Volatile Compounds from Flowers of Elaeagnus x submacrophylla Servett.: Extraction, Identification of Flavonoids, and Antioxidant Capacity

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Volatile Compounds from Flowers of *Elaeagnus x submacrophylla* Servett.: Extraction, Identification of Flavonoids, and Antioxidant Capacity

Isabelle Parrot,*[a] Hélène Bisi,[a] Arnaud Folliard,[b] and Michel Bonnard[a]

Beneficial to the ecosystem and with significant potential in permaculture, *Elaeagnus x submacrophylla* Servett. was studied here mainly for the identification of its floral odorants. After olfactory evaluation and determination of the volatile profile of freshly picked flowers by headspace/solid phase microextraction coupled with gas chromatography/mass spectrometry, an ethanolic extract was prepared and investigated for its antioxidant capacity. Unusual molecules were identified in the floral headspace, such as isochavicol or chrysanthemum acetate. The evaluation of the *in vitro* free radical scavenging capacity (from 0.4 to 1.3 mmol TE/g) and total phenolic content (65.1 mg GAE/g) of the extract pointed out a promising antioxidant activity, potentially related to the identification of several flavonoid glycosides. These results have to be considered in the context of the ever-increasing need to produce innovative natural extracts with notably interesting claims for the cosmetic field.

Introduction

In the Elaeagnaceae family, the genus *Elaeagnus* is commonly found in temperate zones. These include *E. angustifolia* and *E. umbellata*, previously investigated for the production of extracts and the identification of numerous bioactive compounds, such as flavonoids.[1–3] Mainly native to Asia, these evergreen shrubs or trees provide edible fruits and seeds rich in vitamins and fatty acids.[4] In folk medicine, *Elaeagnus* species have been used as analgesic, antiulcer agent, anti-inflammatory or antipyretic due to the presence of bioactive phytoconstituents.[5] Firstly described in 1908,[6] *Elaeagnus x submacrophylla* Servett., (Figure 1) also known as *E. ebbingei* Door. is a cultivar hybrid originating from *E. pungens* and *E. macrophylla*. Now widely planted as an ecosystem-beneficial ornamental hedge with great permaculture potential, it has also recently been reported to accumulate, on and within its leaves, certain air pollutants without being affected,[7] as well as metals such as Cd, Cu, Ni, Pb or Zn.[3,8] In addition to beneficially improve urban air quality, this water-saving shrub does not require regular fertilization or watering, is generally not attacked by pests or diseases, and has a rapid growth, in full sun or in shade while being very tolerant to pruning. The autumn flowering of *E. submacrophylla* provides quantities of highly fragrant white tubular flowers (at least 15 000 flowers per medium size shrub), constituting an additional argument in favor of an optimal candidate for the production of natural flower extracts, without damaging the plant or the environment.

In the quest for innovative natural ingredients for the cosmetic or nutraceutical sectors, it is more than essential today to favor a sustainable exploitation of botanical riches, in a concern of preservation, conservation and rational use of resources. Therefore, in the context of maintaining a fair balance between environmental protection and exploitation of natural resources, with a mode of cultivation, harvesting and processing respectful of the environment, *E. submacrophylla* appears as insufficiently considered, its overall phytochemical composition being not yet defined, whatever the part concerned (flower, leaf, root, etc.).[3]

Intrigued by the fact that the fragrant flower heads of *E. submacrophylla* have never been studied, we aimed to describe the chemical composition of the floral headspace, in connection with the production and analysis of an odorant extract with potential biological activities. Consequently, after an olfactory evaluation, the chemical identification of volatile organic compounds emitted by *E. submacrophylla* flowers was conducted, after collection, by headspace/solid-phase microextraction (HS-SPME) coupled with gas chromatography/mass spectrometry (GC-MS), a method previously employed for the

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Supporting information for this article is available on the WWW under https://doi.org/10.1002/cplu.202100443

Figure 1. Flowers of *E. submacrophylla*. (a) Part of a medium size shrub. (b) Example of flowers collected in this study.

identification and quantification of volatile compounds from the fruits, leafs, fruit peels and flowers of *E. angustifolia*.[9] Gentle maceration at room temperature of the flowers in ethanol, a solvent environmentally preferable,[10] produced, after evaporation, a qualitative ethanolic extract (EE) whose constituents were compared to the odorant compounds of flowers identified in the headspace. Regarding the restriction in the use of synthetic antioxidant ingredients and the need to develop alternative natural sources, a preliminary screening of the antioxidant capacity of *E. submacrophylla* floral extract was finally investigated *in vitro* according to the commonly used DPPH, ABTS and ORAC methods. The observed radical scavenging activity was potentially correlated to the presence of polyphenols such as flavonoids, detected by ultra-performance liquid chromatography/mass spectrometry (UPLC-MS), constituting the first evidence of bioactive phytoconstituents in *E. submacrophylla*.[3]

Results and Discussion

Olfactory evaluation

In the first instance, fresh harvested flowers were submitted to the evaluation of professional perfumers. The aromatic bouquet was described as belonging to the family of "white flower notes", reminiscent of a wide range of facets common to jasmine sambac, cananga or ylang-ylang. More precisely translatable as intensely floral, sweet, jasmine, animal, balsamic and spicy, voluptuous with slightly bitter almond and gaultheria notes, delicately fruity and highly diffusive. Benzyl benzoate, benzyl alcohol, indole, eugenol, methyl salicylate and to a lesser extent benzaldehyde, were the most frequently reported key molecular descriptors.

Although interesting from an olfactive point of view, the olfactory analysis of the ethanolic extract (EE) revealed quite different facets, above all balsamic, caramelized, spicy, and very lightly floral. Furaneol, benzyl benzoate, cinnamic and eugenolic derivatives were proposed as the main molecular descriptors.

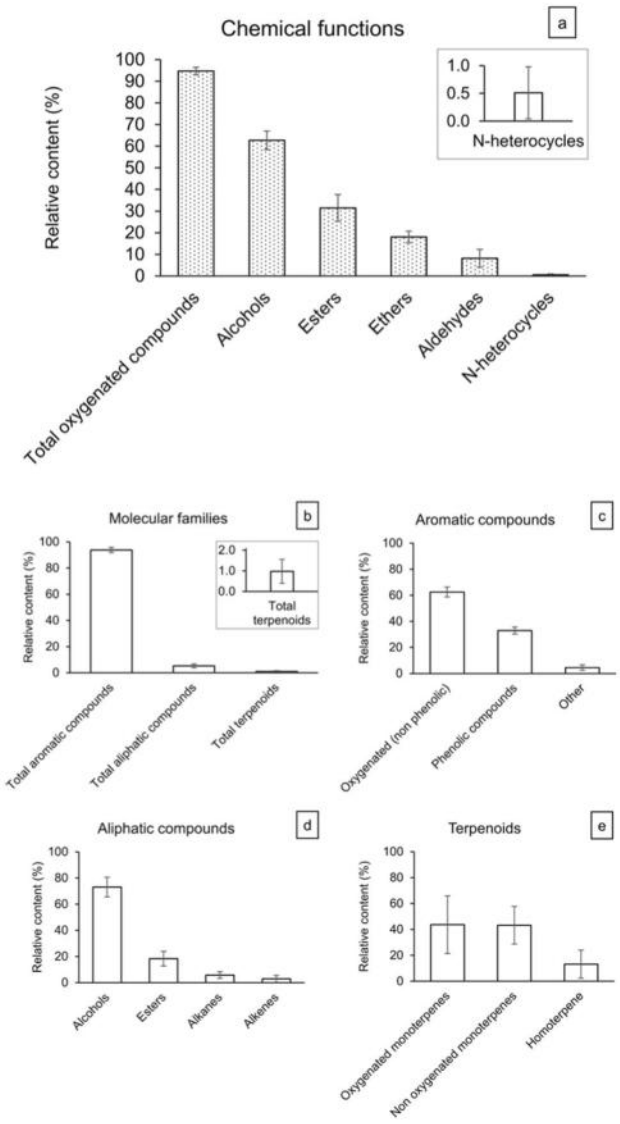


Figure 2. Molecular family and functional groups of volatiles identified in the headspace of flowers of *E. submacrophylla*. (a) Relative content of chemical classes. Molecules containing multiple functional groups were counted in every corresponding group. (b) Relative content of molecular families. (c) Category of aromatic compounds. (d) Category of aliphatic compounds. (e) Category of terpenoids.

Profiles of floral volatile compounds

To the best of our knowledge, floral volatiles of *E. submacrophylla* or either one of its parents, *E. macrophylla* or *E. pungens* have not yet been reported in the scientific literature. The determination of the complex blend of volatile compounds emitted was hence achieved by HS-SPME-GC-MS analysis.[11] As depicted in Table 1, a total of 57 molecules were identified and listed with their relative content and further compared to the richer profile obtained by the GC-MS analysis of EE (see Table S1 for the complete identification of volatiles in EE by GC-MS with polar and non-polar stationary phase). The HS-profile revealed a dominance of aromatic moieties, as well as an imbalance in favor of oxygenates, especially esters and alcohols (Figure 2), which is in agreement with the groups of plant volatiles generally described in the literature.[12,13]

Benzyl alcohol (no. **22**), eugenol (no. **59**), benzyl benzoate (no. **82**), methyl salicylate (no. **43**), benzaldehyde (no. **14**), and methyl benzoate (no. **29**) are among the main compounds identified, which is surprising compared to the floral volatiles emitted by other *Elaeagnus* species. The volatile blend of *E. submacrophylla* differs greatly from the terpenic profile described for *E. angustifolia*,[9] and from the major oxygenated aromatics identified in the floral headspace of *E. umbellata* where benzaldehyde (no. **14**) and benzyl alcohol (no. **22**) were underrepresented in contrast to 4-methylanisole (no. **19**) and *p*-cresol (no. **26**).[1] The presence, absence, and more generally the variation content of these compounds can be related to the flowering period. These observations are consistent with recent work on flower buds of *E. angustifolia*. The authors report that

Table 1. Identification of volatiles in the headspace and the ethanolic extract (EE) of flowers of *E. submacrophylla* using GC-MS.[a]

No.	RI Lit[b]	RI exp[c]	Compound[d]	HS-SPME Relative content [%][e]	EE Relative content [%][e]
1	800	798	Octane	0.31	nd[f]
2	802	801	Ethyl butanoate	0.04	nd
3	810	815	2-Octene[g]	0.15	nd
4	835	846	Ethyl-2-butenoate[g]	0.05	nd
5	856	857	3-Hexen-1-ol[g]	1.20	nd
6	862	867	2-Hexen-1-ol[g]	0.27	nd
7	868	869	1-Hexanol	2.29	nd
8	876	878	Isoamyl acetate	0.03	nd
9	**893**	**889**	**Styrene**	**3.84**	**nd**
10	918	916	2(5*H*)-Furanone	nd	0.07
11	926	925	2-Hydroxycyclopent-2-en-1-one	nd	0.21
12	929	928	α-Thujene	0.02	nd
13	937	935	α-Pinene	0.10	nd
14	**962**	**960**	**Benzaldehyde**	**8.12**	**nd**
15	989	981	2,4-Dihydroxy-2,5-dimethyl-3(2*H*)-furanone	nd	0.20
16	1005	1005	3-Hexen-1-yl acetate[g]	0.15	nd
17	1011	1011	Hexyl acetate	0.52	nd
18	1016	1014	*trans*-2-Hexen-1-yl acetate	0.05	nd
19	1021	1019	4-Methylanisole	0.05	nd
20	1030	1027	2-Ethyl-1-hexanol	0.07	nd
21	1034	1030	Cyclotene (enol form)	nd	0.03
22	**1036**	**1033**	**Benzyl alcohol**	**26.44**	**1.22**
23	1049	1048	*trans*-β-Ocimene	0.30	nd
24	1070	1052	Furaneol	nd	0.20
25	1071	1069	Octanol	0.03	nd
26	1077	1073	*p*-Cresol	0.88	nd
27	1076	1078	2,5-Furandicarboxaldehyde	nd	0.33
28	1087	1086	Furyl hydroxymethyl ketone	nd	0.02
29	**1094**	**1094**	**Methyl benzoate**	**5.50**	**nd**
30	1099	1097	Linalool	0.10	nd
31	1116	1115	*trans*-4,8-Dimethylnona-1,3,7-triene	0.15	nd
32	1139	1140	Methyl nicotinate	0.07	nd
33	1151	1146	3-Hydroxy-2,3-dihydromaltol	nd	0.64
34	1170	1158	Benzoic acid	nd	0.23
35	1164	1164	Benzyl acetate	2.33	nd
36	1170	1167	Lavandulol	0.08	nd
37	1171	1172	Ethyl benzoate	0.42	0.24
38	1186	1184	3-Hexen-1-yl butyrate[g]	0.04	nd
39	1192	1189	Hexyl butanoate	0.02	nd
40	1190	1192	1-Dodecene	nd	0.15
41	1193	1193	2-Methoxy-p-cresol	0.17	nd
42	1208	1196	Catechol	nd	0.06
43	**1192**	**1197**	**Methyl salicylate**	**8.46**	**nd**
44	1209	1212	5-(Hydroxymethyl)dihydrofuran-2(3*H*)-one	nd	0.10
45	1233	1226	5-Hydroxymethylfurfural	nd	1.43
46	1232	1232	Hydrocinnamic alcohol	0.27	0.10
47	1233	1238	1,2-Dimethoxy-4-methylbenzene	0.02	nd
48	1255	1252	Chavicol	2.15	0.87
49	1283	1268	Hydroquinone	nd	0.18
50	1270	1274	*trans*-Cinnamaldehyde	0.11	nd
51	1269	1278	Ethyl salicylate	nd	0.03
52	1280	1282	Chrysanthemyl acetate	0.12	nd
53	1289	1289	Lavandulyl acetate	0.03	nd
54	1295	1297	Indole	0.44	nd
55	1310	1307	*trans*-Cinnamyl alcohol	1.08	0.77
56	1317	1320	2-Methoxy-4-vinylphenol	nd	0.09
57	–	**1340**	***trans*-4-Propenylphenol**	**2.09**	**3.22**
58	1345	1349	Benzyl butyrate	0.39	nd
59	**1357**	**1363**	**Eugenol**	**14.11**	**1.47**
60	1379	1389	*trans*-Methyl cinnamate	0.09	nd
61	1392	1392	1-Tetradecene	nd	0.37
62	1395	1398	Benzyl-3-methylbutanoate	0.11	nd
63	1402	1405	Methyl eugenol	0.24	nd
64	1396	1411	Benzyl-2-butenoate[g]	0.14	nd
65	1408	1413	*cis*-Isoeugenol	0.09	nd
66	1446	1449	*trans*-Cinnamyl acetate	0.10	nd
67	1463	1454	Methyl paraben	nd	0.08
68	1454	1457	*trans*-Isoeugenol	2.12	1.16
69	1500[h]	1500	*trans*-Methylisoeugenol	nd	0.02
70	1498	1505	*trans*-Benzyl tiglate	0.07	nd

Table 1. continued

No.	RI Lit[b]	RI exp[c]	Compound[d]	HS-SPME Relative content [%][e]	EE Relative content [%][e]
71	1508	1511	Lavandulyl isovalerate	0.09	nd
72	1522	1524	Methyl vanillate	nd	0.07
73	1554	1559	Elemicin	1.14	0.99
74	1563	1571	4-Vinylsyringol	nd	0.07
75	1570	1581	3-Hexen-1-yl benzoate[g]	0.02	nd
76	1592	1592	1-Hexadecene	nd	0.44
77	1608	1611	4-Allylsyringol	0.11	1.48
78	1648	1656	Vanillylacetone	nd	0.20
79	1668	1680	*cis*-Coniferyl alcohol	nd	0.13
80	1704	1709	*trans*-4-Propenyl syringol	nd	0.03
81	1743	1747	*trans*-Coniferyl alcohol	nd	0.13
82	**1762**	**1782**	**Benzyl benzoate**	**11.94**	**39.13**
83	1793	1793	1-Octadecene	nd	0.34
84	1855	1849	Methyl ferulate	nd	0.05
85	1880	1881	1-Hexadecanol	nd	0.17
86	**1869**	**1889**	**Benzyl salicylate**	**0.68**	**6.08**
87	1926	1926	Methyl palmitate	nd	1.51
88	**1968**	**1960**	**Palmitic acid**	**nd**	**6.62**
89	1964	1976	Geranyl benzoate	nd	0.05
90	1993	1993	Ethyl palmitate	nd	1.01
91	2000	1998	3-Phenylpropyl benzoate	nd	0.35
92	2016	2018	*cis*-Cinnamyl benzoate	nd	0.02
93	2053	2062	Verimol k	nd	0.17
94	2082	2084	1-Octadecanol	nd	0.07
95	2099	2093	*trans*-Cinnamyl benzoate	nd	1.39
96	2092	2097	Methyl linoleate	nd	2.31
97	2091	2102	Methyl oleate	nd	0.34
98	2098	2104	Methyl linolenate	nd	1.01
99	2102	2110	*trans*-Benzyl cinnamate	nd	0.17
100	2114	2116	Phytol	nd	0.03
101	2128	2127	Methyl stearate	nd	0.13
102	2133	2133	Linoleic acid	nd	0.57
103	2141	2138	Oleic acid	nd	0.38
104	2133	2141	Linolenic acid	nd	0.81
105	2172	2159	Stearic acid	nd	0.06
106	2162	2164	Ethyl linoleate	nd	1.09
107	2173	2170	Ethyl oleate	nd	0.22
108	2169	2172	Ethyl linolenate	nd	0.83
109	2195	2192	Ethyl stearate	nd	0.10
110	2294	2299	Glycidyl palmitate	nd	0.75
111	2329	2326	Methyl eicosanoate	nd	0.07
112	**2493**	**2492**	**Behenic alcohol**	**nd**	**3.19**
113	2498	2514	2-Palmitoylglycerol	nd	0.82
114	**2588**	**2587**	**Benzyl palmitate**	**nd**	**3.73**
115	**2765**	**2770**	**Benzyl linoleate**	**nd**	**5.69**
116	2775	2778	Benzyl linolenate	nd	1.43
117	2794	2793	Benzyl stearate	nd	0.27
118	2864	2878	Cinnamyl palmitate[g]	nd	0.07
119	2924	2934	Geranyl linoleate	nd	0.08
120	3003	3007	Benzyl eicosanoate	nd	0.18
121	3058	3051	Eicosyl benzoate	nd	0.05
122	3062	3067	Cinnamyl linoleate[g]	nd	0.47
123	3072	3080	Cinnamyl linolenate[g]	nd	0.07
124	3150	3167	α-Tocopherol	nd	0.43
125	3264	3262	Behenyl benzoate	nd	0.64

[a] The main volatile components are in bold, the lines with common molecules between the headspace and EE analyses are shaded, phenolic compounds are underlined. Results are expressed as means, detailed data including standard deviation can be found in Supporting Information. [b] RI lit, literature retention indices on semi-standard non-polar columns (NIST,[14] except for compound no. **79**) [c] RI, experimental retention indices on TG-5MS column using a homologous series of *n*-alkanes (C7–C30 or C7–C40). [d] Compounds are listed in order of retention indices. [e] Mean relative content calculated with area ratio. [f] nd, not detected. [g] Geometric isomer could not be distinguished. [h] RI not found in NIST but in Adams, 2007.[15]

benzyl alcohol and benzaldehyde were both identified in flowers collected at the pre-full bloom stage whereas benzaldehyde was absent during the initial bloom, the full bloom and the ending of the bloom stage while benzyl alcohol was only absent during the ending bloom stage.[16] Besides, such variation of the chemical odorants emitted by flowers of *E. submacrophylla* was not clearly and systematically observed in this study from flowers in full bloom stage collected at different anthesis times. Indeed, the analysis of floral volatiles carried out over 4 days at 8:30 a.m., 9:45 a.m., 12:30 p.m.,

1:45 p.m. and 3:00 p.m. shows a stable profile (Table S2 and Figure S1).

To link the structural analysis with the olfactory evaluation, we paid particular attention to the predominant described or identified as emitted by *E. submacrophylla* flowers. The olfactory characteristics of the top three compounds identified during the analysis of the floral headspace (no. **22**, **59** and **82**) are in agreement with the principal molecular odor descriptors highlighted by the perfumer panel. Only few differences should be noted. Despite its low concentration, a jasmine, animal, and indole facet (no. **54**) was described by evaluators that can be explained by a very low detection limit in air (0.03–2 µg/m3).[17] Conversely, benzaldehyde (no. **14**) descriptor was less mentioned, as the almond note, even though its detection limit is higher than most of the major compounds identified by HS-SPME-GC-MS (Table S2). While it is predictable that benzaldehyde is an oxidation-sensitive molecule, its presence in the floral headspace and its detection by GC-MS may be related to the presence of a small amount of benzyl alcohol which can drastically limit its oxidation.[18] The white-floral note depicted here for *E. submacrophylla* flowers is consistent with the chemical composition of the emitted volatiles and also in line with floral fragrances which usually have a voluptuous and heady smell like jasmine. The flowers of *Jasminum sambac* (L.) Ait., *Cananga odorata* or *Lilium 'Belladonna'* all contain several volatiles in common with the major volatiles identified in *E. submacrophylla*,[19–21] and are all considered to be part of the white flower olfactory family with a subtle spicy undertone.

In the floral headspace, the high proportion of aromatics may be explained by exploring the benzenoid and phenylpropanoid pathway (see Figure S2 for a simplified version built as a compilation of different scientific articles).[22,23] Except for styrene (no. **9**), this biosynthetic pathway illustrates the production of the preeminent aromatics. Herein, the enzymatic action of phenylalanine ammonia-lyase (PAL) facilitates the production of (*E*)-cinnamic acid. This key intermediate participates in the generation of various natural products including the benzenoid and phenylpropanoid families such as eugenyl derivatives (no. **59**, **63** and **68**), all descending from coniferyl alcohol, but also benzaldehyde (no. **14**) or benzyl alcohol (no. **22**) which are crucial precursors of benzyl benzoate (no. **82**). All the steps of this pathway have however not yet been identified despite the occurrence and abundance of those compounds in plants.[22,23] The tryptophan pathway could also explain the natural synthesis of indole (no. **54**) via the production of anthranilate instead of phenylalanine.[23]

Considering that styrene (no. **9**) is not commonly found in floral volatiles, rarely above 1% excepted in inflorescence of *Vigna caracalla* (L.) Verdc.[24] and *Rosa x damascena* 'Celsiana',[25] its relative content in the headspace *E. submacrophylla* flowers should be underlined, offering a singular olfactory facet to the "white-floral" note. Likewise, methyl nicotinate (no. **32**), a minor compound identified here is very uncommon in flower extractions by HS-SPME.[26,27]

Beside aromatic structures, a focus on terpenoids reveals a predominance of monoterpenoids (Figure 2e). Commonly found in plants such as ylang-ylang, *trans*-4,8-dimethylnona-1,3,7-triene (no. **31**) is the unique homoterpene detected,[21] being described in the literature as a synomones and released by plants under attack by herbivores.[28] Within the terpenoids (three monoterpenes and five oxygenated monoterpenes), chrysanthemyl acetate (no. **52**) is rarely found in plants, much less in flowers, reported a few times only in essential oils of aerial parts of *Artemisia tridentata* and *A. frigida* for example,[29,30] but to the best of our knowledge, never in a floral headspace.

Regarding aliphatic compounds, a majority of C6 alcohols or C6 alcohol esters (Figure 2d) were identified, respectively 3-hexenol (no. **5**), 2-hexenol (no. **6**), hexanol (no. **7**), 3-hexenyl acetate (no. **16**), hexyl acetate (no. **17**), 2-hexenyl acetate (no. **18**), 3-hexenyl butyrate (no. **38**) and hexyl butanoate (no. **39**). Mostly reported in literature as green leaf volatiles and synomones, they can also be released by healthy plants in a lesser extent.[31] The lipoxygenase pathway (Figure S3) can provide evidence of a common biosynthetic origin for all these oxygenated aliphatic molecules. As reported in literature, all of them possess a common precursor, namely linolenic acid (no. **104**) or linoleic acid (no. **102**),[22] which were further identified in the EE.

Among the 57 molecules detected in the headspace of *E. submacrophylla* flowers, one of them (no. **57**) revealed to be inconsistent with data extracted from various MS libraries. While the first proposal to identify compound **57** by non-polar stationary phase analysis referred to chavicol, this postulate was inconsistent with the experimental RI measured, redirecting our research towards probable isomers since isomerism leads to nearly identical MS spectra, sometimes causing misidentification when only MS spectrum without RI is taken into account.[32] MS spectrum and RI comparison with a *trans*-4-propenylphenol commercial standard allowed us to validate our initial presumption that isochavicol, possessing a variation with chavicol only by the position of the double bond outside the aromatic ring, could be assigned to compound **57**. These observations were in line with the experimental retention index obtained with the polar stationary phase and that described in the literature.[33] Isochavicol is not commonly found in nature, being only reported among four HS-SPME analyses in the literature, *Wisteria sinensis* (Sims) Sweet, *Trachelospermum jasminoides* (Lindl.) Lem., *Jasminum officinale* L.[32] and *Daucus crinitus* Desf.,[34] and also in few essential oils obtained from flowers such as Narcissus 'Trevithian'.[35]

Comparative study of chemical constituents identified in floral volatiles and ethanolic extract

E. submacrophylla floral EE was produced with the ambition to trap the odorous molecules identified in the headspace of flowers, and also less volatile compounds not extractable with HS-SPME. As depicted in Table 1, the examination of GC-MS data obtained from the EE prepared from fresh material allowed us to identify a total of 78 compounds, only 11 being common to the floral headspace, including the three major compounds. The dominant aromatic and oxygenated chemical profile of EE is in agreement with that previously described for HS-SPME

(Figure 2 and 3). Oxygenated compounds identified in EE are mainly mono- and bifunctional, and distributed among esters, alcohols, ethers, carboxylic acids, aldehydes and ketones. The aromatic fragments can be divided into phenolic and non-phenolic oxygenated compounds, among which the presence of fatty acid derivatives, furans and terpenoids has to be noticed.

In comparison with the headspace analysis, the detection of additional molecules in EE, such as benzoic acid (no. **34**), *cis*- and *trans*-coniferyl alcohol (no. **79** and **81**), *trans*-methylisoeugenol (no. **69**) or vanillylacetone (no. **78**) should be highlighted. Their presence can be attributed to their high boiling point and low volatility which makes their adsorption on the SPME fiber unlikely without heating. For a similar reason, we observed the presence of a higher proportion of benzyl benzoate in the EE than in the headspace, a compound of interest, belonging to the WHO Essential Medicines List as insecticide against rabies or lice, also used as a fixative in the perfume industry.[36] Among the phenolic family, it is interesting to notice the presence of a small amount of methylparaben (no. **67**) or methyl-4-hydroxybenzoate. Being generally recognized as safe (GRAS) by the Food and Drug Administration, this molecule is scarcely found in plants and has only been isolated by chloroform extraction of cloudberry (*Rubus chamaemorus* L.)[37] and from vanilla beans (*Vanilla planifolia* Andrews) with solvent-assisted flavor evaporation.[38]

A focus on non-aromatic compounds (Figure 3d) highlights fatty acid derivatives, fatty acids, fatty alcohols, long chain terminal alkenes, one pyran derivative, furan derivatives, and also one terpenoid moiety. We can mention here the presence of pyran and furan derivatives, which were absent in the floral headspace since they result from the degradation of sugars or ascorbic acid.[39,40] Their presence is correlated with the olfactory description of more caramel and balsamic notes. In the category of fatty acid derivatives, 5 fatty acids and 22 fatty esters can be enumerated in EE, such as palmitic acid (C16:0) (no. **88**), which is part of the major compounds, linoleic (no. **102**) and α-linolenic (no. **104**) acids. Both are precursors for the green leaf volatile pathway (Figure S3),[22] most likely responsible for the production of C6 derivatives described in the floral HS-SPME analyses, such as 1-hexanol (no. **7**) and 2-hexenyl acetate (no. **16**).

Finally, among terpenoids, geranyl derivatives were identified (no. **89** and **119**). Interestingly, geranyl pyrophosphate was described as part of the plant secondary metabolites pathway[41] and as a precursor of phytol (no. **100**), and tocopherol derivatives, such as α-tocopherol (no. **124**).

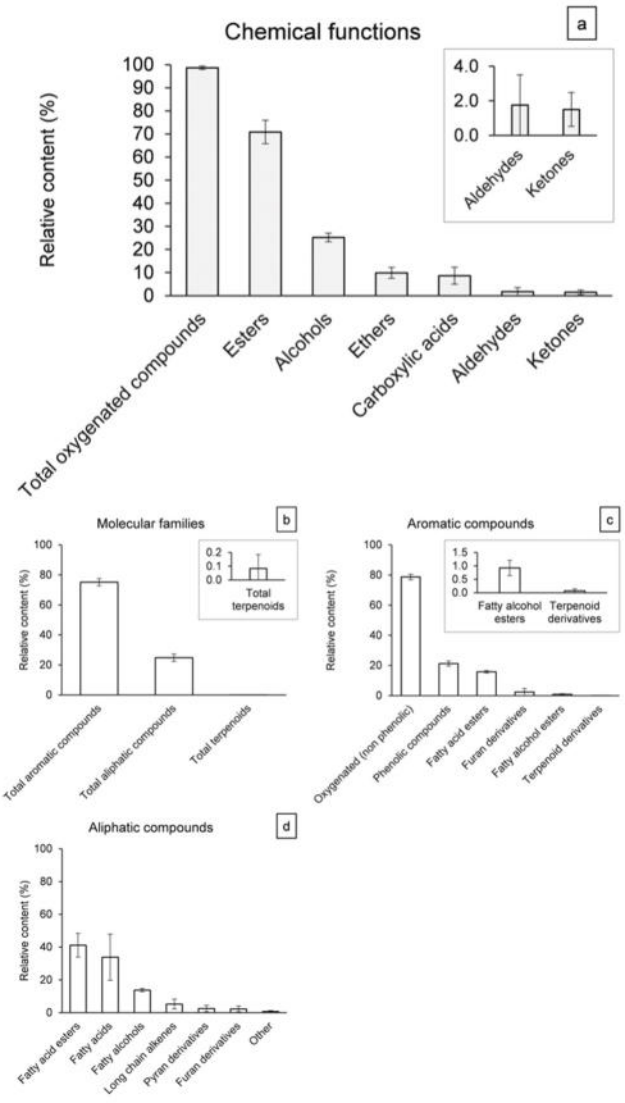


Figure 3. Molecular family and functional groups of volatiles identified in the EE of flowers of *E. submacrophylla*. (a) Relative content of chemical classes. Molecules containing multiple functional groups were counted in every corresponding group. (b) Relative content of molecular families. (c) Category of aromatic compounds. (d) Category of aliphatic compounds.

Evaluation of the antioxidant capacity of floral ethanolic extract

The identification of a high number of phenolic derivatives in the EE of flowers of *E. submacrophylla* led us to explore its *in vitro* antioxidant potential. While many methods have been developed for this type of assessment, results are variable, some having poor reproducibility.[42,43] In the present study, we choose to adopt three different methods, DPPH, ABTS and ORAC, that we expected to be concordant in assessing the antioxidant activity of EE, complemented by an investigation of the phenolic content. Since the ORAC method is generally more considered due to its use of biologically relevant free radicals,[44] the DPPH and ABTS colorimetric methods are easy to implement and cost effective.

The Trolox equivalent antioxidant capacity (TEAC) of EE, with the help of a Trolox calibration curve, were calculated and compared to antioxidant standards and literature data reported for natural extracts (Table 2, details in the experimental section). While the results show a relatively comparable and significant antioxidant potential for EE, its phenolic content, determined by the Folin-Ciocalteu method, is in the same order of magnitude compared to mate, 200 mL of rosé wine, 50 mL of red wine and higher compared to green tea extract (Table 2).

Table 2. Phenolic content and antioxidant activities of EE and commercial standards, compared to results from the literature. The results of the Trolox Equivalent Antioxidant Activity (TEAC) of EE and standards were determined according to their corresponding IC_{50} values, excepted for the TEAC of gallic acid, vitamin C and BHT determined from the ORAC value (data reported from the literature).

Samples	Phenolic content	TEAC [mmol TE/g]		
		DPPH	ABTS	ORAC values
EE	65.1 ± 7.7 [mg GAE/g]	0.4 ± 0.1[a]	0.6 ± 0.1[f]	1.3 ± 0.3[j]
Gallic acid	–	14.5 ± 0.9[b]	19.4 ± 3.8[g]	Ranging from: 6.2 ± 0.9 to 9.0 ± 1.0[45–47]
Quercetin	–	7.9 ± 0.2[c]	10.7 ± 0.8[h]	22.2 ± 3.2[k]
Vitamin C	–	6.5 ± 0.3[d]	4.1 ± 1.0[i]	Ranging from: 1.9 ± 0.3 to 5.4 ± 0.1[45,47,48]
BHT	–	1.2 ± 0.4[e]	4.5 ± 0.2[i]	Ranging from: 0.73 ± 0.05 to 1.22 ± 0.04[49–51]
Samples	Phenolic content	TEAC		
(from literature)		DPPH	ABTS	ORAC values
Mate	Ranging from: 67.5 ± 6.2 to 77.3 ± 2.9 [mg GAE/100 mL][52]	–	Ranging from: 0.28 ± 0.02 to 0.43 ± 0.02 [mmol TE/100 mL][52]	Ranging from: 1.99 ± 0.15 to 2.68 ± 0.17 [mmol TE/100 mL][52]
Green tea extract	149 [mg GAE/g][53]	–	4.60 ± 0.09 [mmol TE/g][53]	4.63 ± 0.37 [mmol TE/100 mL][53]
Red wine	110 ± 1 [mg GAE/100 mL][54]	–	2.36 ± 0.06 [mmol TE/100 mL][54]	–
var. *Tempranillo*	130.2 ± 0.8 [mg GAE/100 mL][55]	–	–	0.31 ± 0.03 [mmol TE/100 mL][55]
var. *Graciano*	146.8 ± 2.3 [mg GAE/100 mL][55]	–	–	0.40 ± 0.02 [mmol TE/100 mL][55]
var. *Cabernet S*	142.8 ± 3.4 [mg GAE/100 mL][55]	–	–	0.35 ± 0.02 [mmol TE/100 mL][55]
Rosé wine				
var. *Cabernet S*	38.9 ± 0.7 [mg GAE/100 mL][55]	–	–	0.089 ± 0.006 [mmol TE/100 mL][55]
var. *Tempranillo*	43.9 ± 1.0 [mg GAE/100 mL][55]	–	–	0.100 ± 0.006 [mmol TE/100 mL][55]
var. *Garnacha*	43.2 ± 0.8 [mg GAE/100 mL][55]	–	–	0.112 ± 0.005 [mmol TE/100 mL][55]
Blueberry	281 ± 16 [mg GAE/100 mL of juice][56]	–	0.0209 ± 0.0007 [mmol TE/g][56]	0.046 ± 0.004 [mmol TE/g][56]

[a–k] all values with different superscript letters are significantly different, $p < 0.05$.

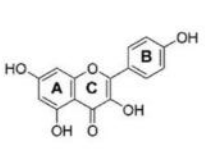
Identification of flavonoids in floral EE

The promising antioxidant activity of EE suggests a high phenolic content as supported by the GC-MS identification of 20 phenols, (mostly monophenolic scaffolds, Table 1, underlined compounds) and the Folin-Ciocalteu assay. Some of which are widely described for their ability to trap free radicals, especially guaiacol derivatives (no. **56**, **59**, **68**, **72**, **78**, **79**, **81**),[57,58] benzyl salicylate (no. **86**),[59] chavicol moieties (no. **48**, **57**),[60] and to a lesser extent syringol derivatives (no. **74**, **77**, **80**), or α-tocopherol (no. **124**). In addition to phenolic compounds, the presence of well-known antioxidant molecules, such as benzyl alcohol (no. **22**)[61,62] and phytol (no. **100**) should also be notice.

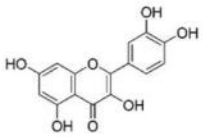
In order to identify more phenolic compounds potentially contributing to the antioxidant activity, the EE was analyzed by UPLC-MS and tandem mass spectrometry (MS/MS), with a focus on flavonoids. Their growing interests have been promoted by their significant health benefits, especially due to their antioxidant activities.[63,64] Representing more than 6,000 compounds, flavonoids naturally occur in the form of aglycones (flavonoid without bonded sugar) and glycosides. Among the 500 flavonoid aglycones isolated from plants, only 8 were described to be more widely disseminated, including quercetin and kaempferol extensively distributed in vegetables and fruits.[65]

In the present study, the structure of 13 glycosylated flavonoids were proposed (Table 3). All flavonoids are flavonols substituted by up to four *O*-hexosyl and/or *O*-rhamnosyl while no *O*-pentosyl forms were observed. Examination of the aglycone moieties reveals abundant quercetin and kaempferol skeletons. Quercetin is distinguished by an additional hydroxyl group present at the 3′ position on the B ring, which is methylated into the isorhamnetin building block, also known as 3-methylquercetin. We can notice that compounds no. **126**–**130**, no. **132** and no. **134** share the same fragmentation pattern with the fragment ion at *m/z* 299 corresponding to a quercetin fragment ion $[Y_0\text{-H}]^-$.[66] Similarly, compounds no. **131** and **133** were subsequently proposed as isorhamnetin glycosides, possessing an additional methylated fragment ion $[Y_0\text{-H}]^-$ at *m/z* 314. Conversely, the absence of a hydroxy group and the

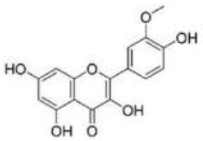
Table 3. Proposed identification by UPLC-MS-MS/MS of flavonoids in the EE of flowers of *E. submacrophylla*.



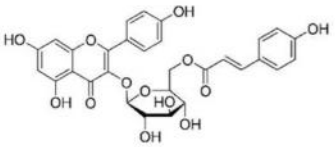
Kaempferol (K)



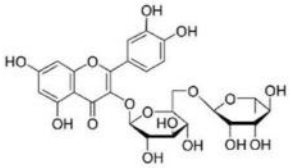
Quercetin (Q)



Isorhamnetin (I)



Tiliroside



Rutin

No.	Flavonoid[a]	Aglycone[b]	RT [min]	Molecular formula	Calcd *m/z* $[M-H]^-$	Obsd *m/z* $[M-H]^-$	Major fragments and adducts[c]
126	Quercetin di *O*-hexosyl-*O*-rhamnoside	Q	8.84	$C_{39}H_{50}O_{25}$	917.2563	917.2568	*963.2617*, *953.2329*, 755.2034, 446.0821, 299.0209, 271.0264, 255.0254, 243.0289
127	Quercetin-*O*-hexosyl-*O*-hexosyl-*O*-rhamnoside (I)	Q	8.87	$C_{33}H_{40}O_{21}$	771.1984	771.1984	*817.2022*, *807.1781*, 446.0821, 299.0171, 271.0228
128	Quercetin-*O*-hexosyl-*O*-hexosyl-*O*-rhamnoside (II)	Q	8.99	$C_{33}H_{40}O_{21}$	771.1984	771.1984	*817.2022*, *807.1781*, 446.0821, 299.0171, 271.0228
129	Rutin or regioisomeric derivative (I)	Q	10.16	$C_{27}H_{30}O_{16}$	609.1456	609.1456	446.0913, 299.0209, 271.0228
130	Quercetin-*O*-rhamnosyl-*O*-rhamnosyl-*O*-hexoside (I)	Q	10.30	$C_{33}H_{40}O_{20}$	755.2035	755.2041	*801.2089*, *791.181*, 446.0867, 299.0209, 271.0264, 255.0324
131	Isorhamnetin di *O*-hexoside	I	10.37	$C_{28}H_{32}O_{17}$	639.1561	639.156	*675.1326*, 314.0413, 299.0171, 271.0228, 255.0324, 243.0255
132	Quercetin-*O*-rhamnosyl-*O*-rhamnosyl-*O*-hexoside (II)	Q	10.44	$C_{33}H_{40}O_{20}$	755.2035	755.2029	*801.2084*, *791.1798*, 609.1456, 446.0867, 299.0209, 271.0264, 255.0324
133	Isorhamnetin-*O*-hexosyl-*O*-rhamnoside	I	11.02	$C_{28}H_{32}O_{16}$	623.1612	623.1616	*659.1377*, 314.0413, 299.0209, 271.0228, 255.0254, 243.0289
134	Rutin or regioisomeric derivative (II)	Q	11.26	$C_{27}H_{30}O_{16}$	609.1456	609.1456	*653.2438*, 463.0862, 300.0272, 271.0228, 255.0289, 243.0289, 227.0359, 215.0320
135	Kaempferol-*O*-hexosyl-*O*-rhamnoside	K	11.28	$C_{27}H_{30}O_{15}$	593.1506	593.1501	430.0876, 285.0399, 283.0247, 255.0254
136	Kaempferol-*O*-rhamnosyl-*O*-rhamnosyl-*O*-hexoside	K	11.35	$C_{33}H_{40}O_{19}$	739.2086	739.2084	593.1531, 283.0247, 255.0289, 227.0326
137	Tiliroside or regioisomeric derivative (I)	K	16.95	$C_{30}H_{26}O_{13}$	593.1295	593.1284	593.1284, 285.0362, 284.0333, 255.0289, 227.0326, 145.0296
138	Tiliroside or regioisomeric derivative (II)	K	17.08	$C_{30}H_{26}O_{13}$	593.1295	593.1294	593.1294, 285.0394, 284.0333, 255.0289, 227.0359, 145.0296

[a] Isomers are represented as (I) and (II). Deoxyhexose was considered to be rhamnose as it is the deoxyhexose found in flavonoid glycosides.[69] [b] Q, Quercetin; K, Kaempferol; I, Isorhamnetin. [c] Adducts (chloride $[M+35]^-$ and/or formic acid $[M-H+46]^-$) are in italics.

presence of a fragment ion $[Y_0\text{-H}]^-$ at *m/z* 283 allowed to categorize compounds no. **135–136** as kaempferol glycosides.

Concerning compounds no. **137** and no. **138** with respect to their very close exact mass of their molecular ion $[M–H]^-$ at *m/z* 593.1284 and 593.1294 respectively, an analogous empirical formula was assigned: $C_{30}H_{26}O_{13}$. Fragmentation gave access to a fragment ion at *m/z* 284.0333, that can be attributed to the kaempferol radical ion $[Y_0–H]^-$, the aglycone being substituted only by a single hydroxy group to a sugar residue. These two compounds have an identical fragmentation pattern with a mass loss of 308 Da starting from the molecular ion, consistent with a loss of a p-coumaroylhexoside moiety. This assignment was thereafter supported by a higher retention time compared to kaempferol-*O*-hexosyl-*O*-rhamnoside (no. **135**), and the presence of a coumaroyl fragment ion at *m/z* 145.0296 corresponding to $C_9H_5O_2^-$. Both compounds, no. **137** and no. **138**, can be potentially proposed as kaempferol-*O*-p-coumaroylhexose, tiliroside isomers or derivatives, tiliroside having already been identified in the flowers of *E. angustifolia*.[67] The acylation position and glycosidic linkage could however not be unambigously identified by UPLC-MS/MS although glycosylation and acylation usually occur respectively on the C3 and C7 of the aglycone, and on the C6 of the ose.[68,69]

This first attempt in the identification of numerous flavonoids supports the promising antioxidant potential previously observed from the floral EE of *E. submacrophylla*. The presence of kaempferol and isorhamnetin derivatives and especially the high proportion of quercetin-type structures are as many clues explaining the significant antioxidant activity observed. In addition, we can mention that the antioxidant capacity of *O*-glycosylated flavonoids is generally less active than the non-glycosylated forms while an opposite effect can be observed for other biological activity such as antiallergic, antirotavirus or antiadipogenic activities.[70] Thus, it could be interesting to include a hydrolysis step to the preparation of EE and to compare their respective antioxidant capacity.

Conclusion

In conclusion, this study reports the first identification of a broad range of odorants detected in the headspace of flowers of *E. submacrophylla* as well as the chemical composition of their ethanolic extract. A set of 57 molecules were identified in the headspace and 78 in the ethanolic extract, such as benzyl alcohol, eugenol or benzyl benzoate, which are part of the 11 compounds commonly detected. A promising antioxidant potential of the flowers ethanolic extract has been pointed out *in vitro*, in line with the description of several flavonol glycosides, that could potentially be enhanced by hydrolysis. This *in vitro* antioxidant capacity would deserve to be complemented by *in vivo* trials. With a need to replace synthetic antioxidants, these results highlight a future potential for flowers of *E. submacrophylla*, a fragrant hedge with multiple flower buds, easy to grow, very resistant, with proven environmental advantages. This shrub would gain from being further studied for its potential health benefits, by considering the whole plant (fruit, root, leaf, etc.), in a permaculture vision, integrating mutually beneficial synergies for man, resources, nature and the environment.

Experimental Section

Plant material. Flowers of *Elaeagnus x submacrophylla* Servett. were harvested from plants growing in ornamental hedges in Montpellier, France (coordinates 43° 38′ 3.99″ N and 3° 51′ 36.81″ E). Botanical identification was confirmed by a local botanist and voucher specimens were deposited at the herbarium of the University of Montpellier, France (MPU814003). Open, complete and freshly-picked flowers at full bloom stage (completely opened perianth)[16] were collected in dry weather condition from November to December 2019. Consisting of pedicel, sepals and reproductive organs. For each sample collection, several shrubs were harvested in order to smooth interindividual variations. Collection time was limited to 1 h in order to prevent excessive degradation of the plant material.

Chemicals. Ethanol (99.8%) was obtained from VWR (Darmstadt, Germany). A standard solution of C7–C30 n-alkanes in hexane (1000 µg/mL each), a standard solution of C7–C40 n-alkanes in hexane (1000 µg/mL each), DPPH• (2,2-diphenyl-1-picrylhydrazyl), 2 M Folin-Ciocalteu phenol reagent, anhydrous sodium carbonate, acetonitrile HPLC grade (>99.5%), Trolox (6-hydroxy-2,5,7,8-tetramethylchroman-2-carboxylic acid), BHT (butylated hydroxytoluene), anhydrous L-ascorbic acid, potassium persulfate, fluorescein sodium salt, AAPH (2,2-Azobis(2-methylpropionamide) dihydrochloride), ABTS (2,2-Azino-bis(3-ethylbenzothiazoline-6-sulfonic acid)) and quercetin hydrate were purchased from Sigma-Aldrich Co. (St. Louis, MO). Sodium phosphate dibasic dihydrate, Sodium phosphate dibasic dodecahydrate were purchased from VWR International bvba (Leuven, Belgium). *trans*-4-Propenylphenol (95%) was purchased from Chemspace (Riga, Latvia). Anhydrous gallic acid was purchased from Merck (Darmstadt, Germany). Helium 5.6 (Linde, Saint-Priest, France) was used for gas chromatography. Formic acid ULC/MS grade (99%) was purchased from Biosolve (Netherlands). Ultrapure water was produced with a Labostar Pro TWF apparatus from Evoqua (Chaville, France) with a conductivity of 0.055 µS/cm.

Sample preparation for HS-SPME. Flowers of *E. submacrophylla* (5 g) were placed immediately after collection in volatile organic analysis vials (40 mL) tightly sealed with a PTFE/silicone septum cap (Phenomenex, Torrance, CA). Five samples were collected per day: Sample A at 8:30 a.m., Sample B at 9:45 a.m., Sample C at 12:30 p.m., Sample D at 1:45 p.m. and Sample E at 3:00 p.m, and repeated for 4 days (Day A, B, C and D).

Sample preparation of flowers ethanolic extract (EE). Approximately 250 g of flowers of *E. submacrophylla* were fully immersed in ethanol in a sealed bottle, for 1 h at 20 °C (controlled temperature room) without stirring (1:6 w/w flowers/ethanol). The mixture was subsequently filtered and the solvent was slowly evaporated under reduced pressure at 3 °C to avoid the loss of the most volatile compounds. The extract was then freeze dried (FreeZone Plus 4.5 Liter Cascade, Labconco, Kansas City, MO) to remove the remaining water, giving a dry extract (10 g), 4.0 ± 0.4 wt.% extraction yield, with respect to the crude material. Several extracts were prepared using the same procedure in order to determine standard deviation and obtain mean results.

Olfactory evaluation. Flowers of *E. submacrophylla* were evaluated in the morning, immediately after collection, to avoid the potential rapid deterioration of the plant material and the loss of volatiles.

Professional perfumers, who regularly practice generating white flower descriptors, were mobilized to assess the olfactory perception of harvested flowers.

HS-SPME. Trapping scent of samples was carried out by HS-SPME at 20 °C (air-conditioned and thermostatically controlled room) to provide the most realistic image of the volatile profile, with a 2 cm StableFlex fiber coated with 50/30 µm divinylbenzene/carboxene/polydimethylsiloxane (DVB/CAR/PDMS; Supelco, Bellefonte, PA). CAR/PDMS fibers are not very efficient with high molecular weight analytes whereas PDMS/DVB fibers are not with low molecular weight analytes. The combination of both and thus bipolar fiber coating therefore enables the adsorption of volatiles and semi-volatiles, giving a broad picture of the flower's scent.[71] Fibers were conditioned for 30 min at 270 °C prior to use. Each sample vial was left closed 1 h to let the headspace equilibrate itself. The fiber was then inserted in the vial for 1 h for molecules adsorption, further removed and placed in the injection port of the GC-MS for desorption at 250 °C for 5 min in split mode (1 : 10).

Gas chromatographic analyses. Non-polar stationary phase: the separation and detection of the analytes was achieved using a TRACE 1300 gas chromatography system coupled to a DSQ II single quadrupole mass spectrometer (Thermo Fisher Scientific, Waltham, MA) in electron impact (EI) ionisation mode (70 eV). Separations were performed on a non-polar TG-5MS stationary phase (30 m × 0.25 mm i.d. × 0.25 µm film thickness; Thermo Fisher Scientific). The oven temperature was kept at 50 °C for 5 min, ramped to 250 °C at 10 °C/min with a final hold time of 5 min. Helium 5.6 (Linde, Saint-Priest, France) was used as the carrier gas at a constant flow rate of 0.9 mL/min. The EE dissolved in EtOH was injected in split ratio 1 : 10 with an injector temperature of 250 °C. The oven temperature was kept at 70 °C for 3 min, ramped to 250 °C at 10 °C/min, held for 3 min and ramped to 330 °C at 25 °C/min and then held at the final temperature for 3 min. MS data were recorded at 6 scans/sec in a range of *m/z* 33–350 amu for SPME analyses and 33–650 amu for EE analyses. Transfer line temperatures was set at 280 °C for SPME analysis and 350 °C for EE. MS ion source temperature was set at 250 °C. The chromatograms were acquired and analysed using Xcalibur 3.0.63 (Thermo Fisher Scientific). Polar stationary phase: the separation and detection were achieved using a 7820A gas chromatography system coupled to a 5977E MSD single quadrupole mass spectrometer (Agilent Technolgies, Palo Alto, CA, USA) in EI ionisation mode (70 eV). Separations were performed on a polar HP-INNOWax stationary phase (20 m x 0.18 mm i.d. × 0.18 µm film thickness; Agilent Technologies). Helium 6.0 (Linde, Saint-Priest, France) was used as the carrier gas at a constant flow rate of 0.9 mL/min. The EE dissolved in EtOH was injected in split ratio 1 : 10 with an injector temperature of 250 °C. The oven temperature was kept at 70 °C for 2 min, ramped to 250 °C at 10 °C/min with a final hold time of 20 min. MS data were recorded at 4.4 scans/sec in a range of *m/z* 33–350 amu. Transfer line and MS ion source temperatures were respectively set at 280 °C and 230 °C. The chromatograms were acquired and analysed using MassHunter GC/MS Acquisition B.07.02.1938 and MSD ChemStation F.01.01.23.17 softwares, respectively (Agilent technologies). For both non-polar and polar GC-MS, the Kováts retention index (RI) of detected compounds was calculated using the C7–C30 and C7–C40 saturated *n*-alkanes analytical standard. Compounds identification was achieved by comparison of their mass spectra (mass spectrum matching score level $\geq$850, corresponding to $\geq$85 % superimposition with mass spectral library) and RI to those reported in a mass spectral library developed at the laboratory with GC-MS data derived from the analysis of commercial and the National Institute of Standards and Technology (NIST) online database the Adams database.[15] Each experiment was performed in triplicate. Relative amounts were determined by area peak normalization and all relative response factors were taken as one.

UPLC-MS-MS/MS analysis. The analyses were performed with an Acquity H-Class UPLC system (Waters, Milford, MA) coupled to a QToF (quadrupole time-of-flight) mass spectrometer (electrospray ionization, ESI). The EE dissolved in EtOH was analysed with 5.0 µL injection volume, preceded by the injection of an EtOH blank. The column used was a reversed-phase Kinetex® EVO C18 (100 Å; 150 mm × 2.1 mm × 2.6 µm; Phenomenex, Torrance, CA) and was thermostated at 30 °C. The mobile phase consisted of ultrapure water (solvent A) and acetonitrile (solvent B) both acidified with 0.1 % formic acid. The separation was achieved at a 0.5 mL/min constant flow rate using a linear gradient: 0 to 100 % B for 60 min. MS detection was performed with a HDMS Synapt G2-S (Waters) in negative ESI mode and parameters: cone voltage, 30 V; capillary voltage, 2 kV; nitrogen desolvation gas flow, 1,000 L/h; desolvation temperature, 450 °C; source temperature, 140 °C. The *m/z* range was 50–1,500. MS/MS was performed with Ar collision gas, cone voltage of 40 V and collision energy of 30 eV. Data acquisition and processing was ensured using MassLynx 4.2 software (Waters).

DPPH radical scavenging ability. The scavenging ability of standards (gallic acid, quercetin, vitamin C, BHT and Trolox) and EE was determined using the method of Bendaikha *et al.*[72] with adjustments. All solutions were prepared in EtOH. 50 µL of EtOH (control), standards or EE solution were added to 950 µL of 75 µM DPPH$^{\bullet}$ and were vortexed thoroughly. Solutions were left 30 min in the dark at 20 °C and the absorbance was read at 515 nm using a UV-1800 spectrophotometer (Shimadzu, Marne-la-Vallée, France). A blank containing 950 µL EtOH instead of DPPH$^{\bullet}$ solution was made for each separate concentration tested. The inhibition percentage was calculated using the following equation: $\%\text{inhibition} = [(A_{control} - A_{sample})/A_{control}] \times 100$. A calibration curve of $\%\text{inhibition} = f(\text{concentration})$ was then drawn for each standard and EE. The IC_{50} value (concentration corresponding to 50 % inhibition) of each sample was then determined. All measures were done in triplicate. The standard calibration curves were linear between: 0.5 and 3.5 mg/L (2.9–20.6 µM) gallic acid, 0.5 and 4.0 mg/L (1.7–13.2 µM) quercetin, 2.0 and 10.0 mg/L (11.3–56.8 µM) vitamin C, 0.5 and 7.8 mg/L (2.0–31.2 µM) Trolox. The BHT calibration curve was polynomial (2nd order) between 5.0 and 32.5 mg/L (22.7–147.4 µM). The EE calibration curve was linear between 10.0 and 380.0 mg/L. The Trolox equivalent antioxidant capacity of standards and EE (TEAC) was obtained according to: $\text{TEAC (mmol TE/g)} = IC_{50(\text{Trolox})}\ (0.0233\ \text{mM})/IC_{50(\text{standards/EE})}\ (\text{g/L})$.

ABTS radical scavenging ability assay. The method of Re *et al.*[73] was used with slight modifications. To generate the ABTS$^{\bullet+}$ radical cations, an equal volume of aqueous 2.45 mM $K_2S_2O_8$ and 6.57 mM ABTS were mixed and allowed to stand for 16 hours in darkness at 20 °C. The working solution was then prepared by diluting the previously mentioned solution in ethanol until the absorbance of the negative control at 752 nm was 0.84 ± 0.02 (usually 1/40). Several dilutions of EE and commercial standards were prepared in ethanol and 20 µL were added to 980 µL of the working solution before homogenization. A negative control containing 20 µL of ethanol was also prepared. A 25/975 (equal to 1/40) H_2O/EtOH solution was used as blank, as all tested materials did not absorb at 752 nm. The absorbance of the samples and negative control at 752 nm (absorption maximum) after 30 min was measured with a UV-1800 (Shimadzu, Tokyo, Japan) with a 1 nm spectral bandwidth. Every experiment was performed in triplicate. The resulting absorbance was used to calculate ABTS$^{\bullet+}$ inhibition, which represents the percentage of ABTS$^{\bullet+}$ having been quenched by the antioxidants present in the solution, and further determine the IC_{50} and TEAC according to the method previously employed for the DPPH assay. The standard calibration curves were linear between:

0.2 and 1.1 mg/L (1.2–6.5 μM) gallic acid, 0.5 and 2.0 mg/L (1.7–6.6 μM) quercetin, 1.0 and 4.5 mg/L (5.7–25.6 μM) vitamin C, 1.0 and 5.0 mg/L (4.5–22.7 μM) BHT, 1.0 and 4.0 mg/L (4.0–16.0 μM) Trolox. The Trolox equivalent antioxidant capacity of standards and EE (TEAC) was obtained according to: TEAC (mmol TE/g) = $IC_{50(Trolox)}$ (0.0137 mM)/$IC_{50(standards/EE)}$ (g/L).

Oxygen Radical Absorbance Capacity (ORAC) assay. The oxygen radical absorbance capacity was assessed using the method of Dudonné *et al.*[74] AAPH and fluorescein solutions were prepared with a 75 mM sodium phosphate buffer (pH 7.4) at final concentrations of 12 mM and 70 nM in the cuvettes. A stock solution of 0.85 mM fluorescein was stored at 4 °C and used to prepare daily fresh working solutions. EE, quercetin and Trolox were prepared in 25:75 (v/v) EtOH/75 mM buffer. 300 μL of standards or EE were mixed with 1.8 mL of fluorescein and let 5 min for incubation at 37 °C. The addition of 900 μL of AAPH started the reaction. The time between the AAPH addition and the actual launch of fluorescence reading was kept alike and as short as possible (30 s). The fluorescence intensity was then measured every 0.5 min for 45 min (excitation 493 nm and emission 511 nm) with a JASCO J-815 CD spectrometer (JASCO, Lisses, France) equipped with a Peltier temperature control system and a fluorescence monochromator. A 1 cm path length fluorometer quartz cuvette and parameters fluorescence detector voltage 700 V, excitation and emission bandwidth 10 nm, digital integration time (D.I.T.) 1 sec were used. The temperature was maintained throughout the whole experiment at 37 °C. A blank sample made with 300 μL 25:75 (v/v) EtOH/75 mM buffer, fluorescein and AAPH was prepared and measured daily. All measurements were made in triplicate. As the reaction is conducted until completion, the area under the curve (AUC) can be measured by integration via the trapezoidal rule:

$$AUC = \sum_{i=1}^{i=90} \frac{(t_{i+1} - t_i)^*(f_{i+1} + f_i)}{2} \quad (1)$$

where t_i is the time at reading i and f_i the fluorescence intensity at reading i. The net AUC was then calculated by subtracting the AUC of the sample to the AUC of the blank. The net AUC = f(Trolox concentration) calibration curve was linear between 0.3 and 1.8 mg/L (1.0–7.0 μM) Trolox ($y = 3.7788x + 3.3591$, r^2 of 0.9932). The ORAC values could then be calculated and expressed in mmol TE/g EE or quercetin: ORAC value = equivalent Trolox concentration/sample concentration. Quercetin was tested at 0.08 and 0.140 mg/L and the EE was tested at 1.3 and 2.0 mg/L.

Folin-Ciocalteu assay. The total phenolic content of EE was assessed using the Folin-Ciocalteu test.[75] Gallic acid (standard) and EE were diluted in 75/25 (v/v) EtOH/H_2O. 100 μL of solvent (blank), EE or gallic acid solution was vortexed with 500 μL of Folin-Ciocalteu reagent (diluted 10-fold) followed by the addition after 5 minutes of 400 μL of 75 g/L Na_2CO_3. After 2 h standing in the dark at 20 °C, the absorbance at 750 nm was read with a UV-1800 spectrophotometer. Each experiment was performed in triplicate. An absorbance = f(gallic acid concentration) calibration curve was drawn (r^2 = 0.9991) and results were then expressed as mg GAE (gallic acid equivalent)/g of floral EE. The gallic acid calibration curve was linear between 1.0 and 11.0 mg/L. The concentration range of EE was tested between 29.5 and 142.5 mg/L.

Statistical analysis. GC-MS analysis and antioxidant assays were conducted at least three times and results are shown as mean ± standard deviation. Statistical significance of the results of floral volatiles (significance level of $p < 0.05$) was calculated using Friedman's test using JASP 0.11.1 (Amsterdam, The Netherlands).

Acknowledgements

This work was supported by the French Ministry of Higher Education, Research and Innovation and the University of Montpellier. We thank the LabEx Chemisyst of the University of Montpellier for analytical facilities, Dr. Yves Caraglio, CIRAD, UMR AMAP, Montpellier for botanical identification, and Elodie Fernandez (junior perfumer) from ARTHUR DUPUY® for having coordinated the olfactory evaluation.

Conflict of Interest

The authors declare no conflict of interest.

Keywords: antioxidants · *Elaeagnus submacrophylla* · flavonoids · phenolic content · volatile compounds

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