (IC_{50} 1.74 × 10^{-5} M). On the other hand, a negligible scavenging activity was observed against the DPPH$^{\cdot}$ radical. As a matter of fact, the compounds *ent*-manoyl oxide (**3**), imbricatolic acid (**1**) and 13-*epi*-cupressic acid (**2**) did not react with DPPH$^{\cdot}$ radical at all, whereas cupressuflavone (**9**) showed an activity that resulted 90 times lower than that of Trolox.

All the isolated compounds exhibited a moderate reducing capacity power measured with FRAP assay, with cupressuflavone (**9**) > imbricataloic acid (**8**) > *ent*-manoyl oxide (**3**) > imbricatolic acid (**1**) ≡ 7α-hydroxysandaracopimaric acid (**4**) > 13-*epi*-cupressic acid (**2**; *Table 5*).

Among the bioactive compounds identified in the *J. turbinata* ethanolic extract, the bioflavonoid cupressuflavone (**9**) seems to be important because of its broad spectrum of biological activities.[68] Cupressuflavone (**9**) proved to be beneficial against oxidative stress by enhancing the antioxidant defense status, reducing lipid peroxidation and protecting against the pathological changes in the liver and kidney tissues.[69] Moreover, it showed neutrophil elastase inhibitory activity[70] and a potential osteoprotective effect.[71] Although imbricatolic acid (**1**) did not show in our assays any notable antioxidant activity, it has been reported to control the cellular cycle progress.[66] Its synthetic derivatives were proved to exert topical anti-inflammatory activity[72] and to inhibit protein tyrosine phosphatase-1B.[73] On the other hand, no information is currently available on the biological properties of imbricataloic acid (**8**).

Conclusions

The phytochemical investigations performed on the population of *J. turbinata* growing in La Maddalena Archipelago evidenced a significant rate of variability compared with other accessions of the same species. As a matter of fact, the essential oil was characterized by a new chemotype (α-terpineol-rich) which was not reported previously in other Mediterranean populations, whereas the ethanolic extract was the source of a new natural product (15-formyloxyimbricatolic acid (**7**)) and showed a phytochemical pattern quite different with respect to other Mediterranean populations, evidencing the influence of the isolated environment on the secondary metabolites expression. In addition, some of the isolated compounds, namely imbricataloic acid (**8**) and cupressoflavone (**9**), showed powerful cytotoxic and antioxidant activities, respectively. These findings support the traditional medical uses of the plant and encourage further studies for the development of plant-borne compounds formulations to be used in pharmaceutical and cosmetic applications.

Experimental Section

General

NMR spectra were recorded on a Varian Mercury 300 MHz instrument and/or on a Bruker Avance III 400 MHz instrument using $CDCl_3$, CD_3OD or (D_6)DMSO as solvents; δ in ppm relative to Me_4Si as internal standard, J in Hz. The internal solvent signal (m5) at 3.31 ppm was set as reference for the spectra in CD_3OD, while the solvent signal (m5) at 2.50 ppm was set as reference for the spectra in (D_6)DMSO.

Plant Material

Aerial parts, including leaves and twigs of *J. turbinata*, were collected in La Maddalena Archipelago (Sardinia) at the beginning of July, 2014. The botanical identification was performed by one of us (M. B.). A voucher specimen (Herbarium CAG 1653/A) has been stored with the General Herbarium of the Department of Life and Environment Sciences, University of Cagliari, Italy.

NMR and MS Experiments

Bidimensional spectra were performed on a Bruker Avance III 400 MHz instrument, operating at 9.4 T at 298 K. HSQC experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon, respectively, an average ^{1}J(C,H) value of 145 Hz, a recycle delay of 2 s and a data matrix of 4K × 256 points. HMBC experiments were acquired with a spectral width of 15 and 250 ppm for the proton and carbon, respectively, a long range coupling constant of ^{n}J(C,H) value of 8 Hz, a recycle delay of 2 s and a data matrix of 4K × 256 points.

MS spectra were performed on a Q-TOF MICRO spectrometer (Waters, Manchester, UK) equipped with an ESI source that was operated in the negative and/or positive ion mode. The flow rate of sample infusion was 10 μL/min with 100 acquisitions per spectrum. Data were analyzed using the MassLynx software developed by Waters.

Solvents of RPE grade were purchased from Sigma–Aldrich (Milan, Italy) or Carlo Erba Reagenti (Milan, Italy); silica gel 60 (70 – 230 mesh ASTM) was from Fluka.

Ethanolic Extracts Analysis

A portion of 260.1 g of fresh plant material, represented by leaves and terminal branches, was consecutively extracted with ethanol 96% (3 times, 48 h extraction, 2.5 L of solvent each extraction). Then, the extracts were filtered and the solvent evaporated under reduced pressure until an aqueous suspension was obtained. The suspension was frozen to −20 °C and then lyophilized at the same temperature. A total amount of 21.3, 7.2 and 1.5 g of crude extract, respectively, was recovered. From the preliminary TLC screening, only the first and the second extract resulted most suitable for fractionation (the three extracts showed the same qualitative composition but the first and the second ones showed more intense spots in respect to the third one) and for this reason we worked on these ones. The first chromatographic separation was conducted on the second extract. A portion of 1.8 g of extract was partitioned over silica gel (33.6 g) using chloroform/methanol as eluting mixture, starting with 9:1 and gradually increasing the polarity to 8:2 during the chromatographic run. From this separation were recovered and identified the following diterpenoids: (Fr. 9A) imbricatolic acid (**1**) and 13-*epi*-cupressic acid (**2**)[52] as a mixture (1:1; quantity not estimated); Fr. 21-23A, a low polar fraction, in which the presence of flavonoidic compounds was firstly evidenced on TLC by fluorescence to UV light and a yellow/orange reaction to 2 N H_2SO_4 reagent after heating at 120 °C. This fraction resulted also positive to $FeCl_3$ reagent. NMR analysis on Fr. 21-22A (39.6 mg) revealed the presence of cupressuflavone (**9**),[53][74] a symmetric 8,8′ dimer of apigenin, as principal component, together with minor amounts of amentoflavone (**10**)[75] and hinokiflavone (**11**)[53] (4:2:1); Fr. 23A (18.8 mg) contained cupressuflavone (**9**) as a quite pure compound.

A portion of the first extract (1.7 g) was partitioned on silica gel (39.3 g) using BuOH saturated with water (82:18, v/v) as first eluting mixture. From this first separation, a less polar fraction (Fr. 2-5B; 0.870 g) was recovered. This fraction was further partitioned on silica gel (36.0 g) using chloroform/methanol as eluting mixture, starting with 98:2 and gradually increasing the polarity to 9:1. According to elution order were obtained the following fractions: (Fr. 14-16C) *ent*-manoyl oxide (**3**)[76] (18.3 mg), (Fr. 32-33C) a mixture of terpenoids (145.2 mg), (Fr. 35-37C) imbricatolic acid (**1**; 20.6 mg), (Fr. 65-66C) 7α-hydroxysandaracopimaric acid (**4**)[77] (10.7 mg). The mixture of terpenoids (Fr. 32-33C; 145.2 mg) was further chromatographed on SiO_2 column chromatography (5.0 g) starting the elution with chloroform and then increased the polarity during the chromatographic run to 99:1 with methanol (v/v). In this manner, the presence of the following diterpenoids was recognized: 13-*epi*-torusolal (**5**),[54][78] sandaracopimaric acid (**6**)[77] and 15-formyloxyimbricatolic acid (**7**)[38] as a mixture (4:3:2; Fr. 34D; 4.3 mg), imbricataloic acid (**8**)[16] (Fr. 38-39D; 8.3 mg), and 13-*epi*-cupressic acid (**2**; Fr. 61-64D; 5.6 mg). All the isolated compounds were identified by comparison of the obtained experimental data with those available in literature.

Imbricatolic Acid (**1**). ^{1}H-NMR (300 MHz, $CDCl_3$): 4.82 (*s*, H_a–C(17)), 4.48 (*s*, H_b–C(17)), 3.67 (*dd*, $^{2}J(H,H)_{gem}$ = 12.8, ^{3}J(H,H) = 6.4, H–C(15)), 2.40 (br. *d*, $^{2}J(H,H)_{gem}$ = 10.5, H_a–C(7)) 2.16 (br. *d*, $^{2}J(H,H)_{gem}$ = 10.5, H_b–C(7)), 1.23 (*s*, Me(18)), 0.89 (*d*, ^{3}J(H,H) = 6.2, Me(16)), 0.58 (*s*, Me(20)). ^{13}C-NMR (100 MHz, $CDCl_3$): 183.3 (COOH); 148.2 (C(8)); 106.4 (CH_2(17)); 61.2

(CH_2(15)); 56.6 (CH(5)); 56.3 (CH(9)); 44.1 (C(4)); 40.5 (C(10)); 39.5 (CH_2(12)); 39.1 (CH_2(1)); 38.7 (CH_2(14)); 38.0 (CH_2(7)); 36.4 (CH_2(3)); 30.2 (CH(13)); 29.0 (Me(18)); 26.0 (CH_2(6)); 21.1 (CH_2(11)); 19.9 (CH_2(2)); 19.8 (Me(16)) 12.7 (Me(20)). ESI-MS: 320.80 ($[M-H]^-$).

13-*epi*-Cupressic Acid (**2**). ^{1}H-NMR, (400 MHz, $CDCl_3$): 5.90 (*dd*, $^3J(H,H)_{trans}$ = 17.4, $^3J(H,H)_{cis}$ = 10.8, H–C(14)), 5.20 (*dd*, $^3J(H,H)_{trans}$ = 17.4, $^2J(H,H)_{gem}$ = 1.2, H_a–C(15)), 5.06 (*dd*, $^3J(H,H)_{cis}$ = 10.8, $^2J(H,H)_{gem}$ = 1.2, H_b–C(15)), 4.83 (br. *s*, H_a–C(17)), 4.49 (br. *s*, H_b–C(17)), 2.39 (*dd*, $^2J(H,H)_{gem}$ = 10.6, $^3J(H,H)$ = 2.7, H_a–C(7)), 2.00 (br. *d*, $^3J(H,H)$ = 10.6, H_b–C(7)), 1.27 (*s*, Me(18)), 1.23 (*s*, Me(16)), 0.59 (*s*, Me(20)). ^{13}C-NMR ($CDCl_3$, 100 MHz): 183.26 (COOH); 148.21 (C(8)); 145.11 (CH(14)); 111.86 (CH_2(15)); 106.67 (CH_2(17)); 73.88 (C(13)); 56.64 (CH(5)); 56.48 (CH(9)); 44.29 (C(4)); 41.48 (CH_2(12)); 40.69 (C(10)); 39.26 (CH_2(1)); 38.86 (CH_2(7)); 36.53 (CH_2(3)); 29.29 (Me(18)); 28.23 (Me(16)); 26.21 (CH2(6)); 20.13 (CH_2(2)); 18.04 (CH_2(11)); 12.89 (Me(20)). ESI-MS: 318.78 ($[M-H]^-$).

***ent*-Manoyl Oxide** (**3**). ^{1}H-NMR, (300 MHz, $CDCl_3$): 5.88 (*dd*, $^3J(H,H)_{trans}$ = 17.3, $^3J(H,H)_{cis}$ = 10.8, H–C(14)), 5.14 (*d*, $^3J(H,H)_{trans}$ = 17.3, H_a–C(15)), 4.92 (*d*, $^3J(H,H)_{cis}$ = 10.8, H_b–C(15)), 1.83 (*d*, $^2J(H,H)_{gem}$ = 12.3, H–C(7)), 1.29 (*s*, Me(16)), 1.27 (*s*, Me(17)), 1.25 (*s*, Me(20)), 0.86 (*s*, Me(19)), 0.79 (*s*, Me(18)). ^{13}C-NMR (75 MHz, $CDCl_3$): 147.9 (CH(14)), 110.2 (CH_2(15)), 75.1 (C(8)), 73.2 (C(13)), 56.4 (CH(9)), 55.6 (CH(5)), 43,2 (CH_2(7)), 42.1 (CH_2(3)), 39.00 (CH_2(1)), 35.7 (CH_2(12)), 33.3 (Me(16)), 33.2 (C(10)), 29.7 (Me(19)), 28.5 (C(4)), 25.5 (CH_2(1)), 21.33 (Me(18)), 19.9 (CH_2(6)), 18.5 (CH_2(2)), 15.4 (CH_2(11)), 15.2 (Me(20)). ESI-MS: 313.21 ($[M+Na]^+$).

7α-Hydroxysandaracopimaric Acid (**4**). ^{1}H-NMR (300 MHz, $CDCl_3$): 5.76 (*dd*, $^3J(H,H)_{trans}$ = 17.4, $^3J(H,H)_{cis}$ = 10.6, H–C(15)), 5.52 (br. *s*, H–C(14)), 4.98 – 4.87 (*m*, H–C(16)), 4.22 (br. *s*, H–C(7)), 2.40 (br. *d*, $^3J(H,H)$ = 10.5, H_a–C(12)), 2.19 (br. *d*, $^3J(H,H)$ = 10.5, H_b–C(12)), 1.18 (*s*, Me(19)), 1.03 (*s*, Me(17)), 0.80 (*s*, Me (20)). ^{13}C-NMR (75 MHz, $CDCl_3$): 182.88 (COOH); 147.89 (CH(15)); 138.33 (C(8)); 134.71 (CH(14)); 110.88 (CH_2(16)); 73.12 (CH(7)); 46.66 (C(4)); 46.03 (CH(9)); 41.77 (CH(5)); 38.09 (CH_2(1)); 37.91 (C(10)); 37.38 (C(13)); 36.76 (CH_2(3)); 34.09 (CH_2(12)); 31.47 (CH_2(6)); 25.65 (Me(17)); 18.07 (CH_2(11)); 18.01 (CH_2(2)); 16.71 (Me(19)); 14.45 (Me(20)). ESI-MS: 317.18 ($[M-H]^-$).

13-*epi*-Torulosal (**5**). ^{1}H-NMR (400 MHz, $CDCl_3$): 9.75 (*d*, $^3J(H,H)$ = 1.4, H–C(19)), 5.91 (*dd*, $^3J(H,H)_{trans}$ = 17.3, $^3J(H,H)_{cis}$ = 10.7, H–C(14)), 5.21 (*dd*, $^3J(H,H)_{trans}$ = 17.3, $^2J(H,H)_{gem}$ = 1.3, H_a–C(15)), 5.07 (*dd*, $^3J(H,H)_{trans}$ = 10.7, $^2J(H,H)_{gem}$ = 1.3, H_b–C(15)), 4.87 (*s*, H_a–C(17)), 4.53 (*s*, H_b–C(17)), 1.28 (*s*, Me(16)), 1.02 (*s*, Me(18)), 0.57 (*s*, Me(20)). ^{13}C-NMR (100 MHz, $CDCl_3$): 205.75 (CHO), 147.50 (C(8)), 144.98 (CH(14)), 111.76 (CH_2(15)), 107.36 (CH_2(17)), 73.62 (C(13)), 56.13 (CH(9)), 55.87 (CH(5)), 48.66 (C(4)), 41.26 (CH_2(12)), 40.55 (C(10)), 38.49 (CH_2(7)), 38.46 (CH_2(3)), 34.47 (CH_2(1)), 28.14 (Me(16)), 24.38 (Me(18)), 24.08 (CH_2(C6)), 19.28 (CH_2(2)), 17.97 (CH_2(11)), 13.54 (Me(20)). ESI-MS: 303.21 ($[M-H]^-$), 327.27 ($[M+Na]^+$).

Sandaracopimaric Acid (**6**). ^{1}H-NMR (400 MHz, $CDCl_3$): 5.78 (*dd*, $^3J(H,H)_{trans}$ = 17.4, $^3J(H,H)_{cis}$ = 10.5, H–C(15)), 5.23 (*s*, H–C(14)), 4.92 (*dd*, $^3J(H,H)_{trans}$ = 17.4, $^2J(H,H)_{gem}$ = 1.4, H_a–C(16)), 4.89 (*dd*, $^3J(H,H)_{cis}$ = 10.5, $^2J(H,H)_{gem}$ = 1.4, H_b–C(16)), 1.24 (*s*, Me(19)), 1.06 (*s*, Me(17)), 0.86 (*s*, Me(20)). ^{13}C-NMR (100 MHz, $CDCl_3$): 180.24 (COOH), 148.18 (CH(15)), 136.60 (C(8)), 129.14 (CH(14)), 110.15 (CH_2(16)), 50.58 (CH(9)), 48.93 (CH(5)), 47.17 (C(4)), 38.30 (CH_2(1)), 37.75 (C(10)), 37.40 (C(13)), 37.00 (CH_2(3)), 35.47 (CH_2(7)), 34.46 (CH_2(12)), 26.08 (Me(17)), 24.89 (CH_2(6)), 18.57 (CH_2(11)), 18.15 (CH_2(2)), 16.87 (Me(19)), 15.21 (Me(20)). ESI-MS: 301.17 ($[M-H]^-$); 325.12 ($[M+Na]^+$).

15-Formyloxyimbricatolic Acid (**7**). ^{1}H-NMR (400 MHz, $CDCl_3$): 8.06 (*s*, H–C(21)), 4.85 (*s*, H_a–C(17)), 4.49 (*s*, H_b–C(17)), 4.22 – 4.17 (*m*, H–C(15)), 1.25 (*s*, Me(19)), 0.93 (*d*, $^3J(H,H)$ = 6.5, Me(16)), 0.62 (*s*, Me(20)). ^{13}C-NMR (100 MHz, $CDCl_3$): 181.37 (COOH), 161.19 (HCOO), 148.90 (C(8)), 106.41 (CH_2(17)), 62.53 (CH_2(15)), 56.59 (CH(5)), 56.32 (CH(9)), 44.09 (C(4)), 40.54 (C(10)), 39.15 (CH_2(1)), 38.74 (CH_2(7)), 38.11 (CH_2(3)), 36.06 (CH_2(12)), 35.14 (CH_2(14)), 30.49 (CH(13)), 29.01 (Me(18)), 26.03 (CH_2(6)), 21.08 (CH_2(11)), 19.93 (CH_2(2)), 19.63 (Me(16)), 12.82 (Me(20)). ESI-MS: 349.18 ($[M-H]^-$); 373.13 ($[M+Na]^+$).

Imbricataloic Acid (**8**). ^{1}H-NMR, (300 MHz, $CDCl_3$): 9.75 (*t*, $^3J(H,H)$ = 2.3, H–C(15)), 4.84 (br. *s*, H_a–C(17)), 4.47 (br. *s*, H_b–C(17)), 1.25 (*s*, Me(19)), 0.97 (*d*, $^3J(H,H)$ = 6.7, H–C(16)), 0.60 (*s*, Me(20)). ^{13}C-NMR (100 MHz, $CDCl_3$): 203.3 (CHO), 182.3 (COOH), 148.21 (C(8)), 106.6 (CH_2(17)), 56.61 (CH(9)), 56.46 (CH(5)), 50.99 (CH_2(14)), 44.28 (C(4)), 40.70 (C(10)), 39.31 (CH_2(1)), 38.87 (CH_2(7)), 38.19 (CH_2(3)), 36.28 (CH(13)), 29.15 (Me(19)), 29.04 (CH_2(12)), 26.21 (CH_2(6)), 21.37 (CH_2(11)), 20.33 (Me(16)), 20.06 (CH_2(2)), 12.93 (Me(20)). ESI-MS: 319.26 ($[M-H]^-$).

Cupressuflavone (**9**). ^{1}H-NMR (300 MHz, CD_3OD): 7.48 (*d*, $^3J(H,H)$ = 8.6, H–C(2′), H–C(6′), H–C(2‴), H–C(6‴)), 6.75 (*d*, $^3J(H,H)$ = 8.6, H–C(3′), H–C(5′), H–C(3‴), H–C(5‴)), 6.60 (*s*, H–C(3), H–C(3″)), 6.48 (*s*, H–C(6), H–C(6″)). ^{1}H-NMR (300 MHz, (D_6)DMSO): 10.32 (br. *s*, 7-OH), 7.50 (*d*, $^3J(H,H)$ = 8.8, H–C(2′), H–C(6′), H–C(2‴), H–C(6‴)), 6.80 (*s*, H–C(3), H–C(3″)), 6.74 (*d*, $^3J(H,H)$ = 8.8, H–C(3′), H–C(5′), H–C(3‴), H–C(5‴)), 6.46 (*s*, H–C(6), H–C(6″)). ^{13}C-NMR (75 MHz, CD_3OD): 184.30 (C(4)=O, C(4″)=O), 166.11 (C(7), C(7′)), 164.22 (C(2), C(2″)), 162.91 (C(4′), C(4‴)), 162.62 (C(5), C(5″)), 156.96 (C(9), C(9″)), 129.16 (CH(2′), CH(2‴),

CH(6′), CH(6‴)), 123.15 (C(1′), C(1‴)), 116.87 (CH(3′), CH (3‴), CH(5′), CH(5‴)), 105.60 (C(10), C(10″)), 103.44 (CH(3), CH(3″)), 99.94 (C(8), C(8′)), 99.83 (CH(6), CH(6″)). ESI-MS: 536.79 ([M − H]$^-$).

Amentoflavone (**10**). ^{1}H-NMR (300 MHz, CD_3OD): 7.93 (br. *s*, H–C(2′)), 7.92 (br. *d*, ^3J(H,H) = 9.0, H–C(6′)), 7.51* (br. *d*, ^3J(H,H) = 8.8, H–C(2‴), H–C(6‴)), 7.48* (br. *d*, ^3J(H,H) = 9.0, H–C(5′)), 7.13 (br. *d*, ^3J(H,H) = 8.7, H–C(3‴), H–C(5‴)), 6.70 (*s*, H–C(3″)), 6.62 (*s*, H–C(3)), 6.41 (*d*, ^4J(H,H) = 1.6, H–C(8)), 6.39 (*s*, H–C(6″)), 6.19 (*d*, ^4J(H,H) = 1.6, H–C(6)); * partially overlapped signals. ESI-MS: 536.99 ([M − H]$^-$); 539.05 ([M + H]$^+$).

Hinokiflavone (**11**). ^{1}H-NMR (300 MHz, CD_3OD): 7.92* (br. *d*, ^3J(H,H) = 8.9, H–C(3′), H–C(5′), H–C(2‴), H–C(6‴)), 7.13 – 6.98* (*m*, H–C(2′), H–C(6′), H–C(3‴), H–C(5‴)), 6.86 (*s*, H–C(3)), 6.76 (*s*, H–C(3″)), 6.73 (*s*, H–C(8″)), 6.48 (br. *s*, H–C(8)), 6.19 (br. *s*, H–C(6″)); * partially overlapped signals. ESI-MS: 536.99 ([M − H]$^-$); 539.05 ([M + H]$^+$).

Hydrodistillation

A portion of fresh plant material (323.7 g), represented by leaves and terminal branches, was subjected to hydrodistillation in a Clevenger-type apparatus for 4 h using 1200 mL of distilled water. The obtained oil was collected in a Clevenger trap, dried on anhydrous Na_2SO_4, stored in hermetically sealed glass vial with rubber lids, protected from light by aluminum foil and kept at −20 °C until analysis. The oil yield, on the fresh-weight basis of the plant material, was 0.046% (w/w).

GC-FID and GC/MS Analysis

Firstly, an Agilent 4890D gas chromatograph equipped with an ionization flame detector (FID) was used. Separation was achieved by using a HP-5 capillary column (5% phenylmethylpolysiloxane, 25 m, 0.32 mm i.d.; 0.17 μm film thickness; J and W Scientific, Folsom, CA). The temperature program of the oven was as follows: 60 °C (5 min) then increased up to 220 °C with a gradient of 4 °C/min, finally to 280 °C at 11 °C/min held for 15 min. The temperature of injector and transfer line was 280 °C. The carrier gas used was He with a flow rate of 1.4 mL/min. The essential oil was diluted in hexane and 1 μL of the solution was injected into the GC system with a split ratio of 1:34. The temperature-programmed retention indices were calculated according to the Van den Dool and Kratz formula[79] using a mixture of $C_8 - C_{30}$ *n*-alkanes (Sigma, Milan, Italy) diluted in hexane. Analysis was run in triplicate and data collected by the HP3398A GC Chemstation software (Hewlett Packard, Rev. A.01.01). Essential oil component percentages were obtained by using a previously developed method by calculating the FID-response factors for the main chemical classes occurring in the essential oil.[80]

Qualitative analysis was performed on an Agilent 6890N gas chromatograph equipped with a 5973N mass spectrometer. Separation of volatiles was achieved on a HP-5 MS (5% phenylmethylpolysiloxane, 30 m, 0.25 mm i.d., 0.1 μm film thickness; J & W Scientific, Folsom) capillary column using the same temperature program reported above. The temperature of injector and detector was set to 280 °C and He was used as carrier gas with a flow rate of 1 mL/min. The essential oil was diluted in hexane (1:100) and injected (2 μL) into the GC/MS system using a split ratio of 1:50. Mass spectra were acquired using electron-impact (EI) mode (ionization voltage: 70 eV) in the range *m/z* 29 – 400. The MSD ChemStation software (Agilent, Version G1701DA D.01.00) and NIST Mass Spectral Search Program for the NIST/EPA/NIH Mass Spectral Library v. 2.0[81] were used to analyze data. The peak assignment was achieved by co-injection with authentic standard whenever possible (see *Table 1*), or by correspondence of the temperature-programmed retention indices (RIs) and acquired mass spectra (MS) with respect to those reported in commercial[81 – 83] and home-made libraries.

Cytotoxicity Assay

A375 human malignant melanoma cell line and MDA-MB 231 human breast adenocarcinoma cell line were maintained in Dulbecco's Modified Eagle's Medium (DMEM). This medium was supplemented with 10% heat-inactivated fetal bovine serum (HI-FBS), 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. HCT116 human colon carcinoma cell line was maintained in RPMI1640 medium supplemented with 10% HI-FBS, 100 IU/mL penicillin, 100 μg/mL streptomycin and 2 mM L-glutamine. The cultures were maintained in a humidified atmosphere at 37 °C in presence of 5% CO_2. Cells were subcultured every 3 – 4 days. The cytotoxicity was assessed using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2*H*-tetrazolium bromide) assay.[84] In brief, cells (2×10^4 cells/mL) were seeded on 96-well plates and incubated for 24 h in a humidified atmosphere of 5% CO_2 at 37 °C. Then, essential oil (1.56 – 400 μg/mL) and polar compounds (0.001 – 100 μM) were added to the supernatant and the samples were incubated for a further 72 h. At the end of incubation, each well received 10 μL of MTT (5 mg/mL in phosphate-buffered saline, PBS) and the

plates were incubated for 4 h at 37 °C. After removal of the supernatant, DMSO was added to solubilize water-insoluble dark blue formazan crystals formed in viable cells and the absorbance was measured at 540 nm using a Titertek Multiscan microElisa (Labsystems, FI-Helsinki). The cytotoxicity was expressed as the concentration of compound inhibiting cell growth by 50% (IC_{50}) and was calculated with GraphPad Prism 4 computer program (GraphPad Software, S. Diego, CA, USA). Experiments were conducted in triplicate.

Antioxidant Activity

Free radical scavenging activity ($DPPH^{\cdot}$) was evaluated on a microplate analytical assay according to the procedures previously described by Srinivasan and co-workers.[85] The stock solution was prepared by dissolving $DPPH^{\cdot}$ in methanol and then stored at −20 °C until use. The working solution was obtained by mixing stock solution with methanol to obtain an absorbance of 1 unit at 517 nm. Discoloration was measured at 517 nm after incubation for 30 min in the dark. The free radical-scavenging activity of each solution was then calculated as percent inhibition according to the following equation:

$$\% \text{ Inhibition} = 100 \times (A_{\text{blank}} - A_{\text{sample}})/A_{\text{blank}}$$

Antioxidant activity of the essential oil and isolated compounds was expressed as IC_{50}, defined as the concentration of the test material required to cause a 50% decrease in initial $DPPH^{\cdot}$ concentration. Trolox was used as reference. Results were expressed in μM Trolox equivalents (TE)/g of essential oil.

The $ABTS^{\cdot+}$ assay was performed following the procedure described previously,[86] applied to a 96-well microplate assay.[87] The $ABTS^{\cdot+}$ stock solution was prepared by mixing the two solutions of $ABTS^{\cdot+}$ (7.4 mM) and potassium persulfate (2.6 mM) in equal quantities and allowing them to react for 12 h at room temperature and in the dark. The working solution was then obtained by mixing $ABTS^{\cdot+}$ stock solution with methanol (or ethanol) to obtain a final solution with absorbance about 1 unit at 734 nm. Absorbance values were measured with a Varian Cary 1 spectrophotometer and Trolox was used as reference compound. Results were expressed in μM Trolox equivalents (TE)/g of product or essential oil. The capacity of free radical scavenging (IC_{50}) was determined using an analogous equation to those previously used in the $DPPH^{\cdot}$ method. All data of antioxidant activity were expressed as means ± standard deviations (SD) of triplicate measurements. The confidence limits were set at $P < 0.05$. SD did not exceed 5% for the majority of the values obtained.

The reducing capacity power (FRAP) assay was carried out according to Firuzi and co-workers,[88] with minor modifications. The stock solutions included 300 mM acetate buffer, pH 3.6, 10 mM TPTZ (2,4,6-tripyridyl-*S*-triazine) solution in 40 mM HCl, and 20 mM $FeCl_3 \cdot 6\ H_2O$ solution. The fresh working solution was prepared by mixing 25 mL acetate buffer, 2.5 mL TPTZ solution, and 2.5 mL $FeCl_3 \cdot 6\ H_2O$ solution and then warmed at 37 °C before use. Aliquots of diluted essential oil and isolated compounds in methanol allowed to react with 500 μL of the FRAP solution for 30 min in the dark. Samples were centrifuged at 11'500 *g* and the withdrawn aliquots of solutions containing the colored product (ferrous tripyridyltriazine complex; 280 μL) were read in 96-well microplates at 593 nm. The standard curve was linear between 25 and 800 μM Trolox used as positive control. Results were expressed in μM TE/g of product or essential oil. Additional dilution was applied when the measured FRAP value resulted over the linear range of the standard curve.

Supplementary Material

Supporting information for this article is available on the WWW under https://doi.org/10.1002/cbdv.201800148.

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Author Contribution Statement

A. Venditti conceived and designed the experiments, performed the chromatographic separation of the ethanol fraction, analyzed the data, performed the structure elucidation of isolated compounds, supervise the hydrodistillation of the essential oil and wrote the article. *F. Maggi* analyzed the essential oil and conceived the experiments. *L. Quassinti* and *M. Bramucci* performed cytotoxic assays. *G. Lupidi* carried out the antioxidant assays. *L. Ornano* collected the plant materials, obtained the ethanolic extracts and performed the hydrodistillation. *M. Ballero* and *C. Sanna* performed the botanical identification of plant materials. *M. Bruno* and *S. Rosselli* contributed to the structure identification of isolated compounds. *A. Bianco* supervised the experiments and contributed to organic solvents, reagents, materials, analysis tools, analyzed the

data and checked the final version of the article. All the co-authors contributed to the writing of the article on the basis of their competence.

Conflict of Interest

All the authors declare no conflict of interest.

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