

Bioprospecting of *Artemisia* genus: from artemisinin to other potentially bioactive compounds

Stefano Negri

University of Verona

Fabio Pietrolucci

University of Verona

Sebastiano Andreatta

Natural History Museum, verona

Ruth Njoku

University of Verona

Carolina Antunes Silva Nogueira Ramos

University of Verona

Massimo Crimi

University of Verona

Mauro Commisso

University of Verona

Flavia Guzzo

University of Verona

Linda Avesani

`linda.avesani@univr.it`

University of Verona

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Abstract

Species from genus *Artemisia* are widely distributed throughout temperate regions of the northern hemisphere and many cultures have a long-standing traditional use of these plants as herbal remedies, liquors, cosmetics, spices, etc. Nowadays, the discovery of new plant-derived products to be used as food supplements or drugs has been pushed by the exploitation of bioprospection approaches. Often driven by the knowledge derived from the ethnobotanical use of plants, bioprospection explores the existing biodiversity through integration of modern omics techniques with targeted bioactivity assays.

In this work we set up a bioprospection plan to investigate the phytochemical diversity and the potential bioactivity of five *Artemisia* species with recognized ethnobotanical tradition (*A. absinthium*, *A. alba*, *A. annua*, *A. verlotiorum* and *A. vulgaris*), growing wild in the natural areas of the Verona province. We characterized the specialized metabolomes of the species (including sesquiterpenoids from the artemisinin biosynthesis pathway) through an LC-MS based untargeted approach and, in order to identify potential bioactive metabolites, we correlated their composition with the *in vitro* antioxidant activity. We propose as potential bioactive compounds several isomers of caffeoyl and feruloyl quinic acid esters (e.g. dicaffeoylquinic acids, feruloylquinic acids and caffeoylferuloylquinic acids), which strongly characterize the most antioxidant species *A. verlotiorum* and *A. annua*. Moreover, in this study we report for the first time the occurrence of sesquiterpenoids from the artemisinin biosynthesis pathway in the species *A. alba*.

Introduction

Bioprospection is defined as the systematic and organized exploration of natural resources and products derived from microorganisms, plants, fungi and animals, whose exploitation has the potential to generate significant economic and social benefits [1]. This approach has been applied for centuries by humans, who have always looked at plants as a crucial source of botanical drugs and other compounds for their well-being. Nowadays, about 25% of the drugs prescribed in modern medicine come from plants or are synthetic derivatives obtained from molecular plant precursors [2], while, on the other side, a great part of the population living in developing countries still relies on traditional herbal remedies for their primary healthcare (WHO reports). However, recent literature estimates that only 15% of the species that are used for the treatment of diseases (about 70,000) have been studied for their medical use [2], emphasizing the imperative for additional exploration. The discovery of new plant-derived drugs has classically relied on ethnobotanical information [3], as in the case of artemisinin discovery from the herbaceous plant *Artemisia annua* [4, 5]. Nonetheless, with the rise of *omics* sciences, new perspectives have emerged about the combination of ethnobotanical, phytochemical, and molecular phylogenetic data, and promising predictions of medicinal plant uses have been developed [3, 6]. For instance, the improvement of DNA sequencing techniques enabled the phylogenetic reconstruction of plant clusters that include species with a prominent medicinal use and demonstrated bioactivities, allowing the acceleration for the discovery of new potential bioactive compounds in related taxa [3].

The *Artemisia* genus (family Asteraceae) comprises about 500 species of herbs and shrubs that are either biannual, annual or perennial [7] and that are widely distributed in temperate regions of Europe, Asia, North Africa and North America. In many cultures, several species from this genus are characterized by a long-standing ethnobotanical use including preparation of herbal remedies for treatment of major and minor ailments (e.g. fever, hypertension, diabetes and malaria), preparation of herbal teas, alcoholic beverages, tonics and cosmetics or cultivation as crops [8, 9]. Many recent studies have unveiled the pleiotropic pharmacological profile of the genus reporting a broad spectrum of bioactivities reflecting the huge number and variety of phytochemicals found in each species. These include anthelmintic, antimalarial, antitubercular, antiviral, antihyperlipidemic, antiemetic, antidepressant, anticancer, anti-asthmatic, antihypertensive, antidiabetic, anxiolytic, hepatoprotective, gastroprotective, and insecticidal action [7, 10–12].

In this work we set up a bioprospection plan based on literature of local flora to investigate the phytochemical diversity and the potential bioactivity of five *Artemisia* species with recognized ethnobotanical tradition: *A. absinthium* L., *A. alba* Turra, *A. annua* L., *A. verlotiorum* Lamotte, *A. vulgaris* L. (Table 1). These species grow spontaneously in the natural areas within the province of Verona and within the Lessinia regional park, an area featured by hilly and mountainous environments, where the climate is classified as cold with no dry season and warm summer [13]. In this bioprospection work, we combined a sampling plan covering different growing seasons with an untargeted metabolomics approach to profile the specialized metabolomes of the five *Artemisia* species (including sesquiterpenoids from the artemisinin biosynthesis pathway) and correlate them with the *in vitro* antioxidant activity measured by FRAP and DPPH assays.

Table 1
Five selected *Artemisia* spp. used in this work with their ascribed medicinal properties

Species	Geographic distribution	Growing season	Medicinal use/bioactivities	References
<i>A. absinthium</i> (wormwood)	Europe, Middle East, North Africa, Asia; Italy: indigenous entity	perennial shrub	treatment of gastrointestinal problems, anorexia, and indigestion; antiparasitic effects, pain; antispasmodic, febrifuge, stomachic, cardiac stimulant and anthelmintic effects	[23, 57, 69, 70]
<i>A. alba</i> (white wormwood)	Europe south-east; Italy: indigenous entity	perennial shrub	Burns, contusion, digestive antimicrobial activity	[45, 64, 71]
<i>A. annua</i> (sweet wormwood)	Asia, Europe, North Africa and North America Italy: neophyte allochthonous invasive species (Galasso et al., 2018).	annual herb	antimalarial, anthelmintic, antipyretic, antiseptic, antispasmodics activities	[23, 56, 72, 73]
<i>A. verlotiorum</i> (Chinese mugwort)	Eastern Asia, South central Europe; Italy: neophyte allochthonous invasive species (Galasso et al., 2018).	perennial herb	treatment of hypertension; fever; psoriasis; circulatory, digestive, genito-urinary and respiratory disorders;	[74, 75]
<i>A. vulgaris</i> (common mugwort)	Asia, Europe, North America; Italy: indigenous entity	perennial herb	antioxidant, hypolipidemic, hepatoprotective, antispasmodic, analgesic, estrogenic, cytotoxic, antibacterial, antifungal, hypotensive, and broncholytic effects	[70, 76–78]

Results and Discussion

6.1 Metabolic profiles of *Artemisia* spp. methanolic extracts

In this work, the high sensitivity and wide analytical range of an LC-MS-based untargeted approach was used to profile nonvolatile medium-polar metabolites extracted from the aerial organs of five *Artemisia* species (*A. absinthium*, Aab; *A. alba*, Aal; *A. annua*, Aan; *A. verlotiorum*, Ave; *A. vulgaris*, Avu) collected throughout three sampling seasons (2019–2021) in the natural areas around Verona (Fig. 1).

Methanolic extracts of *Artemisia* spp. leaves and stems were produced and analyzed by untargeted UPLC-ESI-MS leading to the detection of 226 *m/z* features in negative ionization mode (Supplementary File 1) and to the identification of 80 secondary metabolites. The latter are listed in Table 2 together with their chromatographic and spectral features. Representative chromatographic profiles of leaf and stem methanolic extracts are shown in Fig. 2, together with pie charts depicting the metabolome composition according to the percentage of each metabolite class with respect to the total LC-MS signal detected. Moreover, to describe single compounds characterizing the various *Artemisia* species, a relative comparison of the percentages of all identified metabolites, grouped according to the respective metabolite class, is reported in the heatmap of Fig. 3.

Table 2

Secondary metabolites identified in *Artemisia* spp. samples by UPLC-ESI-MS[−] analysis. Peak IDs refer to peak numbers represented in Fig. 2. Rt, retention time. MS/MS diagnostic fragments detected; na, not available. Identification level was established according to metabolomics standards initiative (MSI) [79]: unambiguous identifications (level 1), comparison with reference standards analyzed under equal experimental conditions; putative assignments (level 2), MS data similarity with literature data or public databases; level 3 was established by spectral similarity to chemical class of compounds and chemotaxonomic data when no literature/database data are available for proposed structures, level 4 unidentified

	Rt (min)	m/z (-) detected	Putative identification	formula	ESI [−] detected ion	m/z (-) expected	mass error (ppm)	MS/MS	λ max (nm) UV- vis	MSI	Ref.
1	3.54	315.0716	Dihydroxybenzoic acid hexoside isomer	C ₁₃ H ₁₆ O ₉	[M-H] [−]	315.0710	1.766	108.022; 153.018	na	2	
2	3.92	315.0717	Dihydroxybenzoic acid hexoside isomer	C ₁₃ H ₁₆ O ₉	[M-H] [−]	315.0710	2.110	108.022; 153.018	na	2	
3	4.09	353.0872	Neochlorogenic acid (5-caffeoylquinic acid)	C ₁₆ H ₁₈ O ₉	[M-H] [−]	353.0873	-0.072	135.044; 179.035; 191.057	245, 326	1	
4	4.27	315.0719	Dihydroxybenzoic acid hexoside isomer	C ₁₃ H ₁₆ O ₉	[M-H] [−]	315.0710	2.935	108.022; 153.018	na	2	
5	4.31	285.0619	Dihydroxybenzoic acid pentoside	C ₁₂ H ₁₄ O ₈	[M-H] [−]	285.0610	-0.308	108.022; 153.018	na	2	
6	4.33	339.0715	Esculetin hexoside	C ₁₅ H ₁₆ O ₉	[M-H] [−]	339.0716	-0.290	177.0194	na	2	[15]
7	4.41	515.1400	Caffeoylquinic acid hexoside isomer	C ₂₂ H ₂₈ O ₁₄	[M-H] [−]	515.1401	-0.082	na	na	2	
8	4.50	369.0820	Coumarin-O-hexoside	C ₁₅ H ₁₆ O ₈	[M + FA-H] [−]	369.0822	-0.412	133.024; 162.025	246, 317	2	[80]
9	4.76	353.0875	Chlorogenic acid (3-caffeoylquinic acid)	C ₁₆ H ₁₈ O ₉	[M-H] [−]	353.0872	-0.073	135.044; 179.035; 191.057	242, 325	1	
10	4.86	399.0926	Methoxycoumarin-O-hexoside	C ₁₆ H ₁₈ O ₉	[M + FA-H] [−]	399.0927	-0.268	191.034; 176.010	247, 290, 336	2	
11	4.88	189.0767	Unidentified	C ₈ H ₁₄ O ₅	[M-H] [−]	189.0763	-	115.040; 99.081	na		
12	4.89	325.0923	<i>trans</i> -Melilotoside	C ₁₅ H ₁₈ O ₈	[M-H] [−]	325.0923	-0.126	163.039	275, 310	2	[19]
13	5.12	429.1033	Dimethoxycoumarin-O-hexoside	C ₁₇ H ₂₀ O ₁₀	[M + FA-H] [−]	429.1033	-0.061	221.046; 206.021	246, 293, 330	2	[81]
14	5.16	581.1511	Unidentified	C ₂₆ H ₃₀ O ₁₅	[M-H] [−]	581.1506	0.772	239.056; 209.046; 167.035; 269.067	na	4	
15	5.39	353.0876	Caffeoylquinic acid isomer	C ₁₆ H ₁₈ O ₉	[M-H] [−]	353.0872	-0.115	135.044; 179.035; 191.057	243, 319	2	
16	5.40	387.1656	Tuberonic acid hexoside	C ₁₈ H ₂₈ O ₉	[M-H] [−]	387.1655	0.258	207.102; 163.112	na	2	[18]
17	5.52	311.0771	Caffeoylpentoside	C ₁₄ H ₁₆ O ₈	[M-H] [−]	311.0766	-0.159	179.035	246, 323	2	
18	5.57	515.1184	Dicafeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] [−]	515.1189	0.095	135.044; 179.035; 191.057; 353.085		2	
19	5.81	367.1026	Feruloylquinic acid isomer	C ₁₇ H ₂₀ O ₉	[M-H] [−]	367.1029	-0.932	191.057; 193.051	246, 325	2	
20	5.91	325.0923	<i>cis</i> -Melilotoside	C ₁₅ H ₁₈ O ₈	[M-H] [−]	325.0923	-0.190	163.039	261	2	[19]

	Rt (min)	m/z (-) detected	Putative identification	formula	ESI ⁻ detected ion	m/z (-) expected	mass error (ppm)	MS/MS	λ max (nm) UV- vis	MSI	Ref.
21	6.26	367.1028	Feruloylquinic acid isomer	C ₁₇ H ₂₀ O ₉	[M-H] ⁻	367.1029	-0.159	191.057; 193.051	246, 325	2	
22	6.34	609.1455	Quercetin-3-O-rutinoside	C ₂₇ H ₃₀ O ₁₆	[M-H] ⁻	609.1450	0.790	300.029; 301.033	254, 348	1	
23	6.44	593.1512	Kaempherol-O-hexoside-deoxyhexoside	C ₂₇ H ₃₀ O ₁₅	[M-H] ⁻	593.1506	0.974	na	na	2	
24	6.57	463.0874	Quercetin-3-O-glucoside	C ₂₁ H ₂₀ O ₁₂	[M-H] ⁻	463.0876	-0.452	300.027; 271.024; 255.029; 301.035	254, 353	1	
25	6.61	677.1730	Dicaffeoylquinic acid hexoside	C ₃₁ H ₃₄ O ₁₇	[M-H] ⁻	677.1718	1.748	na	na	2	
26	6.64	493.0978	Mearnsetin-O-hexoside	C ₂₂ H ₂₂ O ₁₃	[M-H] ⁻	493.0982	-0.893	315.015; 316.022; 330.039; 331.043	253, 339	2	[82]
27	6.78	389.1082	Unidentified (hexoside)	C ₁₅ H ₂₀ O ₉	[M + FA-H] ⁻	389.1084	-0.637	343.104; 181.052	282, 340	4	
28	6.83	515.1187	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.044	135.044; 179.035; 191.057; 353.085	246, 326	2	
29	6.83	593.1512	Kaempherol-3-O-rutinoside	C ₂₇ H ₃₀ O ₁₅	[M-H] ⁻	593.1506	0.915	285.041; 284.032; 255.027; 227.034	364, 346	1	
30	6.93	623.1610	Isorhamnetin-O-hexoside-deoxyhexoside	C ₂₈ H ₃₂ O ₁₆	[M-H] ⁻	623.1612	-0.298	315.051	357	2	
31	7.00	577.1565	Apigenin-O-hexoside-deoxyhexoside	C ₂₇ H ₃₀ O ₁₄	[M-H] ⁻	577.1557	1.263	269.046	na	2	
32	7.00	653.1719	Eupatolitin-O-hexoside-O-deoxyhexoside	C ₂₉ H ₃₄ O ₁₇	[M-H] ⁻	653.1718	0.181	345.083	na	2	[15]
33	7.05	515.1180	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.175	135.044; 179.035; 191.057; 353.085	244, 325	2	
34	7.11	515.1188	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.029	135.044; 179.035; 191.057; 353.085	244, 325	2	
35	7.14	695.1242	Tricaffeoylhexaric acid isomer	C ₃₃ H ₂₈ O ₁₇	[M-H] ⁻	695.1248	0.092	533.083; 371.061	247, 325	2	
36	7.18	477.1028	Isorhamnetin-3-O-glucoside	C ₂₂ H ₂₂ O ₁₂	[M-H] ⁻	477.1033	-0.998	271.023; 243.029; 314.037; 299.018; 285.037; 215.031	na	1	
37	7.25	515.1180	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.175	135.044; 179.035; 191.057; 353.085	246, 326	2	
38	7.27	515.1190	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	-0.021	135.044; 179.035; 191.057; 353.085	243, 326	2	

	Rt (min)	m/z (-) detected	Putative identification	formula	ESI ⁻ detected ion	m/z (-) expected	mass error (ppm)	MS/MS	λ max (nm) UV- vis	MSI	Ref.
39	7.29	431.0976	Apigenin-7-O-glucoside	C ₂₁ H ₂₀ O ₁₀	[M-H] ⁻	431.0970	-0.142	268.035; 269.045	266, 332	1	
40	7.41	515.1188	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.013	135.044; 179.035; 191.057; 353.085	246, 326	2	
41	7.45	515.1187	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.048	135.044; 179.035; 191.057; 353.085		2	
42	7.47	693.2023	Ferulic acid derivative	-	[M-H] ⁻	-	-	193.052; 175.038; 160.016	246, 328	2	[83]
43	7.53	625.1197	Quercetin-O-caffeoylhexoside	C ₃₀ H ₂₆ O ₁₅	[M-H] ⁻	625.1193	0.568	300.029; 301.033; 463.086		2	
44	7.53	695.1261	Tricaffeoylhexaric acid isomer	C ₃₃ H ₂₈ O ₁₇	[M-H] ⁻	695.1248	-0.178	371.061; 209.031; 191.057; 191.020; 179.035; 161.022; 135.044	247, 325	2	
45	7.66	529.1340	Caffeoylferuloylquinic acid isomer	C ₂₆ H ₂₆ O ₁₂	[M-H] ⁻	529.1340	0.076	191.057	246, 326	2	
46	7.66	549.1975	Tracheloside isomer	C ₂₇ H ₃₄ O ₁₂	[M-H] ⁻	549.1972	0.538	387.1650; 161.025	247, 323	2	[15]
47	7.68	499.1235	Coumaroylcaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₁	[M-H] ⁻	499.1240	0.099	191.057; 179.034; 163.042	247, 323	2	
48	7.71	695.1271	Tricaffeoylhexaric acid isomer	C ₃₃ H ₂₈ O ₁₇	[M-H] ⁻	695.1248	-0.328	371.061; 209.031; 191.057; 191.020; 179.035; 161.023; 135.044	247, 325	2	
49	7.73	461.1084	Dihydroxy-methoxy-flavone-O-hexoside isomer	C ₂₂ H ₂₂ O ₁₁	[M-H] ⁻	461.1080	-0.094	na	na	2	
50	7.92	529.1343	Caffeoylferuloylquinic acid isomer	C ₂₆ H ₂₆ O ₁₂	[M-H] ⁻	529.1346	-0.551	191.057	246, 326	2	
51	8.02	529.1343	Caffeoylferuloylquinic acid isomer	C ₂₆ H ₂₆ O ₁₂	[M-H] ⁻	529.1346	-0.551	191.057	246, 326	2	
52	8.05	499.1238	Coumaroylcaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₁	[M-H] ⁻	499.1240	0.038	191.057; 179.035; 163.042	247, 323	2	
53	8.05	549.1972	Tracheloside isomer	C ₂₇ H ₃₄ O ₁₂	[M-H] ⁻	549.1972	-0.053	na	na	2	
54	8.09	529.1350	Caffeoylferuloylquinic acid isomer	C ₂₆ H ₂₆ O ₁₂	[M-H] ⁻	529.1340	1.951	191.057	246, 326	2	
55	8.09	515.1186	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	0.053	135.044; 179.035; 191.057; 353.085	246, 326	2	
56	8.23	461.1083	Dihydroxy-methoxy-flavone-O-hexoside isomer	C ₂₂ H ₂₂ O ₁₁	[M-H] ⁻	461.1080	-0.065	na	na	2	

	Rt (min)	m/z (-) detected	Putative identification	formula	ESI ⁻ detected ion	m/z (-) expected	mass error (ppm)	MS/MS	λ max (nm) UV- vis	MSI	Ref.
57	8.37	695.1267	Tricaffeoylhexaric acid isomer	C ₃₃ H ₂₈ O ₁₇	[M-H] ⁻	695.1248	-0.270	371.061; 209.031; 191.057; 191.020; 179.035; 161.022; 135.044	247, 325	2	
58	8.58	735.2127	Ferulic acid derivative isomer	-	[M-H] ⁻	-	-	175.040; 193.051; 160.016; 539.137	247, 329	2	
59	8.77	529.1344	Caffeoylferuloylquinic acid isomer	C ₂₆ H ₂₆ O ₁₂	[M-H] ⁻	529.1340	0.795	191.057	246, 326	2	
60	8.78	677.1507	Tricaffeoylquinic acid isomer	C ₃₄ H ₃₀ O ₁₅	[M-H] ⁻	677.1500	-0.103	191.057; 515.118	248, 329	2	
61	8.82	515.1186	Dicaffeoylquinic acid isomer	C ₂₅ H ₂₄ O ₁₂	[M-H] ⁻	515.1189	-0.671	135.044; 179.035; 191.057; 353.085	246, 326	2	
62	8.82	735.2133	Ferulic acid derivative isomer	-	[M-H] ⁻	-	-	175.040; 193.051; 160.016; 539.137	247, 329	3	
63	8.88	543.1500	Diferuloylquinic acid isomer	C ₂₇ H ₂₈ O ₁₂	[M-H] ⁻	543.1502	-0.477	191.057; 193.051	249, 327	2	
64	8.97	735.2124	Ferulic acid derivative isomer	-	[M-H] ⁻	-	-	175.040; 193.051; 160.016; 539.137	247, 329	3	
65	9.04	677.1509	Tricaffeoylquinic acid isomer	C ₃₄ H ₃₀ O ₁₅	[M-H] ⁻	677.1500	1.382	191.057; 515.118	na	2	
66	9.26	345.0612	Tetrahydroxydimethoxyflavone isomer	C ₁₇ H ₁₄ O ₈	[M-H] ⁻	345.0610	0.518	327.012; 315.015	250, 340	2	[15]
67	9.34	487.2530	Farnesane sesquiterpene acetylhexose isomer	C ₂₃ H ₃₈ O ₈	[M + FA-H] ⁻	487.2543	-2.66	441.248; 221.066; 161.045; 149.047; 131.032; 113.028; 101.026	na	4	
68	9.36	677.1530	Tricaffeoylquinic acid isomer	C ₃₄ H ₃₀ O ₁₅	[M-H] ⁻	677.1500	4.427	191.057; 515.118	na	2	
69	9.45	765.1673	Isobutyryl-tricaffeoylhexaric acid isomer	C ₃₇ H ₃₄ O ₁₈	[M-H] ⁻	765.1660	-0.17	279.071; 441.104; 603.137; 191.023		2	
70	9.70	765.1702	Isobutyryl-tricaffeoylhexaric acid isomer	C ₃₇ H ₃₄ O ₁₈	[M-H] ⁻	765.1660	-0.55	279.071; 441.104; 603.137; 191.023		2	[20]
71	9.76	487.2532	Farnesane sesquiterpene acetylhexose isomer	C ₂₃ H ₃₈ O ₈	[M + FA-H] ⁻	487.2543	-2.29	441.248; 221.066; 161.045; 149.047; 131.032; 113.028; 101.026	248, 341	4	

	Rt (min)	m/z (-) detected	Putative identification	formula	ESI ⁻ detected ion	m/z (-) expected	mass error (ppm)	MS/MS	λ max (nm) UV- vis	MSI	Ref.
72	9.92	765.1683	Isobutyryl-tricaffeoylhexaric acid isomer	C ₃₇ H ₃₄ O ₁₈	[M-H] ⁻	765.1660	-0.31	279.071; 441.104; 603.137; 191.023		2	[20]
73	10.05	779.1798	Acylated tricaffeoylhexaric acid isomer	C ₃₈ H ₃₆ O ₁₈	[M-H] ⁻	779.1820	0.28	293.087; 455.118; 617.148		3	[20]
74	10.10	345.0606	Tetrahydroxydimethoxyflavone isomer	C ₁₇ H ₁₄ O ₈	[M-H] ⁻	345.0610	-1.266	na	na	2	[15]
75	10.48	955.4918	Ginsenoside-like saponin	C ₄₈ H ₇₆ O ₁₉	[M-H] ⁻	955.4902	1.605	793.441; 731.434; 613.374; 569.381; 523.377	na	3	[84]
76	10.51	779.1781	Acylated tricaffeoylhexaric acid isomer	C ₃₈ H ₃₆ O ₁₈	[M-H] ⁻	779.1820	0.50	293.087; 455.118; 617.148		3	[20]
77	10.54	359.0765	Trihydroxytrimethoxyflavone isomer	C ₁₈ H ₁₅ O ₈	[M-H] ⁻	359.0767	-0.518	286.013; 344.053; 329.030; 314.007; 258.017; 230.022; 202.027	250, 350	2	[85]
78	11.10	793.4369	Calenduloside-like saponin	C ₄₂ H ₆₆ O ₁₄	[M-H] ⁻	793.4374	-0.702	631.381; 613.374; 569.386; 455.354	na	3	[86]
79	11.35	487.2536	Farnesane sesquiterpene acetylhexose isomer	C ₂₃ H ₃₈ O ₈	[M + FA-H] ⁻	487.2543	-1.458	441.248; 221.066; 161.045; 149.047; 131.032; 113.028; 101.026	na	4	
80	11.83	373.0923	Casticin	C ₁₉ H ₁₈ O ₈	[M-H] ⁻	373.0923	-0.202	358.071; 343.043; 285.003; 257.009; 229.015	254, 350	2	[15]
81	11.85	541.2799	Absinthin	C ₃₀ H ₄₀ O ₆	[M + FA-H] ⁻	541.2801	-0.404	495.272; 451.284; 433.274; 337.804; 293.820	251, 353	1	
82	12.66	265.1443	Unidentified	C ₁₅ H ₂₂ O ₄	[M-H] ⁻	265.1440	1.146	233.115; 189.126; 177.093	na	4	
83	12.92	571.2543	Unidentified	-	-	-	-	na	na		
84	13.76	539.2639	Putative guaiane-type sesquiterpenoid dimer	C ₃₀ H ₃₈ O ₆	[M + FA-H] ⁻	539.2645	-1.113	493.269; 471.232; 293.105;	251, 353	4	[17]
85	14.86	507.2379	Putative guaiane-type sesquiterpenoid dimer	C ₃₀ H ₃₆ O ₇	[M-H] ⁻	507.2383	-0.79	463.246; 445.243; 259.099; 215.108	na	4	[16]

The main specialized metabolites composing the metabolomes of *Artemisia* spp. comprise derivatives of hydroxycinnamic acid (HCA), mainly caffeic and ferulic acid differentially esterified with one or multiple organic acid moieties, O-glycosylated flavonoids (mainly flavonols and flavones together with their O-methylated derivatives), glycosylated coumarins and a few other metabolites identified as glycosides of hydroxybenzoic acids,

lignans, saponins and sesquiterpenes. In general, a high level of metabolome characterization was achieved, resulting in a metabolite annotation range of 85–97% of total LC-MS signal, with *A. annua* and *A. absinthium* being the species with the higher proportion of unidentified metabolites left. The HCA derivatives represented the most abundant metabolite class, ranging from 62 to 94% of total LC-MS signal in *A. absinthium* and *A. verlotiorum*, respectively. As can be observed in the chromatograms of Fig. 2, in all five species this class was dominated by the caffeic acid esters of quinic acid, in particular two caffeoylquinic acid isomers (compounds **9**, **15**) and many isomers of dicaffeoylquinic acid (**28**, **33**, **34**, **37**, **38**, **40**, **41**). The prevalence of caffeic acid derivatives in the *Artemisia* metabolome is well pronounced in *A. absinthium*, *A. verlotiorum* and *A. vulgaris*, in which they represent the main peaks, whereas a more diversified profile is observed in favor of other compound classes in *A. alba* and *A. annua* (e.g., other HCA derivatives, coumarins and flavonoids).

A detailed description of the metabolic profiles of leaves and stems of the five *Artemisia* species is reported below according to data presented in Figs. 2 and 3.

A. absinthium (Fig. 2A) leaves are mainly characterized by the presence of two isomers (**9**, **15**) of caffeoylquinic acid, one of them identified as 3-O-caffeoylquinic acid (chlorogenic acid; **15**) and of two abundant isomers of dicaffeoylquinic acid (**34**, **38**); also caffeoylquinic acid hexoside was best represented in this species (**7**). The leaves of *A. absinthium*, compared to the other species, present higher proportion of the flavonols kaempferol-O-hexoside-deoxyhexoside (**23**), isorhamnetin-O-hexoside-deoxyhexoside (**30**), eupatolitin-O-deoxyhexoside-O-hexoside (**32**) and isorhamnetin-3-O-glucoside (**36**). Moreover, in leaves and, at a lower level, in stems of this species only, we detected high amounts of the dimeric guaianolide absinthin (**81**), which is described as a specific marker of *A. absinthium* and is responsible for the bitterness of absinth [7, 14, 15]. In addition, two other compounds annotated as guaiane-type sesquiterpene dimers (**84**, **85**) were putatively identified according to recent literature data and *in silico* fragmentation analysis [16, 17]. The stems of *A. absinthium* showed a much more simplified profile, including the same caffeoylquinic acid isomers found in the leaves together with two less represented dicaffeoylquinic acid isomers (**40**, **55**). Furthermore, in both leaves and stems we observed two unidentified metabolites with a molecular ion of 189.07 *m/z* (**11**) and 571.25 *m/z* (**83**) characterized, respectively, by higher and lower polarity.

Leaves and stems of *A. alba* (Fig. 2B) were strongly characterized by the glycosides of different coumarins including esculetin hexoside (**6**), coumarin-O-hexoside (**8**), methoxycoumarin-O-hexoside (**10**) and dimethoxycoumarin-O-hexoside (**13**). These organs present major peaks attributed to caffeoylquinic acids (**9**, **15**) and various isomers of dicaffeoylquinic acid (**28**, **34**, **37**, **38**, **40**). Less represented hydroxycinnamic acid derivatives include dicaffeoylquinic acid hexoside (**25**), caffeoylpentoside (**17**) and three isomers of tricaffeoylquinic acid (**60**, **65**, **68**), which were mostly abundant in the leaves. Moreover, this species is strongly characterized by the presence of four ferulic acid derivatives, one with a molecular ion of 693.20 *m/z* (**42**) and three isomers showing a molecular ion of 735.21 *m/z* (**58**, **62**, **64**); the resulting neutral loss of 42.01 Da and the higher retention time strongly suggest that the latter could be the acetylated forms of compound **42**. With respect to stems, *A. alba* leaves presented higher levels of one isomer of a dihydroxybenzoic acid hexoside (**4**) and various flavonoids glycosides including quercetin-3-O-rutinoside (**22**), kaempferol-O-hexoside-deoxyhexoside (**23**), kaempferol-3-O-rutinoside (**29**), isorhamnetin-O-hexoside-deoxyhexoside (**30**) and apigenin-O-hexoside-deoxyhexoside (**31**). Interestingly, according to literature [18], we detected high levels in the leaves of *A. alba* of tuberonic acid hexoside (**16**), which belong to the class of jasmonate glycosylated derivatives, i.e. phospholipid-derived hormones that regulate plant development and responses to environmental stress.

A. annua (Fig. 2C) was the species presenting the most diversified profile of its secondary metabolome, being characterized by many major compounds belonging to different metabolite classes. In general, the highest diversification was observed within the class of hydroxycinnamic acid derivatives and their esters. In particular, two isomers (**12**, **20**) of *o*-coumaric acid glucoside (*trans*- and *cis*-melilotoside), previously reported in *A. annua* tea infusions [19], were detected at high levels in the leaves together with other hydroxycinnamate esters that include feruloyl moieties, such as two feruloylquinic acid isomers (**19**, **21**), one diferuloylquinic acid isomer (**63**) and five caffeoylferuloylquinic acid isomers, one characterizing mostly the leaves (**50**) and the other ones the stems (**45**, **51**, **54**, **59**). Also, caffeoyl- (**9**, **15**), dicaffeoyl- (**33**, **34**, **37**, **38**, **40**, **61**) and tricaffeoyl- (**60**, **65**, **68**) quinic acids were well represented in both leaves and stems within this species. Among the coumarins, the stems reported the highest levels of methoxycoumarin-O-hexoside (**10**). Leaves, on the other hand, were characterized by high amounts of flavonoids, especially methoxylated and often in aglycone form, such as casticin (**80**), which is described as a marker of *A. annua* [15] and a trihydroxytrimethoxyflavone isomer (**77**). The latter, together with the methoxylated flavonol mearnsetin-O-hexoside (**26**), represented the major flavonoid peaks of the *A. annua* leaf chromatograms. Other characteristic flavonoids included two isomers of a tetrahydroxydimethoxyflavone (**66**, **74**), quercetin-3-O-glucoside (**24**) and apigenin-7-O-glucoside (**39**).

Finally, three unidentified metabolites (**14**, **27**, **82**) were detected at high levels in *A. annua*. In particular, compound **27** forms a molecular adduct with formic acid at 389.11 *m/z* under negative ionization conditions; its fragmentation results in the molecular ion at 343.10 *m/z* and in the fragment 181.05 *m/z*, which is indicative of the neutral loss of a hexose (-162.05 Da). Moreover, the UV-vis absorbance at 282 and 340 nm together with the fact that it is detected as formic acid adduct, supports the hypothesis that this metabolite could belong to the class of coumarins.

A. verlotiorum (Fig. 2D) and *A. vulgaris* (Fig. 2E) presented much simpler profiles respect to the other species.

A. verlotiorum was distinct from all the other species for the higher content of two lignans (tracheloside isomers; **46**, **53**) and three compounds that were putatively annotated as farnesane sesquiterpene acetyl-hexose isomers according to their fragmentation pattern (**67**, **71**, **79**). Another peculiarity of this species was the high relative levels of various tricaffeoylhexaric acid isomers (**35**, **44**, **48**, **57**), including those acylated to isobutyric acid moieties commonly found in other Asteraceae species (**69**, **70**, **72**); [20] and other tricaffeoyl acid esters (**73**, **76**) that were particularly high in the stems. The leaves and stems were also characterized by many different isomers of dicaffeoyl (**18**, **28**, **33**, **34**, **37**, **38**, **40**, **41**, **55**) and tricaffeoyl (**60**, **65**, **68**) quinic acid, by dicaffeoylquinic acid hexoside (**25**) and coumaroylcaffeoylquinic acid isomers (**47**, **52**), while neochlorogenic acid (**3**) levels were higher in the leaves of this species. Other characteristic metabolites of *A. verlotiorum* leaves were represented by hexose (**1**) and pentose (**5**) esters of a dihydroxybenzoic acid, by quercetin-O-caffeoylhexoside (**43**) and by four flavone glycosides, which included apigenin-O-hexoside-deoxyhexoside (**31**), apigenin-7-O-glucoside (**39**) and two dihydroxymethoxyflavone-O-hexoside isomers (**49**, **56**), the latter best represented in the stems.

A. vulgaris, within the five species, contained lower levels of flavonoids but its stems were characterized by the presence of ginsenoside-like (**75**) and calenduloside-like (**78**) saponins. Moreover, leaves were characterized by three dicaffeoylquinic acid isomers (**34**, **37**, **38**), which were much more represented in this species respect to other ones, yet at lower amounts. In proportion, also neochlorogenic acid (**3**), caffeoyl pentoside (**17**), two dihydroxybenzoic acid hexosides (**2**, **4**) and one sesquiterpene diol monohexoside derivative (**79**) characterize *A. vulgaris* aerial organs.

The HCA derivatives, in particular the esters of caffeic acid, were the most characterizing compounds in all the five *Artemisia* species considered in this work. All these metabolites showed accumulation patterns similar to those already reported in literature for several *Artemisia* species by using similar extraction methods and identified with different techniques from LC-MS such as NMR and HPLC-DAD [21–23]. The degree and the nature of esterification determines the type of the specific ester isomer that is produced and, for several HCA derivatives, we observed species-specific esterification patterns. This indicates a diversification in the late enzyme of the pathway, among *Artemisia* spp., such those involving the esterases [24]. It is interesting to notice how distribution of isomers of different esters of caffeic acid changes also between different organs (stems or leaves) of each species. For instance, various tricaffeoylhexaric acid isomers, a molecule that has been already detected in various Asteraceae [25, 26], were found at high levels in *A. verlotiorum* stems, while tricaffeoylquinic acids were located mostly in the leaves. This is probably due to a different spatial distribution in the enzyme involved in the esterification of HCA biosynthesis between the different organs in each species.

As already reported by the literature, flavonoids are another widely represented class of metabolites in the *Artemisia* genus [10, 22, 27]. Ubiquitous presence of different glycosylated and methoxy-glycosylated flavonoids among all the five species investigated has been observed. Interestingly, in *A. annua*, and to a lesser extent also in *A. absinthium* and *A. alba*, aglycone form of different flavonoids, including the methoxylated flavonol casticin, were observed. Within plant cells, most flavonoids are present as O- or C-glycosides [28, 29], while the aglycones are more typical of extracellular exudates [30]. The presence of different flavonoid aglycones has been reported in plant exudates of different *Artemisia* species [31, 32]. For instance, in *A. absinthium*, *A. alba* and *A. vulgaris*, different polymethoxylated flavonoid aglycones were found on the surface of aerial parts, in the extracellular environment, predominantly in leaves and floral buddings, where they probably have protective roles and/or allelopathic functions [12, 33]. The occurrence of these compounds is probably related to the presence on the leaf surface of epidermal trichomes, which can synthesize and store large quantities of specialized metabolites [34]. Trichomes are epidermis appendages and can be divided into glandular trichomes (GTs) and non-glandular (NGTs) according to their morphology [35]. In particular, glandular trichomes can synthesize, store, and secrete large amounts of exudates, including alkaloids, polysaccharides, terpenoids, polyphenols, organic acids, and defensive proteins. In turn, these exudates can entrap or poison herbivores and prevent pathogen infection [36, 37]. Usually Asteraceae, harbors mainly GTs, where high-value secondary metabolites, including artemisinin in *A. annua*, are produced and then stored, ready to be used in plant defensive mechanism against both biotic and abiotic stress [35, 38]. Presence of glandular trichomes, has been reported for all the species investigated in this work, and can explain the observation of the above mentioned flavonoid aglycones in our samples [39, 40].

Considering the three different growing seasons, the strongest differences were observed for *A. annua* and *A. verlotiorum*. In *A. annua*, the relative levels of many flavonoids and hydroxycinnamic acid derivatives during 2019 was lower than 2020 and 2021. In *A. verlotiorum*, some flavonoids and various hydroxycinnamic acid derivatives showed higher relative level in 2021 compared with 2019 and 2020. This variation could be expected for herbaceous annual species [23], such as *A. annua*, if we consider that the sampling of potentially distinct individuals over three years could have increased the genotypic variability of the samples. It is not excluded that some *Artemisia* species are more sensitive to environmental conditions than others and modulate the levels of single or groups of metabolites in response to different stimuli. However, the type of experimental design that we adopted in this work does not allow us to precisely dissect the effects of specific climate or geographic conditions and, thus, is not suitable to investigate such complex environment-metabolome interactions.

6.2 Antioxidant assays of *Artemisia* spp. methanolic extracts

In this work we performed *in vitro* antioxidant assays as low cost and easy to use high-throughput screening systems for the identification of potential sources of antioxidants [41, 42]. These should then be followed by confirmatory *in vivo* biological tests with simulated digestion samples [43] to assess the antioxidant activity in a more physiological context. It is commonly accepted that antioxidant activity must not be tested on the basis of a single method [44] given the involvement of different antioxidant mechanisms by the molecules present in a phytocomplex. Thus, we

used FRAP and DPPH to assess the reducing capacity and radical scavenging activity, respectively, of leaf and stem methanolic extracts of the five *Artemisia* species.

In general, extracts from leaves showed higher antioxidant capacity compared with extracts from stems in both assays (Fig. 4).

Within the five species, *A. absinthium* showed the lowest antioxidant activity (up to 30.0 and 24.8 mmolTE/Kg fr. wt in FRAP and DPPH), while *A. verlotiorum* showed the highest antioxidant power for both FRAP (121.2 mmolTE/Kg fr. wt) and DPPH (up 88.7 mmolTE/Kg fr.wt), about four times higher than *A. absinthium*. In between these two species, *A. annua*, *A. alba* and *A. vulgaris* displayed a medium to high antioxidant activity (see Supplementary Table 1).

Many studies report the antioxidant activity of different *Artemisia* spp. extracts assayed with a broad panel of methods but a comparison with the results presented in this work is challenged by non-homogeneous expression of data (e.g. TEAC, IC₅₀, percentage of radical scavenging, etc.) or the use of reference compounds other than Trolox. A few recent studies report the antioxidant activity of methanolic extracts of various *Artemisia* species in comparison to Trolox[15, 45, 46] (Supplementary Table 2). Our results are in line with the trend observed by Trifan and colleagues for FRAP assay, in which *A. absinthium* displayed the lowest antioxidant activity. On the other hand, we did not observe higher antioxidant activity for *A. vulgaris*, as reported by the authors. In general, the TEAC values reported in all these studies for the five *Artemisia* species are five to ten times higher than our results, but this is justified by the fact that dried instead of fresh plant material was used to produce the extracts, thus resulting in higher concentrations of antioxidant compounds.

In Fig. 5 the antioxidant activity in each of the three years of sampling is shown (see also Supplementary Table 1). In some cases, a clear impact of the specific growing season on antioxidant activity was observed. For example, *A. verlotiorum* extracts showed higher antioxidant activities in 2021 than 2019 and 2020 in both FRAP (Fig. 5G) and DPPH (Fig. 5H), while *A. annua* and *A. vulgaris* showed lower antioxidant activities in 2019 compared with 2020 and 2021 (Fig. 5E, F, I, L). On the other hand, the antioxidant activity of the leaves of *A. absinthium* (Fig. 5A, B) and *A. alba* (Fig. 5C, D), did not vary significantly throughout the three years. According to these data, the antioxidant activity of species like *A. absinthium* and *A. alba* seems to be less influenced by the growing season as it occurs in the case of *A. annua*, *A. vulgaris* and *A. verlotiorum*.

6.3 Correlation analysis of antioxidant data and metabolic profiles of *Artemisia* spp.

In order to obtain information on which metabolites may be responsible for antioxidant activity of *Artemisia* spp. methanolic extracts, a statistical tool of multivariate analysis (OPLS) was used to find linear relations between the metabolite levels (whose *m/z* features were assigned as X variables) and the antioxidant capacity (whose mmolTE/Kg were assigned as Y variables). The score scatter plots of Fig. 6 show a good, yet not too strong, linear correlation between the metabolite levels (t, x axis) and the antioxidant activity (u, y axis), for both FRAP and DPPH ($0.86 < R^2 < 0.90$), thus recalling the need for an independent OPLS analysis in each different species; this is expected, since different set of metabolites could be responsible for the overall antioxidant activity of each species. In this analysis, samples that displayed the highest mmol TE/Kg of fresh plant material clustered on the top right corner of the graph and those with the lower values in the left-down corner.

The same analysis was applied to each of the individual species. The OPLS individual models for leaves and stem extracts of each of the species are shown in Fig. 7. The *loadings* of these OPLS analyses can be used to evaluate the contribution of each *m/z* feature, *i.e.* of each detected metabolite, to the observed antioxidant activity (Tables 3, 4).

Table 3
Lists of metabolites that correlate with FRAP activity in *Artemisia* spp. samples. Only metabolites with $pq(\text{corr}) > 0.85$ are reported.

species	ID	FRAP- correlating metabolites	pq(corr)
Aab	15	Caffeoylquinic acid isomer	0.96
	9	Chlorogenic acid	0.95
	81	Absinthin	0.94
	29	Kaempferol-3-O-rutinoside	0.93
	80	Casticin	0.91
	77	Trihydroxytrimethoxyflavone	0.89
	23	Kaempferol-O-hexoside-deoxyhexoside	0.89
	85	Putative guaiane-type sesquiterpenoid dimer	0.88
	30	Isorhamnetin-O-hexoside-deoxyhexoside	0.87
	84	Putative guaiane-type sesquiterpenoid dimer	0.87
Aal	22	Quercetin-3-O-rutinoside	0.91
	23	Kaempferol-O-hexoside-deoxyhexoside	0.88
	29	Kaempferol-3-O-rutinoside	0.87
Aan	37	Dicaffeoylquinic acid isomer	0.99
	51	Caffeoylferuloylquinic acid isomer	0.98
	34	Dicaffeoylquinic acid isomer	0.98
	21	Feruloylquinic acid isomer	0.96
	15	Caffeoylquinic acid isomer	0.95
	19	Feruloylquinic acid isomer	0.95
	40	Dicaffeoylquinic acid isomer	0.94
	9	Chlorogenic acid	0.94
	65	Tricaffeoylquinic acid isomer	0.91
	38	Dicaffeoylquinic acid isomer	0.91
	50	Caffeoylferuloylquinic acid isomer	0.91
	60	Tricaffeoylquinic acid isomer	0.89
	63	Diferuloylquinic acid isomer	0.88
Ave	34	Dicaffeoylquinic acid isomer	1.00
	37	Dicaffeoylquinic acid isomer	0.99
	40	Dicaffeoylquinic acid isomer	0.97
	55	Dicaffeoylquinic acid isomer	0.94
	3	Neochlorogenic acid	0.91
	33	Dicaffeoylquinic acid isomer	0.91
	5	Dihydroxybenzoic acid pentoside	0.88
	39	Apigenin-7-O-glucoside	0.88
	38	Dicaffeoylquinic acid isomer	0.88
	26	Mearnsetin-O-hexoside	0.86
	21	Feruloylquinic acid isomer	0.86
	23	Kaempferol-O-hexoside-deoxyhexoside	0.86

species	ID	FRAP- correlating metabolites	pq(corr)
	43	Quercetin-O-caffeoylhexoside	0.86
Avu	38	Dicaffeoylquinic acid isomer	1.00
	37	Dicaffeoylquinic acid isomer	1.00
	34	Dicaffeoylquinic acid isomer	0.99
	33	Dicaffeoylquinic acid isomer	0.96

Table 4
Lists of metabolites that correlate with DPPH activity in *Artemisia* spp. samples. Only metabolites with $pq(\text{corr}) > 0.85$ are reported.

species	ID	DPPH – correlating metabolites	pq(corr)
Aab	15	Caffeoylquinic acid isomer	0.85
	9	Chlorogenic acid	0.85
Aal	38	Dicaffeoylquinic acid isomer	0.90
	37	Dicaffeoylquinic acid isomer	0.90
	34	Dicaffeoylquinic acid isomer	0.89
	9	Chlorogenic acid	0.86
Aan	34	Dicaffeoylquinic acid isomer	0.98
	40	Dicaffeoylquinic acid isomer	0.98
	21	Feruloylquinic acid isomer	0.97
	19	Feruloylquinic acid isomer	0.97
	50	Caffeoylferuloylquinic acid isomer	0.96
	37	Dicaffeoylquinic acid isomer	0.96
	9	Chlorogenic acid	0.96
	51	Caffeoylferuloylquinic acid isomer	0.96
	15	Caffeoylquinic acid isomer	0.96
	33	Dicaffeoylquinic acid isomer	0.94
	65	Tricaffeoylquinic acid isomer	0.94
	63	Diferuloylquinic acid isomer	0.93
	26	Mearnsetin-O-hexoside	0.92
	24	Quercetin-3-O-glucoside	0.90
	36	Isorhamnetin-3-O-Glucoside	0.88
	61	Dicaffeoylquinic acid isomer	0.87
	59	Caffeoylferuloylquinic acid isomer	0.86
Ave	34	Dicaffeoylquinic acid isomer	0.99
	37	Dicaffeoylquinic acid isomer	0.98
	40	Dicaffeoylquinic acid isomer	0.97
	33	Dicaffeoylquinic acid isomer	0.92
	55	Dicaffeoylquinic acid isomer	0.92
	31	Apigenin-O-hexoside-deoxyhexoside	0.90
	3	Neochlorogenic acid	0.89
	19	Feruloylquinic acid isomer	0.88
	26	Mearnsetin-O-hexoside	0.86
	38	Dicaffeoylquinic acid isomer	0.86
Avu	38	Dicaffeoylquinic acid isomer	0.86
	37	Dicaffeoylquinic acid isomer	0.86

Consistently to what observed in the previous paragraphs, the antioxidant activity of leaf extracts is in general higher than those of stem extracts (Fig. 7). This comparative analysis between the species, showed that the higher antioxidant activity of *A. verlotiorum*, described in the previous paragraph, may be mainly due to caffeic and ferulic acid derivatives and flavonoids; also, various unidentified metabolite showed high correlation

with antioxidant activity (data not shown). In *A. vulgaris* the antioxidant activity correlated with caffeic acid derivatives, in *A. annua*, with coumaric, caffeic and ferulic acid derivatives. In *A. alba*, which showed the lowest linear correlation between metabolome composition and antioxidant activity (Fig. 7), also flavonoids were found to strongly correlate with antioxidant activity in FRAP assay, while the scavenging activity measured by DPPH was mostly correlated with dicaffeoylquinic acid isomers. Interestingly, coumarins accumulated at high levels in *A. alba* but did not strongly correlate with the antioxidant activity. Finally, in *A. absinthium* the antioxidant activity strongly correlated with caffeoylquinic acids, absinthin and flavonoids.

The hydroxycinnamates esterified with quinic acid, in particular some isomers of dicaffeoylquinic acid, were found to be metabolites with the strongest correlation with antioxidant activity in all species. This class of molecules has been extensively studied in the past years for their potential use in medicine. Caffeoylquinic acid derivatives are natural compounds isolated from a variety of traditional medicinal plants and possess a wide range of pharmacological properties, including antioxidant, hepatoprotective, antibacterial, antihistaminic and other biological effects [47]. Currently, in literature, caffeoyl and dicaffeoylquinic acids have been widely tested through *in vitro* and *in vivo* assays to evaluate their bioactive properties. Two caffeoylquinic acids extracted from *Aronia melanocarpa* berries, *i.e.* 3-caffeoylquinic acid and 4-caffeoylquinic acid, were identified as inhibitor of the dipeptidyl peptidase IV, an enzyme involved in the development of type 2 diabetes mellitus [48]. The protective effect of chlorogenic acid against neurotoxic effect of arsenic poisoning was demonstrated in mice model [49]. Potential benefits with therapeutic applications were reported also for dicaffeoylquinic acids. For example, Kim and collaborators demonstrated the neuroprotective effect of 3,5-dicaffeoylquinic acid and 3,4-dicaffeoylquinic acid from *Dipsacus asper* on hydrogen peroxide-induced cell death in SH-SY5Y human cells [50]. In another study it is reported that 1,5-dicaffeoylquinic acid (cynarin) downregulates the expression of inducible nitric oxide synthase, expressed under conditions of inflammation, sepsis, or oxidative stress, in human coronary smooth muscle cells [51]. In addition, the dicaffeoyl quinic acid cynarin affects the survival, growth, and stress response of normal, immortalized, and cancerous human cells [52]. Protective effects of cynarin against hepatotoxicity effects of cyclophosphamide, an important anticancer drug which belongs to the class of alkylating agents, as well as its antihypertensive and vasodilator effects have been observed in *in vivo* studies [53, 54]. *Artemisia* spp., which were able to accumulate various isomers of caffeoyl- and dicaffeoylquinic acid, could thus represent a valuable source of these potentially bioactive compounds. A further characterization to reveal the precise identity of the various isomers accumulated by the five species is thus required.

6.4 Looking for a new artemisinin source

The sesquiterpene lactone artemisinin and its semi-synthetic derivatives are very important from a pharmaceutical perspective for their anti-malarial properties. Isolated from *A. annua* plants, artemisinin earned in short time the status of most potent antimalarial drug and recently new evidence of many other bioactivities (e.g, anticancer, anti-inflammatory and antiviral) have emerged [55]. For this reason, a great interest arose in the search for artemisinin-rich *A. annua* ecotypes and towards the manipulation of its biosynthetic pathway through different biotechnological tools [56]. Moreover, since antimalarial activity was reported for different *Artemisia* species [57], many studies have been conducted to find alternative natural sources for artemisinin within the *Artemisia* genus. Despite artemisinin was demonstrated to occur in different amounts in *A. dubia* [58], *A. scoparia* [59], *A. cina* [60], *A. vachanica* and *A. dracunculus* [61], *A. verlotiorum* and *A. vulgaris* [62], the major source of this metabolite still remains *A. annua* [63].

In this work we explored the capacity of the *Artemisia* plants collected in the province of Verona to produce the antimalaria lead drug artemisinin and related compounds from its biosynthesis pathway. We therefore performed an LC-MS analysis in positive ionization mode, which is more suitable for the ionization of sesquiterpenoid molecules, and we searched for the final products of the pathway (artemisinin and arteannuin B) and their immediate precursors (dihydroartemisinic acid and artemisinic acid, respectively). Their identification was made through the comparison of *m/z* values, fragmentation patterns and retention times with those of the respective reference standards (Table 5). The relative comparison of their levels within the leaves of the five *Artemisia* species is reported in Fig. 8.

Table 5
Metabolites from the artemisinin pathway with their MS features searched in LC-MS ESI⁺ analysis.

Metabolite	Formula	Rt (min)	Neutral mass (Da)	[M + H] ⁺	[M + Na] ⁺	MS/MS
Arteannuin B	C ₁₅ H ₂₀ O ₃	12.14	248.141	249.14	271.131	231.138 [M + H-H ₂ O]; 185.134 [M + H-H ₂ O-2CH ₃ -O]
Artemisinin	C ₁₅ H ₂₂ O ₅	12.98	282.147	283.154	305.136	247.133 [M + H-2H ₂ O]; 265.144 [M + H-H ₂ O]
Artemisinic acid	C ₁₅ H ₂₂ O ₂	14.93	234.162	235.169		217.159 [M + H-H ₂ O]; 199.149 [M + H-2H ₂ O]
Dihydroartemisinic acid	C ₁₅ H ₂₄ O ₂	14.75	236.178	237.185		219.175 [M + H-H ₂ O]

A. annua is the only species reporting detectable levels of artemisinin. Interestingly, the precursor of artemisinin, dihydroartemisinic acid, is present not only in *A. annua* but also in *A. alba* and *A. verlotiorum*. Arteannuin B, the final metabolite of a parallel pathway that originates from artemisinic aldehyde, was detected at high levels in *A. annua* and at considerably lower levels in *A. absinthium* and *A. alba* but was absent in *A. verlotiorum*. The precursor of both arteannuin B and artemisinin, artemisinic acid, is present in *A. annua*, as expected, but we did not detect it in *A. absinthium* nor *A. alba*, despite the fact that arteannuin B has been detected in both species. *A. vulgaris* does not produce any of the metabolites from the selected pathway.

According to the literature, the presence of artemisinin has been recently reported in *A. verlotiorum* [62], *A. absinthium* [57] and *A. vulgaris* [62]. On the other hand, we reported for the first time the presence of artemisinin intermediates in *A. alba*, thus furtherly increasing the number of sesquiterpene compounds (e.g. germacrane and eudesmane) that were previously reported in this species [64]. Although we did not detect traces of artemisinin, the presence of the precursor, dihydroartemisinic acid, and side product of the pathway, arteannuin B, indicates that genes of artemisinin biosynthetic pathway are all expressed in these plants, since the last step of artemisinin biosynthesis is a non-enzymatic photooxidative process [65]. One possible explanation for the absence of artemisinin could be the sampling time. It has been reported that peak in artemisinin content in *A. annua* and *A. vulgaris* corresponds to the budding stage, while for *A. absinthium* maximum accumulation is reached during the flowering stage [66–68]. All the plants used in this work were sampled during their vegetative stage. This could have prevented the artemisinin detection in species with lower levels of this metabolite. However, results obtained in this work strengthen the hypothesis that the artemisinin biosynthetic pathway may be an ancestral characteristic shared within plants belonging to *Artemisia* [57].

Experimental section

7.1 Plant material

The five selected *Artemisia* species were sampled independently throughout three growing seasons in June of the years 2019, 2020, 2021 from three hills and mountain areas in the province of Verona. The formal identification of plant material was performed by Sebastiano Andreatta (from the “Museo di Storia Naturale” of Verona), the plants were given the barcode number and a voucher specimen of each species is available at the publicly available herbarium present in the “Museo di Storia Naturale” of Verona (Supplementary Table 3). Sampling complied with relevant institutional, national and international guidelines and legislations.

In each sampling site, plants were collected from three distinct spots (*i.e.* three different plant populations representing three biological replicates), far enough to avoid the sampling of plant populations deriving from the same genetic source. For each replicate, leaves and stems were collected from at least 5 individuals and pooled together according to the organ. The samples were immediately frozen in dry ice and then stored at -80°C. The frozen plant material was homogenized in liquid nitrogen using an IKA A11 basic mill (IKA, Germany).

7.2 Chemicals and reagents

Reference standard of artemisinin was purchased from Sigma-Aldrich (St. Louis, USA). Reference standards of arteannuin B and artemisinic acid were purchased from Biosynth® Carbosynth (Bratislava, Slovakia). Reference standard of dihydroartemisinic acid was purchased from Toronto Reasearch Chemical (Toronto, Canada). Methanol, acetonitrile and water (all LC-MS grade) were purchased from Honeywell (Charlotte, USA). Formic acid (LC-MS grade) was purchased from Biosolve Chimie (Dieuze, France). Trolox and DPPH were purchased, respectively, from Sigma-Aldrich and Thermo Fisher Scientific.

7.3 Metabolites extraction

100 mg of frozen powder were extracted in 1.5 ml methanol, mixed vigorously for 30 sec, sonicated at 40 KHz in an ultrasonic bath Sonica® Ultrasonic Cleaner (SOLTEC, Milan, Italy) at 4°C for 15 min and centrifuged (16000 *g*, 15 min, 4°C). The supernatants were opportunely diluted in 100% methanol. Just before the analysis, the samples were diluted 1:2 (V:V) with ultrapure water (Honeywell, USA). The final mixtures were passed through Minisart RC4 filters (0.2 µm pores) (Sartorius, Göttingen, Germany) and 1 µl was injected into the UPLC device.

7.4 UPLC-ESI-MS analysis

An Acquity I Class UPLC system (Waters, Milford, USA) with a BEH C18 column (Waters), coupled online with a PDA (photo-diode array) and to a Xevo G2-XS qTOF mass spectrometer (Waters), equipped with an electrospray ionization (ESI) source were used. The extracts were injected through a cooled autosampler (8°C) and a flow rate of 0.350 ml/min was used. The mobile phases were 0.1% formic acid in water (solvent A) and acetonitrile (solvent B), and the elution gradient was as follows: 0-1min, 1% B; 1–10 min, 1 to 40% B; 10-13.50 min, 40 to 70% B; 13.50–15.00 min, 70 to 90% B; 15.00-16.50 min, 90 to 100% B; 16.50–20 min 100% B; 20-20.1, 100 to 1% B; 20.1–25 min, 1% B (initial conditions).

The sample analysis sequence was randomized. A quality control (QC) prepared by mixing equal part of all the extracts was analyzed along the whole experiment every ten sample analysis. The ion source parameters were the following: capillary voltage 0.8 Kv, sampling cone voltage 40 V, source offset voltage 80 V, source temperature 120°C, desolvation temperature 500°C, cone gas flow rate 50 l/h and desolvation gas flow rate 1000 l/h. Nitrogen gas was used for the nebulizer and in desolvation whereas argon was used to generate collision-induced dissociation. MS data were acquired in continuum in both negative and positive ionization mode within the range 50-2000 *m/z* using a fixed collision energy of 35 V. Data were acquired through the Mass Lynx v4.2 software (Waters).

7.5 Processing of LC-MS data and metabolites identification

The chromatograms were manually inspected through Mass Lynx software. Metabolites were identified by relying on *m/z* value of the monoisotopic molecular ion, retention time and MS/MS fragmentation pattern by comparison with an in-house library of authentic standard. When no standard compounds were available, the identification was tentatively assigned comparing *m/z*, isotopic ratio, fragmentation pattern and UV/vis absorbance spectra with those reported in scientific literature and public databases (Chemspider, Human Metabolome Database, Lotus Natural Products, MassBank, MoNA, Pubchem, etc.). In particular, for the characterization of caffeoyl ester derivatives and various glycosides the following neutral losses (Da) were considered: 132.042 (pentose), 146.058 (deoxyhexose), 162.032 (caffeic acid moiety), 162.053 (hexose),

The chromatograms acquired in negative ionization mode were processed with Progenesis Q1 software (Waters) to obtain the Feature Quantification Matrix (FQM; Supplementary File 1).

7.6 Antioxidant assays

The same methanolic extracts used for UPLC-ESI-MS analysis were used for determination of antioxidant activity *in vitro* by FRAP and DPPH assays in transparent 96-well microplates.

A FRAP solution was prepared mixing in a ratio of 10:1:1 (V:V:V) the following reagents: FRAP buffer (3.1 g/l sodium acetate trihydrate, 16 ml/l acetic acid pH 3.6), 10 mM TPTZ (2,4,6-tri(2-pyridyl)-1,2,5-triazine) in HCl 40 Mm, FeCl₃*6H₂O 20Mm, The test was carried out mixing 200 µl of the FRAP solution to 20 µl of the sample, or solutions of Trolox at different concentrations or methanol (blank). Methanolic extracts of samples were diluted 1:20 for leaves and in a range from 1:3 to 1:10 for stems. Each sample was tested in three technical replicates. The microplate was incubated at 37°C in the dark for 15 min and then kept cooling at room temperature for 4 min. The absorbance was measured at 593 nm using the Infinite 200 PRO plate reader (Tecan, Männedorf, Switzerland).

1 mM DPPH stock solution was freshly prepared in methanol at least 2 hours before the assay. 100 µM of working solution was prepared diluting 1:10 (V:V) in 70% methanol the DPPH solution. 200 µl of the DPPH solution were added to 20 µl of the sample, *i.e.* diluted plant extracts or solutions of Trolox at different concentrations or methanol (blank). Methanolic extracts were diluted in a range from 1:10 to 1:20 and from 1:3 to 1:10 for leaves and stems, respectively. Each sample was tested in three technical replicates. The microplate was incubated at 25°C in the dark for 30 min and then the absorbance was measured at 517 nm using the Infinite 200 PRO plate reader (Tecan).

The compound Trolox, a water-soluble Vitamin E analogue, was used as reference antioxidant in order to express the antioxidant power of the plant extracts, expressed as Trolox Equivalent Antioxidant Capacity (TEAC), whose unit is mmol of Trolox Equivalent for Kg (mmol/kg). 20 µl of Trolox solutions with concentrations spanning from 500 µM to 5 µM was added to 200 µl of FRAP or DPPH solution to generate a Trolox calibration curve in each assay.

7.7 Statistical analysis

The FQM and antioxidant (TEAC values) data were analyzed with SIMCA-P software (Umetrics, Sweden) for multivariate statistical analysis in order to look for relationships among the in-vitro antioxidant activity of the plant extracts and their metabolite composition. The *m/z* features (*i.e.* the metabolites) of the dataset were assigned as X variables (Pareto scaling) and the antioxidant activity as Y variables (UV scaling). Orthogonal Partial Least Square (OPLS) analysis was used. Metabolites putatively responsible for the antioxidant activity were identified by inspection of the column loading plot; only metabolites showing a *pq*(corr) value > 0.8 (arbitrary threshold) were considered correlated with antioxidant activity. All

statistical calculations were performed using the GraphPad Prism version 8.0 software (GraphPad Software, San Diego, California USA). The means values \pm SD ($n = 3$) are reported in the figures. Statistical analyses were conducted using One or Two-way Anova followed by Tukey's Test.

Declarations

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Conflict of interest

The authors declare no conflict of interest.

Data Availability

All data generated or analysed during this study are included in this published article [and its supplementary information files]

References

1. Oyemitan IA. African medicinal spices of genus Piper. Medicinal Spices and Vegetables from Africa, Elsevier; 2017, p. 581–97.
2. Süntar I. Importance of ethnopharmacological studies in drug discovery: role of medicinal plants. *Phytochemistry Reviews* 2020;19:1199–209.
3. Garnatje T, Peñuelas J, Vallès J. Ethnobotany, phylogeny, and 'omics' for human health and food security. *Trends in Plant Science* 2017;22:187–91.
4. Su X-Z, Miller LH. The discovery of artemisinin and the Nobel Prize in Physiology or Medicine. Springer; 2015.
5. Tu Y. The discovery of artemisinin (qinghaosu) and gifts from Chinese medicine. *Nature Medicine* 2011;17:1217–20.
6. Rønsted N, Symonds MR, Birkholm T, Christensen SB, Meerow AW, Molander M, et al. Can phylogeny predict chemical diversity and potential medicinal activity of plants? A case study of Amaryllidaceae. *BMC Evolutionary Biology* 2012;12:1–12.
7. Bora KS, Sharma A. The genus Artemisia: A comprehensive review. *Pharmaceutical Biology* 2011;49:101–9. <https://doi.org/10.3109/13880209.2010.497815>.
8. Koul B, Khatri T. The Artemisia genus: Panacea to several maladies. *Bioactive Natural Products in Drug Discovery* 2020;3–95.
9. Martínez MJA, Del Olmo LMB, Ticona LA, Benito PB. The Artemisia L. genus: a review of bioactive sesquiterpene lactones. *Studies in Natural Products Chemistry* 2012;37:43–65.
10. Bisht D, Kumar D, Kumar D, Dua K, Chellappan DK. Phytochemistry and pharmacological activity of the genus artemisia. *Archives of Pharmacol Research* 2021;44:439–74.
11. Pandey AK, Singh P. The genus Artemisia: A 2012–2017 literature review on chemical composition, antimicrobial, insecticidal and antioxidant activities of essential oils. *Medicines* 2017;4:68.
12. Trendafilova A, Moujir LM, Sousa PM, Seca AM. Research advances on health effects of edible Artemisia species and some sesquiterpene lactones constituents. *Foods* 2020;10:65.
13. Chen L, Qiu J, Tarolli P. Water resources management of grasslands under climate change in the historic rural landscape of Lessinia Regional Park (Veneto, Italy). *EGU General Assembly Conference Abstracts*, 2022, p. EGU22-7963.
14. Aberham A, Cicek SS, Schneider P, Stuppner H. Analysis of sesquiterpene lactones, lignans, and flavonoids in wormwood (*Artemisia absinthium* L.) using high-performance liquid chromatography (HPLC)-mass spectrometry, reversed phase HPLC, and HPLC-solid phase extraction-nuclear magnetic resonance. *J Agric Food Chem* 2010;58:10817–23. <https://doi.org/10.1021/jf1022059>.
15. Trifan A, Zengin G, Sinan KI, Sieniawska E, Sawicki R, Maciejewska-Turska M, et al. Unveiling the Phytochemical Profile and Biological Potential of Five Artemisia Species. *Antioxidants* 2022;11:1017. <https://doi.org/10.3390/antiox11051017>.
16. Dong W, Ma W-J, Ma Y-B, Li F-J, Li T-Z, Wang Y-C, et al. Guaiane-type sesquiterpenoid dimers from Artemisia zhongdianensis and antihepatoma carcinoma activity via the p38MAPK pathway. *Chinese Journal of Chemistry* 2023.
17. Su L-H, Ma W-J, Ma Y-B, Li T-Z, Geng C-A, Dong W, et al. Artemiprincepsolides A– F, novel germacrane-guaiane and eudesmane-guaiane sesquiterpenoid dimers from Artemisia princeps and their antihepatoma activity. *Chinese Journal of Chemistry* n.d.
18. Melguizo-Melguizo D, Diaz-de-Cerio E, Quirantes-Piné R, Švarc-Gajić J, Segura-Carretero A. The potential of Artemisia vulgaris leaves as a source of antioxidant phenolic compounds. *Journal of Functional Foods* 2014;10:192–200. <https://doi.org/10.1016/j.jff.2014.05.019>.
19. Mouton J, Kooy F van der. Identification of cis-and trans-melilotoside within an Artemisia annua tea infusion. *European Journal of Medicinal Plants* 2014;4:52–63.

20. Kłeczek N, Malarz J, Gierlikowska B, Kiss AK, Stojakowska A. Constituents of *Xerolekia speciosissima* (L.) Anderb.(Inuleae), and anti-inflammatory activity of 7, 10-diisobutyryloxy-8, 9-epoxythymyl isobutyrate. *Molecules* 2020;25:4913.
21. Bora KS, Sharma A. The Genus *Artemisia*: A Comprehensive Review. *Pharmaceutical Biology* 2011;49:101–9. <https://doi.org/10.3109/13880209.2010.497815>.
22. Carvalho IS, Cavaco T, Brodelius M. Phenolic composition and antioxidant capacity of six artemisia species. *Industrial Crops and Products* 2011;33:382–8. <https://doi.org/10.1016/j.indcrop.2010.11.005>.
23. Nigam M, Atanassova M, Mishra AP, Pezzani R, Devkota HP, Plygun S, et al. Bioactive compounds and health benefits of *Artemisia* species. *Natural Product Communications* 2019;14:1934578X19850354.
24. El-Seedi HR, Taher EA, Sheikh BY, Anjum S, Saeed A, AlAjmi MF, et al. Hydroxycinnamic acids: natural sources, biosynthesis, possible biological activities, and roles in Islamic medicine. *Studies in Natural Products Chemistry* 2018;55:269–92.
25. Kłeczek N, Michalak B, Malarz J, Kiss AK, Stojakowska A. *Carpesium divaricatum* Sieb. & Zucc. Revisited: Newly Identified Constituents from Aerial Parts of the Plant and Their Possible Contribution to the Biological Activity of the Plant. *Molecules* 2019;24:1614. <https://doi.org/10.3390/molecules24081614>.
26. Kramberger K, Barlič-Maganja D, Bandelj D, Baruca Arbeiter A, Peeters K, Miklavčič Višnjevec A, et al. HPLC-DAD-ESI-QTOF-MS Determination of Bioactive Compounds and Antioxidant Activity Comparison of the Hydroalcoholic and Water Extracts from Two *Helichrysum italicum* Species. *Metabolites* 2020;10:403. <https://doi.org/10.3390/metabo10100403>.
27. Aberham A, Cicek SS, Schneider P, Stuppner H. Analysis of Sesquiterpene Lactones, Lignans, and Flavonoids in Wormwood (*Artemisia absinthium* L.) Using High-Performance Liquid Chromatography (HPLC)–Mass Spectrometry, Reversed Phase HPLC, and HPLC–Solid Phase Extraction–Nuclear Magnetic Resonance. *J Agric Food Chem* 2010;58:10817–23. <https://doi.org/10.1021/jf1022059>.
28. Slámová K, Kapešová J, Valentová K. “Sweet Flavonoids”: Glycosidase-Catalyzed Modifications. *International Journal of Molecular Sciences* 2018;19:2126. <https://doi.org/10.3390/ijms19072126>.
29. VISKUPIČOVÁ J, ONDREJOVIČ M, ŠTURDÍK E. Bioavailability and metabolism of flavonoids. *Journal of Food & Nutrition Research* 2008;47.
30. Wollenweber E, Dörr M, Christ M. Flavonoid Aglycones from the Leaf and Stem Exudates of Some Geraniaceae Species. *Natural Product Communications* 2011;6:1934578X1100600105. <https://doi.org/10.1177/1934578X1100600105>.
31. Olennikov DN, Kashchenko NI, Chirikova NK, Vasil’eva AG, Gadimli AI, Isaev JI, et al. Caffeoylquinic Acids and Flavonoids of Fringed Sagewort (*Artemisia frigida* Willd.): HPLC-DAD-ESI-QQQ-MS Profile, HPLC-DAD Quantification, in Vitro Digestion Stability, and Antioxidant Capacity. *Antioxidants* 2019;8:307. <https://doi.org/10.3390/antiox8080307>.
32. Valant-Vetschera KM, Fischer R, Wollenweber E. Exudate flavonoids in species of *Artemisia* (Asteraceae–Anthemideae): new results and chemosystematic interpretation. *Biochemical Systematics and Ecology* 2003;31:487–98. [https://doi.org/10.1016/S0305-1978\(02\)00178-3](https://doi.org/10.1016/S0305-1978(02)00178-3).
33. Nikolova M, Velickovic D. Phenological Variations in the Surface Flavonoids of *Artemisia vulgaris* L. and *Artemisia absinthium* L. n.d.
34. Tissier A. Glandular trichomes: what comes after expressed sequence tags? *The Plant Journal* 2012;70:51–68. <https://doi.org/10.1111/j.1365-313X.2012.04913.x>.
35. Xiao L, Tan H, Zhang L. *Artemisia annua* glandular secretory trichomes: the biofactory of antimalarial agent artemisinin. *Science Bulletin* 2016;61:26–36.
36. Huchelmann A, Boutry M, Hachez C. Plant Glandular Trichomes: Natural Cell Factories of High Biotechnological Interest1[OPEN]. *Plant Physiol* 2017;175:6–22. <https://doi.org/10.1104/pp.17.00727>.
37. Tissier A. Harnessing plant trichome biochemistry for the production of useful compounds. *Molecular Pharming: Applications, Challenges, and Emerging Areas* 2018:353–82.
38. Liu Y, Jing S-X, Luo S-H, Li S-H. Non-volatile natural products in plant glandular trichomes: chemistry, biological activities and biosynthesis. *Nat Prod Rep* 2019;36:626–65. <https://doi.org/10.1039/C8NP00077H>.
39. Minarchenko V, Karpiuk U, Kovalska N, Tymchenko I, Dvirna T, Cholok I. Diagnostic micromorphological features of leaf surface of some *Artemisia* species (Asteraceae). *Hacquetia* 2023;22:131–41.
40. Peron G, Baldan V, Sut S, Faggian M, Roccabruna L, Zanini D, et al. Phytochemical investigations on *Artemisia alba* Turra growing in the North-East of Italy. *Nat Prod Res* 2017;31:1861–8. <https://doi.org/10.1080/14786419.2016.1263845>.
41. Pisoschi AM, Negulescu GP. Methods for Total Antioxidant Activity Determination: A Review. *Biochem & Anal Biochem* 2012;01. <https://doi.org/10.4172/2161-1009.1000106>.
42. Tan JBL, Lim YY. Critical analysis of current methods for assessing the in vitro antioxidant and antibacterial activity of plant extracts. *Food Chem* 2015;172:814–22. <https://doi.org/10.1016/j.foodchem.2014.09.141>.
43. Granato D, Shahidi F, Wrolstad R, Kilmartin P, Melton LD, Hidalgo FJ, et al. Antioxidant activity, total phenolics and flavonoids contents: Should we ban in vitro screening methods? *Food Chemistry* 2018;264:471–5. <https://doi.org/10.1016/j.foodchem.2018.04.012>.
44. Munteanu IG, Apetrei C. Analytical Methods Used in Determining Antioxidant Activity: A Review. *International Journal of Molecular Sciences* 2021;22:3380. <https://doi.org/10.3390/ijms22073380>.

45. Ickovski JD, Mitić M, Stojković M, Stojanović G. Comparative analysis of HPLC profiles and antioxidant activity of *Artemisia alba* Turra from two habitats in Serbia. *Chemia Naissensis* 2020;3:89–95.
46. Suroowan S, Llorent-Martínez EJ, Zengin G, Dall'Acqua S, Sut S, Buskaran K, et al. Phytochemical characterization, anti-oxidant, anti-enzymatic and cytotoxic effects of *Artemisia verlotiorum* Lamotte extracts: A new source of bioactive agents. *Molecules* 2022;27:5886.
47. Alcázar Magaña A, Kamimura N, Soumyanath A, Stevens JF, Maier CS. Caffeoylquinic acids: chemistry, biosynthesis, occurrence, analytical challenges, and bioactivity. *Plant J* 2021;107:1299–319. <https://doi.org/10.1111/tpj.15390>.
48. Imai M, Yamane T, Kozuka M, Takenaka S, Sakamoto T, Ishida T, et al. Caffeoylquinic acids from aronia juice inhibit both dipeptidyl peptidase IV and α -glucosidase activities. *LWT* 2020;129:109544. <https://doi.org/10.1016/j.lwt.2020.109544>.
49. Metwally DM, Alajmi RA, El-Khadragy MF, Yehia HM, AL-Megrin WA, Akabawy AMA, et al. Chlorogenic acid confers robust neuroprotection against arsenite toxicity in mice by reversing oxidative stress, inflammation, and apoptosis. *Journal of Functional Foods* 2020;75:104202. <https://doi.org/10.1016/j.jff.2020.104202>.
50. Kim S-S, Park R-Y, Jeon H-J, Kwon Y-S, Chun W. Neuroprotective effects of 3,5-dicaffeoylquinic acid on hydrogen peroxide-induced cell death in SH-SY5Y cells. *Phytother Res* 2005;19:243–5. <https://doi.org/10.1002/ptr.1652>.
51. Xia N, Pautz A, Wollscheid U, Reifenberg G, Förstermann U, Li H. Artichoke, Cynarin and Cyanidin Downregulate the Expression of Inducible Nitric Oxide Synthase in Human Coronary Smooth Muscle Cells. *Molecules* 2014;19:3654–68. <https://doi.org/10.3390/molecules19033654>.
52. GEZER C, YÜCECAN S, RATTAN S. Artichoke compound cynarin differentially affects the survival, growth, and stress response of normal, immortalized, and cancerous human cells. *Turkish Journal of Biology* 2015;39:299–305. <https://doi.org/10.3906/biy-1407-67>.
53. Hakkou Z, Maciuk A, Leblais V, Bouanani NE, Mekhfi H, Bnouham M, et al. Antihypertensive and vasodilator effects of methanolic extract of *Inula viscosa*: Biological evaluation and POM analysis of cynarin, chlorogenic acid as potential hypertensive. *Biomedicine & Pharmacotherapy* 2017;93:62–9. <https://doi.org/10.1016/j.biopha.2017.06.015>.
54. Tong J, Mo Q-G, Ma B-X, Ge L-L, Zhou G, Wang Y-W. The protective effects of *Cichorium glandulosum* seed and cynarin against cyclophosphamide and its metabolite acrolein-induced hepatotoxicity in vivo and in vitro. *Food Funct* 2017;8:209–19. <https://doi.org/10.1039/c6fo01531j>.
55. Zyad A, Tilaoui M, Jaafari A, Oukerrou MA, Mouse HA. More insights into the pharmacological effects of artemisinin. *Phytotherapy Research* 2018;32:216–29. <https://doi.org/10.1002/ptr.5958>.
56. Kayani WK, Kiani BH, Dilshad E, Mirza B. Biotechnological approaches for artemisinin production in *Artemisia*. *World Journal of Microbiology and Biotechnology* 2018;34:1–14.
57. Pellicer J, Saslis-Lagoudakis CH, Carrió E, Ernst M, Garnatje T, Grace OM, et al. A phylogenetic road map to antimalarial *Artemisia* species. *Journal of Ethnopharmacology* 2018;225:1–9. <https://doi.org/10.1016/j.jep.2018.06.030>.
58. Kiani BH, Safdar N, Mannan A, Mirza B. Comparative Artemisinin analysis in *Artemisia dubia* transformed with two different *Agrobacteria* harbouring rol ABC genes. *Plant Omics* 2012;5:386–91.
59. Singh A, Sarin R. *Artemisia scoparia*: A new source of artemisinin. ||| *Bangladesh Journal of Pharmacology* ||| 2010;5:17–20.
60. Ermayanti TM, Bintang M, Mariska I. Production of antileukemic agent in untransformed and transformed root culture of *Artemisia cina*. *Annales Bogorienses*: Vol 2001;8.
61. Numonov S, Sharopov F, Salimov A, Sukhrobov P, Atolikshoeva S, Safarzoda R, et al. Assessment of artemisinin contents in selected *Artemisia* species from Tajikistan (Central Asia). *Medicines* 2019;6:23.
62. Singh P, Bajpai V, Khandelwal N, Varshney S, Gaikwad AN, Srivastava M, et al. Determination of bioactive compounds of *Artemisia* Spp. plant extracts by LC–MS/MS technique and their in-vitro anti-adipogenic activity screening. *Journal of Pharmaceutical and Biomedical Analysis* 2021;193:113707. <https://doi.org/10.1016/j.jpba.2020.113707>.
63. Ranjbar M, Naghavi MR, Alizadeh H, Soltanloo H. Expression of artemisinin biosynthesis genes in eight *Artemisia* species at three developmental stages. *Industrial Crops and Products* 2015;76:836–43.
64. Todorova M, Trendafilova A, Danova K, Simmons L, Wolfram E, Meier B, et al. Highly oxygenated sesquiterpenes in *Artemisia alba* Turra. *Phytochemistry* 2015;110:140–9. <https://doi.org/10.1016/j.phytochem.2014.12.008>.
65. Zhao L, Zhu Y, Jia H, Han Y, Zheng X, Wang M, et al. From Plant to Yeast—Advances in Biosynthesis of Artemisinin. *Molecules* 2022;27:6888. <https://doi.org/10.3390/molecules27206888>.
66. Mannan A, Ahmed I, Arshad W, Hussain I, Mirza B. Effects of vegetative and flowering stages on the biosynthesis of artemisinin in *Artemisia* species. *Archives of Pharmacal Research* 2011;34:1657–61.
67. Ranjbar M, Naghavi MR, Alizadeh H, Soltanloo H. Expression of artemisinin biosynthesis genes in eight *Artemisia* species at three developmental stages. *Industrial Crops and Products* 2015;76:836–43.
68. Towler MJ, Weathers PJ. Variations in key artemisinic and other metabolites throughout plant development in *Artemisia annua* L. for potential therapeutic use. *Industrial Crops and Products* 2015;67:185–91. <https://doi.org/10.1016/j.indcrop.2015.01.007>.

69. Bora KS, Sharma A. Evaluation of antioxidant and free-radical scavenging potential of *Artemisia absinthium*. *Pharmaceutical Biology* 2011;49:1216–23. <https://doi.org/10.3109/13880209.2011.578142>.
70. Obistioiu D, Cristina RT, Schmerold I, Chizzola R, Stolze K, Nichita I, et al. Chemical characterization by GC-MS and in vitro activity against *Candida albicans* of volatile fractions prepared from *Artemisia dracunculus*, *Artemisia abrotanum*, *Artemisia absinthium* and *Artemisia vulgaris*. *Chemistry Central Journal* 2014;8:6. <https://doi.org/10.1186/1752-153X-8-6>.
71. Peron G, Baldan V, Sut S, Faggian M, Roccabruna L, Zanini D, et al. Phytochemical investigations on *Artemisia alba* Turra growing in the North-East of Italy. *Natural Product Research* 2017;31:1861–8.
72. Martínez MJA, Del Olmo LMB, Ticona LA, Benito PB. The *Artemisia* L. genus: a review of bioactive sesquiterpene lactones. *Studies in Natural Products Chemistry* 2012;37:43–65.
73. Crespo-Ortiz MP, Wei MQ. Antitumor activity of artemisinin and its derivatives: from a well-known antimalarial agent to a potential anticancer drug. *BioMed Research International* 2012;2012.
74. Bittencourt De Souza LF, Laughinghouse HD, Pastori T, Tedesco M, Kuhn AW, Canto-Dorow TSD, et al. Genotoxic potential of aqueous extracts of *Artemisia verlotorum* on the cell cycle of *Allium cepa*. *International Journal of Environmental Studies* 2010;67:871–7. <https://doi.org/10.1080/00207233.2010.520457>.
75. Mootoosamy A, Fawzi Mahomoodally M. Ethnomedicinal application of native remedies used against diabetes and related complications in Mauritius. *Journal of Ethnopharmacology* 2014;151:413–44. <https://doi.org/10.1016/j.jep.2013.10.069>.
76. Ekiert H, Pajor J, Klin P, Rzepiela A, Ślesak H, Szopa A. Significance of *Artemisia Vulgaris* L. (Common Mugwort) in the History of Medicine and Its Possible Contemporary Applications Substantiated by Phytochemical and Pharmacological Studies. *Molecules* 2020;25:4415. <https://doi.org/10.3390/molecules25194415>.
77. Natividad GM, Broadley KJ, Kariuki B, Kidd EJ, Ford WR, Simons C. Actions of *Artemisia vulgaris* extracts and isolated sesquiterpene lactones against receptors mediating contraction of guinea pig ileum and trachea. *Journal of Ethnopharmacology* 2011;137:808–16. <https://doi.org/10.1016/j.jep.2011.06.042>.
78. Lee S-J, Chung H-Y, Maier CG-A, Wood AR, Dixon RA, Mabry TJ. Estrogenic flavonoids from *Artemisia vulgaris* L. *Journal of Agricultural and Food Chemistry* 1998;46:3325–9.
79. Sumner LW, Amberg A, Barrett D, Beale MH, Beger R, Daykin CA, et al. Proposed minimum reporting standards for chemical analysis. *Metabolomics* 2007;3:211–21. <https://doi.org/10.1007/s11306-007-0082-2>.
80. Mamatova AS, Korona-Glowniak I, Skalicka-Woźniak K, Józefczyk A, Wojtanowski KK, Baj T, et al. Phytochemical composition of wormwood (*Artemisia gmelinii*) extracts in respect of their antimicrobial activity. *BMC Complementary and Alternative Medicine* 2019;19:288. <https://doi.org/10.1186/s12906-019-2719-x>.
81. El-Wassimy MTM, Ahmed MM, Younes SHH, Hegazy M-EF. Antimicrobial and Antiproliferative activity of Tomenin compound isolated from *Seriphidium Herba-Alba*. *Sohag Journal of Sciences* 2018;3:1–8. <https://doi.org/10.21608/sjsci.2018.233272>.
82. Han J, Ye M, Qiao X, Xu M, Wang B, Guo D-A. Characterization of phenolic compounds in the Chinese herbal drug *Artemisia annua* by liquid chromatography coupled to electrospray ionization mass spectrometry. *Journal of Pharmaceutical and Biomedical Analysis* 2008;47:516–25. <https://doi.org/10.1016/j.jpba.2008.02.013>.
83. Ghareeb MA, Sobeh M, Rezaq S, El-Shazly AM, Mahmoud MF, Wink M. HPLC-ESI-MS/MS Profiling of Polyphenolics of a Leaf Extract from *Alpinia zerumbet* (Zingiberaceae) and Its Anti-Inflammatory, Anti-Nociceptive, and Antipyretic Activities In Vivo. *Molecules* 2018;23:3238. <https://doi.org/10.3390/molecules23123238>.
84. Zhang C, Zuo T, Wang X, Wang H, Hu Y, Li Z, et al. Integration of Data-Dependent Acquisition (DDA) and Data-Independent High-Definition MSE (HDMSE) for the Comprehensive Profiling and Characterization of Multicomponents from *Panax japonicus* by UHPLC/IM-QTOF-MS. *Molecules* 2019;24:2708. <https://doi.org/10.3390/molecules24152708>.
85. Boukhalkhal S, Gourine N, Pinto DCGA, Silva AMS, Yousfi M. UHPLC-DAD-ESI-MSn profiling variability of the phenolic constituents of *Artemisia campestris* L. populations growing in Algeria. *Biocatalysis and Agricultural Biotechnology* 2020;23:101483. <https://doi.org/10.1016/j.bcab.2019.101483>.
86. Grati W, Samet S, Bouzayani B, Ayachi A, Treilhou M, Téné N, et al. HESI-MS/MS Analysis of Phenolic Compounds from *Calendula aegyptiaca* Fruits Extracts and Evaluation of Their Antioxidant Activities. *Molecules* 2022;27:2314. <https://doi.org/10.3390/molecules27072314>.

Figures

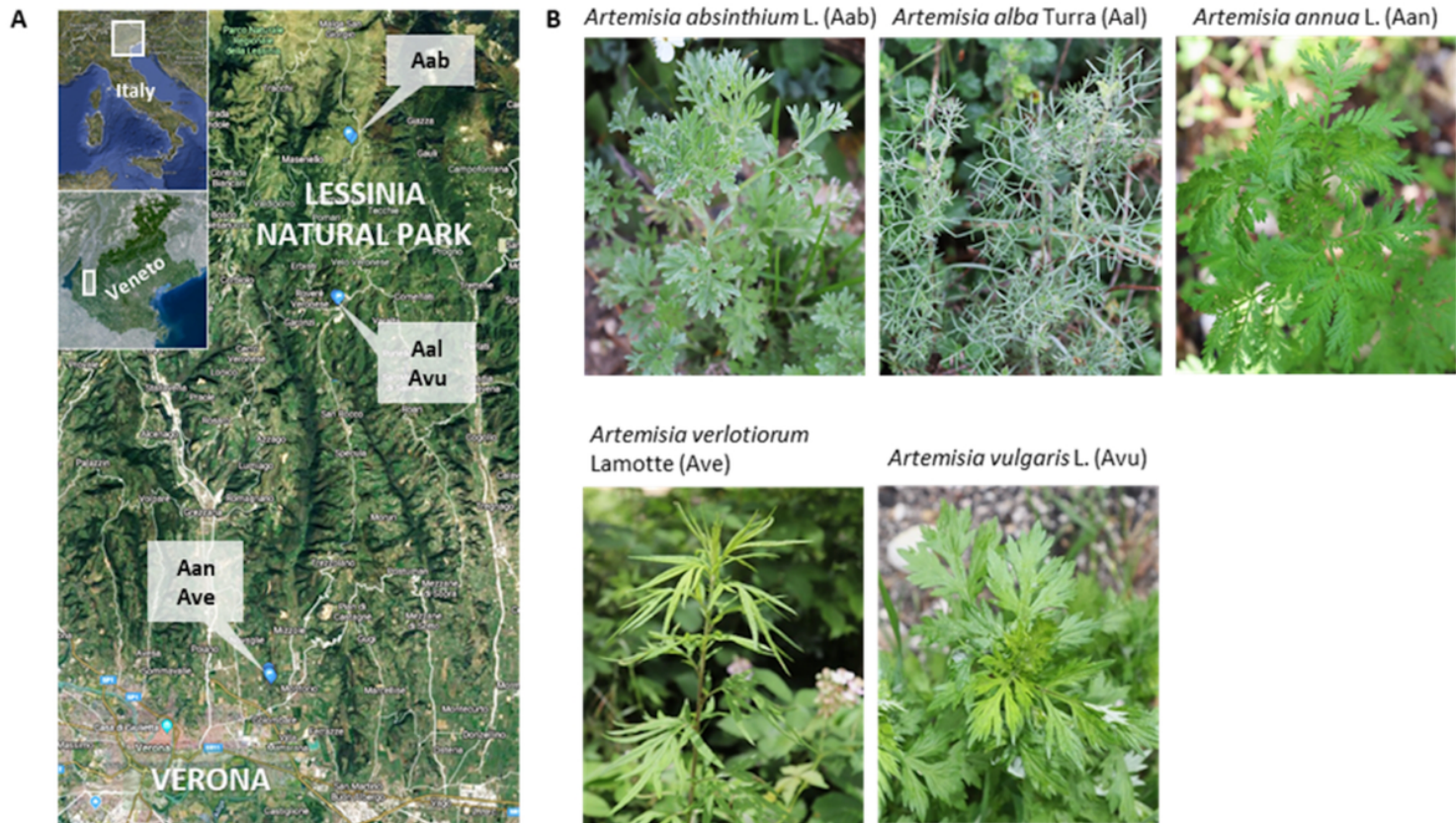


Figure 1

Geolocation of the sampling spots in the northern area of the Verona province (A) and pictures of the five *Artemisia* spp. plants collected (B).

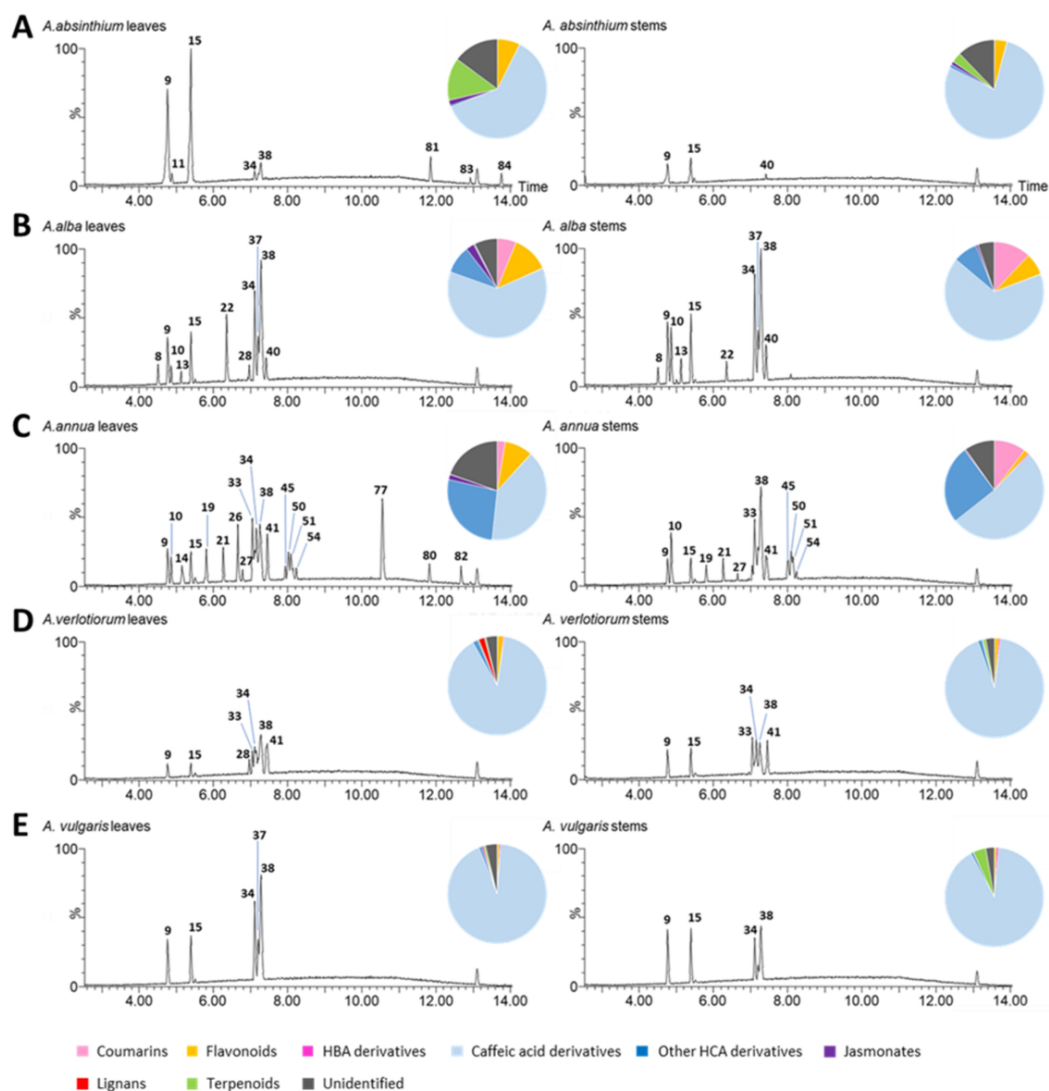


Figure 2

Secondary metabolomes of *Artemisia* spp. Exemplificative base peak chromatograms recorded in LC-MS-ESI⁺ (intensity scaled to 2.5×10^5) of leaves (left) and stems (right) are shown together with pie charts representing the metabolite classes according to the total LC-MS signal detected. Peak annotation numbers refer to Table 2. HBA, hydroxybenzoic acid, HCA, hydroxycinnamic acid derivatives.

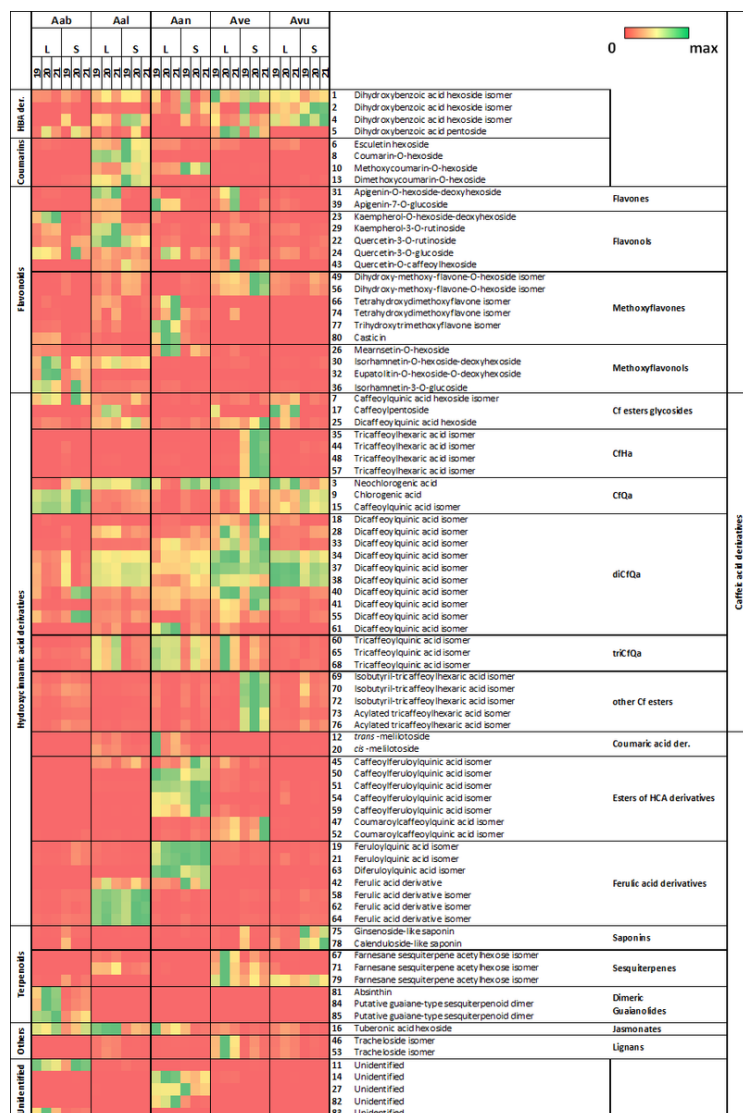


Figure 3

Heat map showing the average relative percentage abundance (respect to the total LC-MS signal of each metabolome) of all metabolites tentatively identified in *Artemisia* spp. Leaves (L) and stems (S) over a three-year sampling period (2019, 2020, 2021). The metabolite identification numbers match those in the dataset. Aab, *A. absinthium*; Aal, *A. alba*; Aan, *A. annua*; Ave, *A. verlotiorum*; Avu, *A. vulgaris*. Cf, caffeoyl; CfQa, caffeoylquinic acids; CfHa, caffeoylhexaric acids; HBA, hydroxybenzoic acid; HCA, hydroxycinnamic acid; der, derivatives.

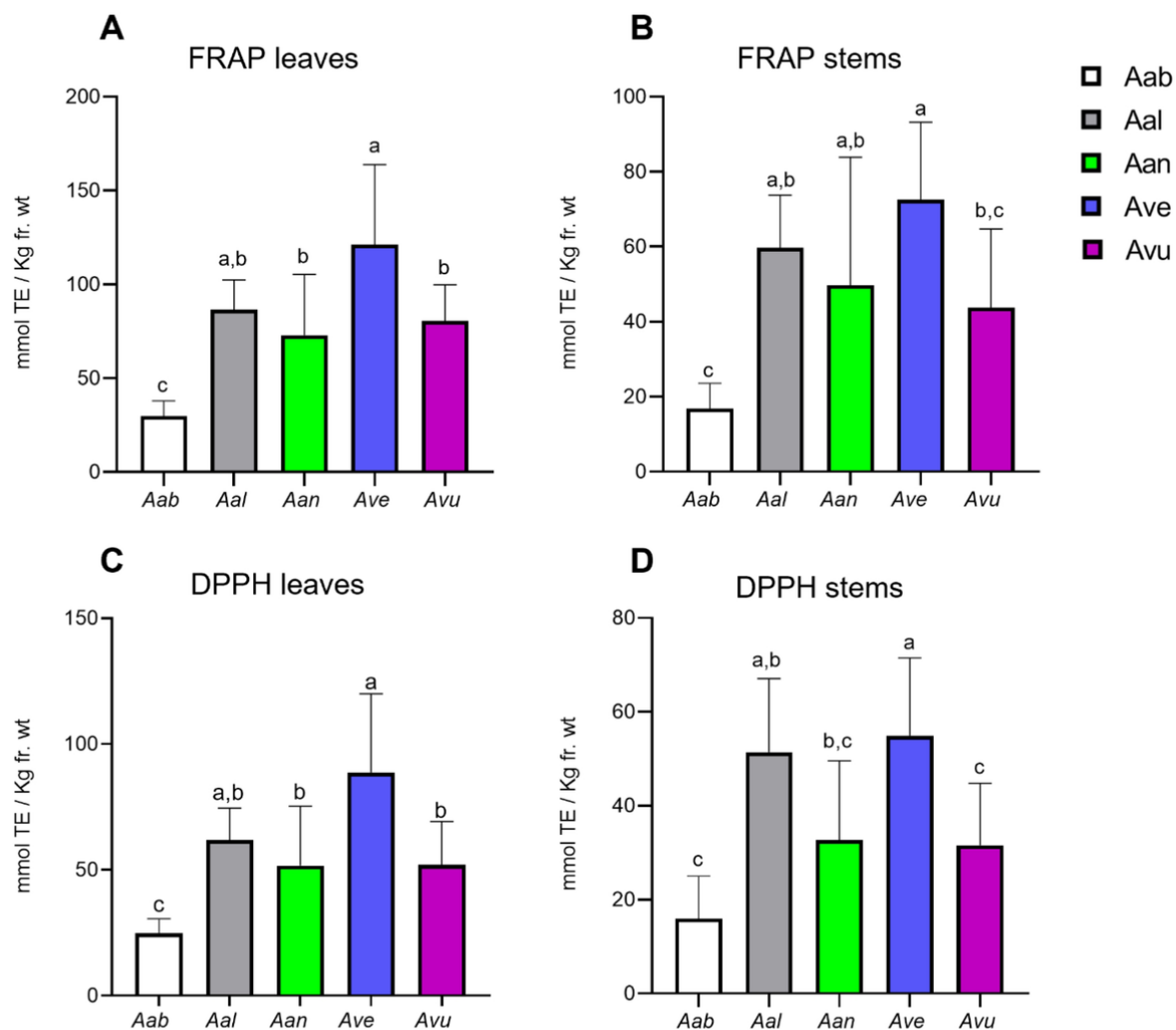


Figure 4

Antioxidant activity of extracts from *Artemisia* spp. leaves and stems, sampled in three independent growing seasons, and determined by FRAP (A,B) and DPPH (C,D) assays, and expressed as Trolox Equivalent Antioxidant Capacity (TEAC), in millimoles of Trolox Equivalents/kg of tissue (leaves or stem), fr. wt. Values are expressed as mean \pm standard deviation (n=9). Significant differences were calculated with one-way ANOVA.

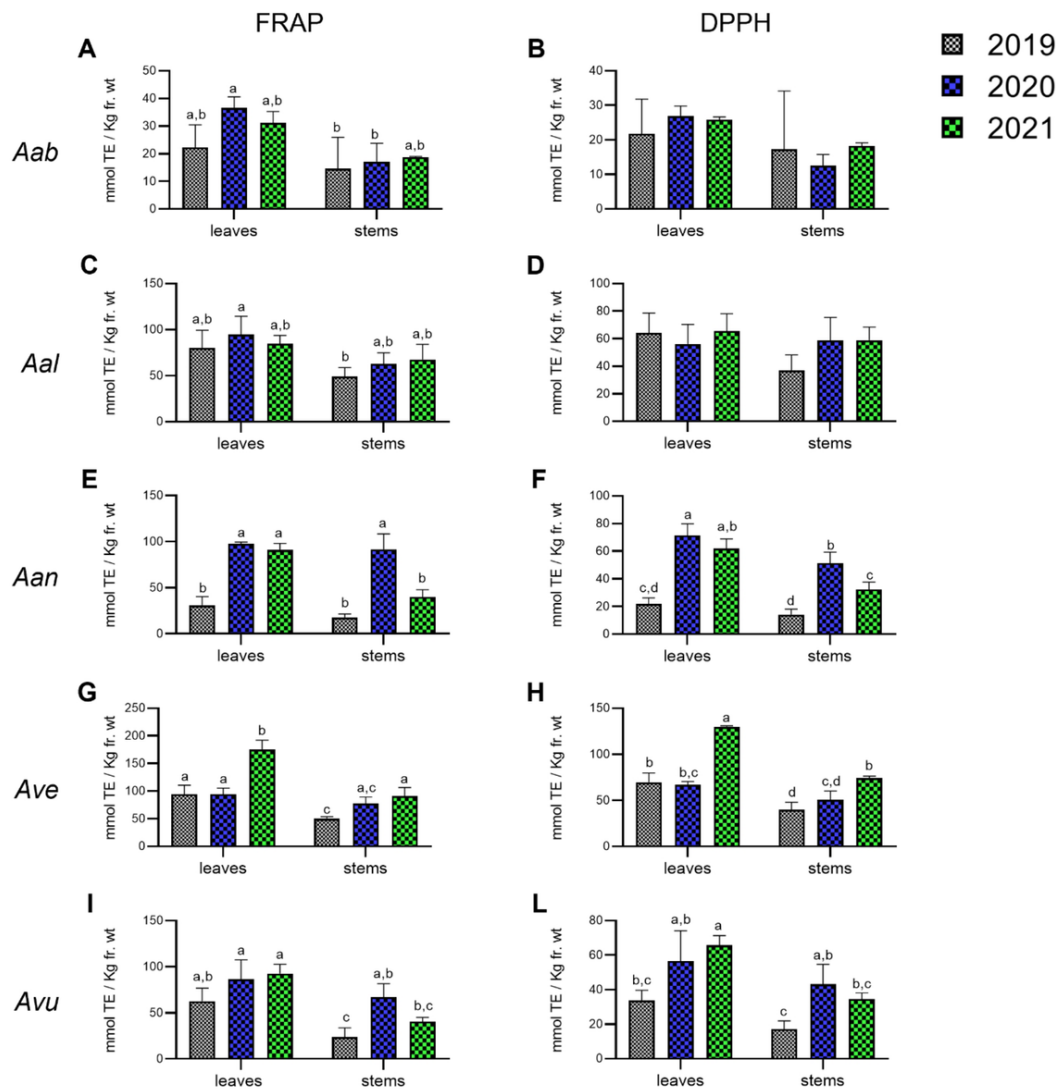


Figure 5

Antioxidant activity of extracts from *Artemisia* spp. leaves and stems, sampled in three independent growing seasons, and determined by FRAP (A, C, E, G, I) and DPPH (B, D, F, H, L) assays, and expressed as Trolox Equivalent Antioxidant Capacity (TEAC), in millimoles of Trolox Equivalents/kg of tissue (leaves or stem), fresh weight. Values are expressed as mean \pm standard deviation (n=9). Aab, *absinthium*; Aal, *alba*; Aan, *annua*; Ave, *verlotiorum*; Avu, *vulgaris*. Significant differences calculated with two-way ANOVA.

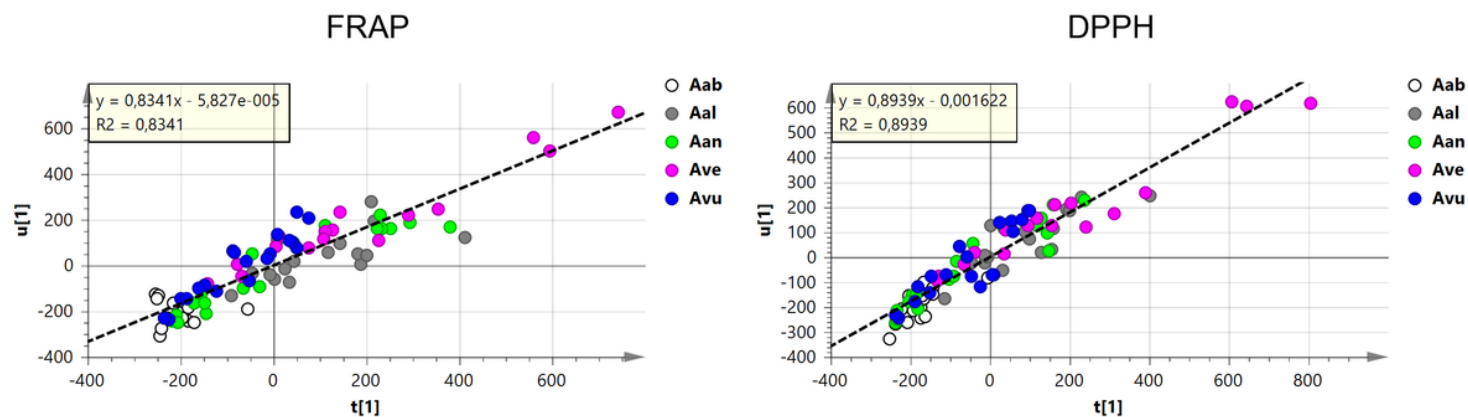


Figure 6

Scatter plot of OPLS analysis that correlates antioxidant activity (u) (FRAP on the left and DPPH on the right) with metabolic composition (t). Samples are colored according to the species.

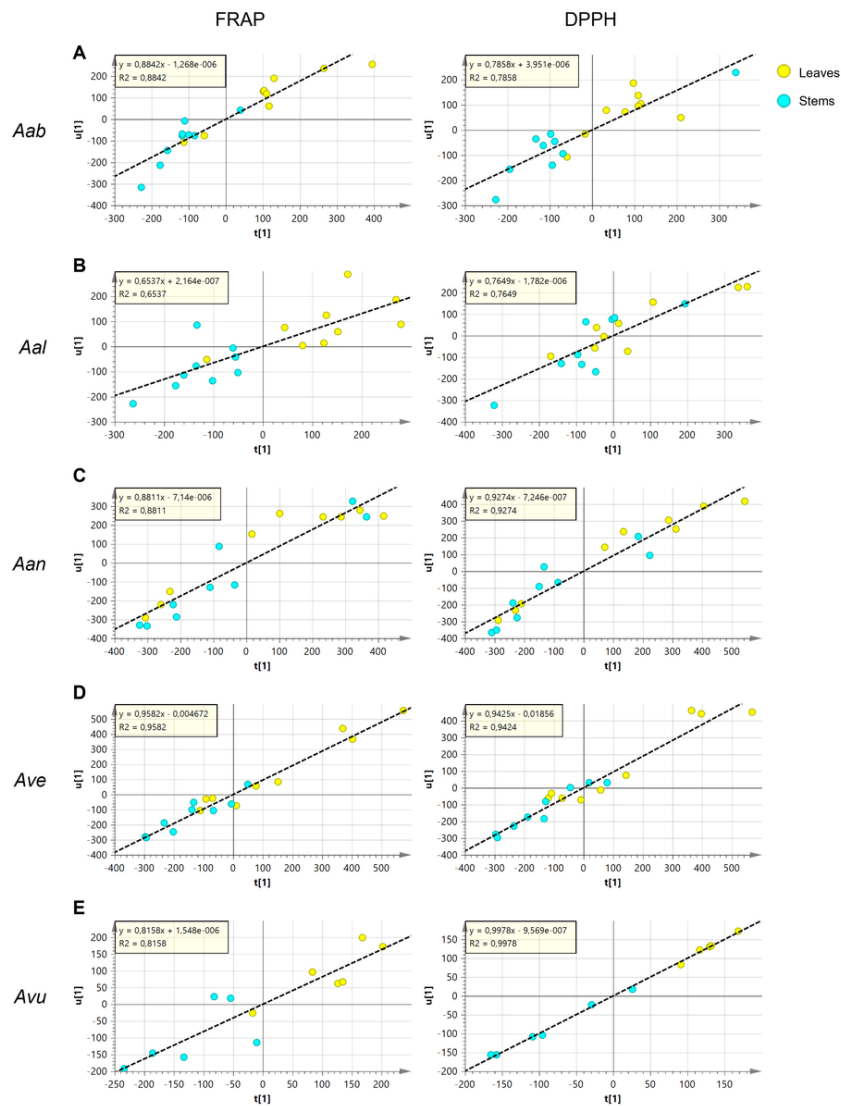


Figure 7

Scatter plot of OPLS analysis that correlates antioxidant activity (u) (FRAP on the left and DPPH on the right) with metabolic composition (t). Samples are colored according to the organs. **A)** *A. absinthium* (Aab); **B)** *A. alba* (Aal); **C)** *A. annua* (Aan); **D)** *A. verlotiorum* (Ave); **E)** *A. vulgaris* (Avu).

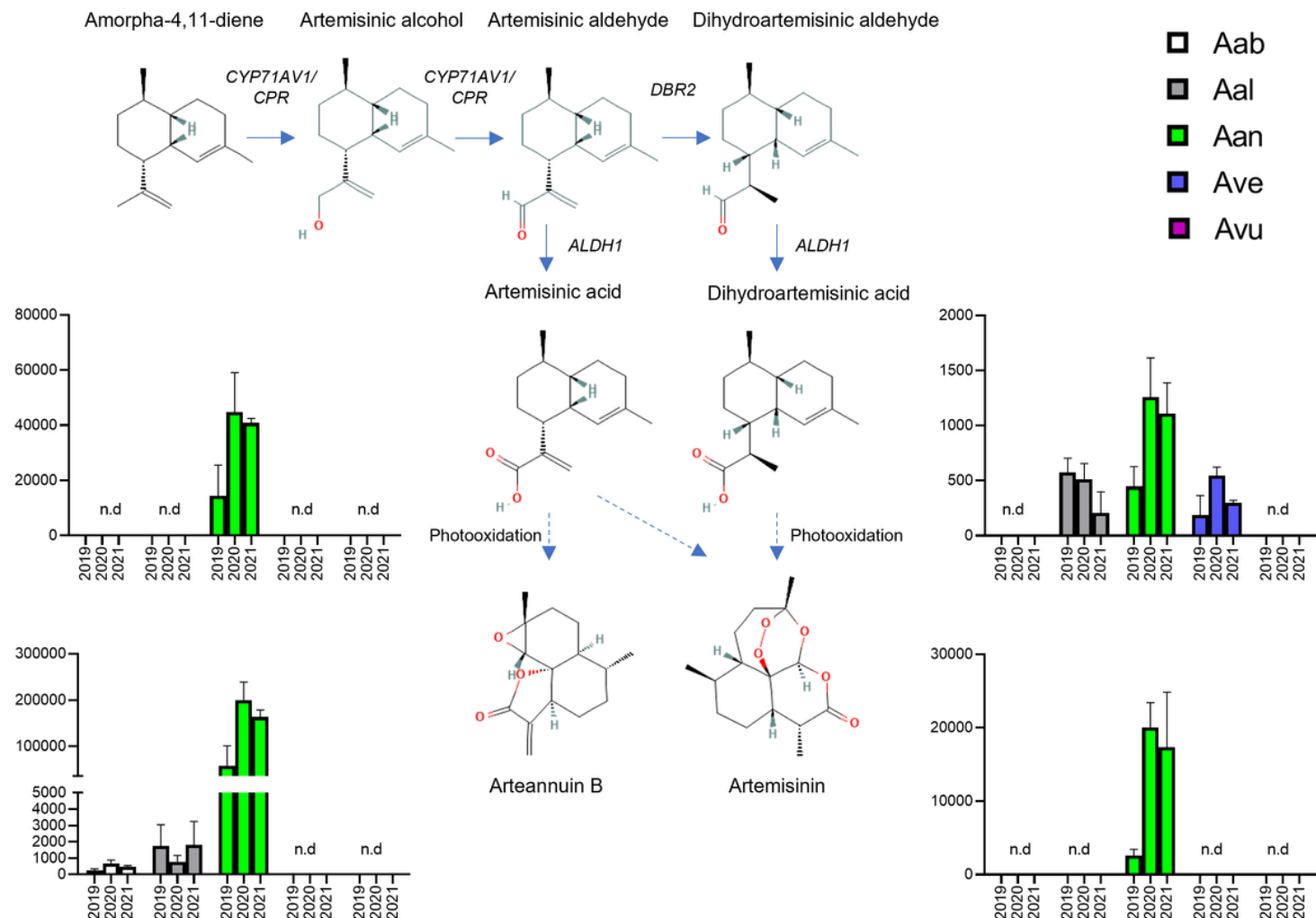


Figure 8

Relative comparison of the final sesquiterpenoid products from the artemisinin biosynthesis pathway in the five *Artemisia* species. Y axis: peak area arbitrary units. Bars represent SD (n=3)

Supplementary Files

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