scientific reports

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Bioprospecting of six polyphenol-rich Mediterranean wild edible plants reveals antioxidant, antibiofilm and bactericidal properties against Methicillin resistant *Staphylococcus aureus*

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Plant biodiversity offers a valuable source of bioactive molecules to address critical global challenges including antimicrobial resistance (AMR), a major health threat. Wild edible plants (WEPs) have recently gained attention for their ability to accumulate specialized metabolites that are emerging for their efficacy against AMR. Although many studies suggest their potential use in combating infectious diseases, knowledge about the biochemical properties of these plants, their chemical profile and antibacterial activities, remains highly limited. In this scenery, the aim of this study was a bioprospecting of the chemical and antioxidant profile, the antibiofilm and bactericidal properties of six WEPs, largely distributed in Italy and historically used as food, namely: *Silene alba*, *Silene vulgaris*, *Chenopodium album*, *Sonchus oleraceus*, *Glechoma hederacea* and *Diplotaxis erucoides*. We applied an integrated approach, combining analytical chemistry, plant biochemistry and microbiology. These WEPs revealed notable antibiofilm and bactericidal abilities, anti-adherence and cell wall damage properties. These activities were strongly linked to the presence of phenolic compounds and to the antioxidant abilities of these plants. *S. alba*, *S. oleraceus*, and *G. hederacea* showed the highest efficacy. Our findings might encourage their consumption or use, which could improve dietary plant biodiversity, human health, and fight the rise of AMR.

Keywords *Silene alba*, *Silene vulgaris*, *Chenopodium album*, *Sonchus oleraceus*, *Glechoma hederacea*, *Diplotaxis erucoides*, AMR, *S. aureus*, Wild edible plants, Antimicrobial properties, Polyphenols

Abbreviations

AMR	Antimicrobial resistance
MSSA	Methicillin susceptible *S. aureus*
MRSA	Methicillin resistant *S. aureus*
MBIC	Minimal biofilm inhibiting concentration
MIC	Minimal inhibiting concentration
MBC	Minimal bactericidal concentration
WEP	Wild edible plant
ATCC	American type culture collection

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TPC	Total phenolic content
TEAC	Trolox equivalent antioxidant capacity
DPPH	2 2 Diphenyl 1 picrylhydrazyl
FRAP	Ferric reducing antioxidant power
ASC	Ascorbate
BSL2	Bio safety level 2
UPLC-PDA	Ultra high performance liquid chromatography-photodiode array detector
LC-HRMS:	Ultra high performance liquid chromatography-high resolution mass spectrometry

The Mediterranean countries hosts a huge plant biodiversity, with Wild Edible Plants (WEPs), also referred to as Phytoalimurgic plants, constituting a significant component[1]. These plants represent an important cultural heritage at regional level, and they have historically served as vital food resources during periods of famine, owing to their high content of micronutrients essential for human health[2]. Moreover, their documented resilience to climate change underscores their adaptive potential[3]. Recently the biodiversity of plant specialized metabolites[4] (also known as secondary metabolites) has received growing attention as a promising source of new solution to today's health challenges, such as the antimicrobial resistance (AMR) which poses a critical emerging global threat[5,6]. More and more frequently pathogenic and opportunistic bacteria are increasingly responsible for complications in the postoperative course of nosocomial infections, primarily due to the formation of bacterial biofilm, colonizing the implantable medical devices[7,8]. This issue is of particular concern for several reasons: i) modern medicine practices often involve immune suppression; ii) the misuse and overuse of antibiotics over the past five decades have accelerated resistance development; iii) the dietary imbalances prevalent in Western diets negatively affect immune health. Further, a recent study revealed that certain antidepressant drugs might exacerbate the development of AMR[9]. Additionally, climate changes, like the increase of temperatures, may contribute to the spread of AMR, as it has been recently suggested[10,11].

Within this framework, phytotherapy may offer a promising adjunct to conventional antibiotics for the treatment of both susceptible and resistant strains of microorganisms.

In Italy and in other Mediterranean countries, the antimicrobial properties of WEPs are known since ancient times, and today a growing number of people are rediscovering them as natural remedies to the most common infections[12,13].

The six WEP species examined in this work, namely *Silene alba*, *Silene vulgaris*, *Chenopodium album*, *Sonchus oleraceus*, *Glechoma hederacea* and *Diplotaxis erucoides*, have been described in ethnobotanical studies for their traditional use in rural regions (Table 1), mostly Mediterranean[3], and for their known medicinal properties.

Previous studies have shown that extracts from *S. alba*[14], *S.vulgaris*[15], *C. album*[16], *S. oleraceus*[17] and *G. hederacea*[18] display variable values of Minimal Inhibitory Concentration (MIC) and Minimal Bactericidal Concentration (MBC) only against planktonic *S. aureus.* Differently, *D. erucoides* has been studied for its potential inhibitory activity against SARS-CoV-2[19].

However, none of these plants was investigated for their capacity to inhibit *Staphylococcus aureus* wild type and Methicillin Resistant *S. aureus* (MRSA) mature biofilm formation, nor for their potential to damage the bacterial cell wall. Notably, biofilm formation is an important virulence determinant, and allows bacteria to give rise to AMR colonies, even in opportunistic human pathogens[20].

The discovery of new drugs from natural sources inevitably should rely on the application of a multifaceted approach, combining botanical, phytochemical, biological, and molecular techniques[21]. Therefore, in this work we further chacterized the overall metabolic and antioxidant profiles of six WEP species, along with their antibiofilm and antimicrobial properties. Antibacterial properties were thus connected to the chemical composition of each species, as well as their total phenolic content and antioxidant capacity.

This multifaceted bioprospecting highlighted that *S. alba, S. vulgaris, C. album, S. oleraceus* and *G. hederacea* possess notable antibiofilm and antimicrobial properties, which appear to be closely correlated with their phenolic composition and antioxidant activities. In contrast, *D. erucoides* and *C. album*, displayed only antibiofilm properties, consistent with their lower phenolic composition and antioxidant capacity.

This work could be included in the strategies for the valorization of WEPs, an essential activity to preserve edible plant biodiversity. In addition, these data might help improving the western eating habits and propose these species both as a daily diet healthy component, and as a valuable phytotherapeutic tool to contribute to counteract the onset of antibiotic-resistance in pathogenic bacteria.

Family	Species	Common name	Life cycle	Reference
Caryophyllaceae	*Silene alba*	White Campion	biennial	Zengin, 2018
Caryophyllaceae	*Silene vulgaris*	Red Campion	perennial	Zengin, 2018
Amaranthaceae	*Chenopodium album*	White goosefoot	annual	Akgunlu, 2016
Asteraceae	*Sonchus oleraceus*	Common sowthistle	biennial	Xia, 2011
Brassicaceae	*Diplotaxis erucoides*	White wall rocket	annual	Guijarro-Real, 2021
Lamiaceae	*Glechoma hederacea*	Ground Ivy	perennial	El-Aasr, 2022

Table 1. List of the six species analyzed. List of the six Wild Edible Plants (WEPs) species analyzed in this study. For each species, we report the botanical Family, the English common name, the life cycle and the first reference cited in the text.

Results

Total phenolic and ascorbate content

Although the underlying mechanisms are not fully understood, several phenolic compounds may have significant antibacterial properties, even though they are well-known for their antioxidant activity[22]. In this context, we monitored the content of total phenolic compounds in the considered plant extracts. The total phenolic content revealed that the extract obtained from *G. hederacea* leaves presents the highest phenolic content, followed by the extracts obtained from *S. oleraceus* and *S. alba* (Fig. 1a). On the other hand, *S. vulgaris*, *C. album* and *D. erucoides* showed low levels of total phenolic compounds, with *S. vulgaris* having the lowest amount. In particular, the phenolic content of *S. vulgaris* was approximately 6% of that observed in *G. hederacea* (Fig. 1a).

The content of total ASC in the plant extracts revealed that *S. alba*, *S. oleraceus* and G. *hederacea* show significantly higher levels of total ASC in comparison to *S. vulgaris*, *C. album* and *D. erucoides* (Fig. 1b).

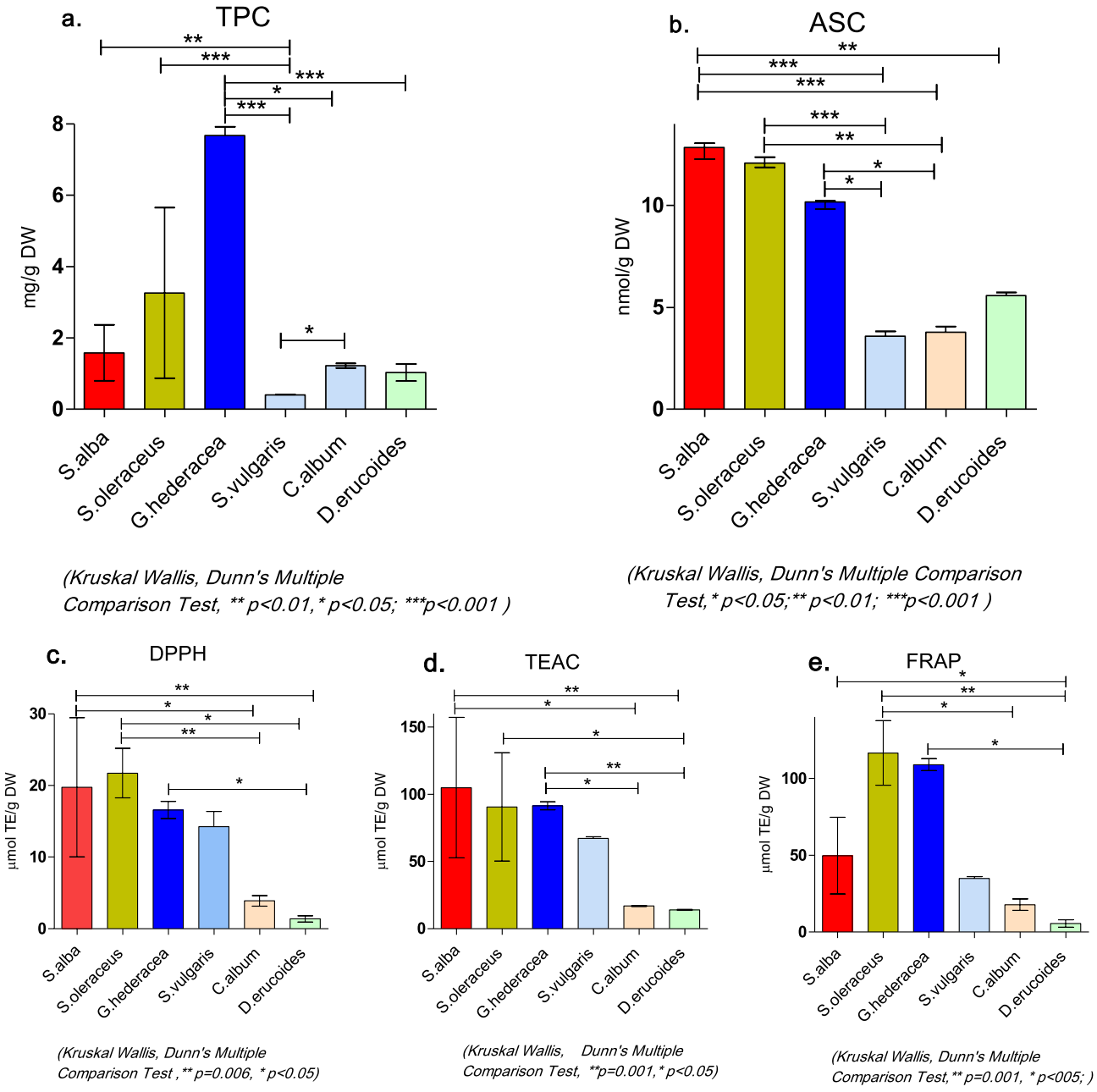


Fig. 1. Antioxidant related analysis of the six WEPs extracts. **a.** Total phenol content (n = 5); **b.** Total ascorbate content (n = 5); **c.** DPPH scavenging capacity (n = 6); **d.** TEAC scavenging capacity (n = 6); **e.** FRAP scavenging capacity (n = 6); Values are means ± SD. Different statistical difference ($p < 0.05$) are based on Kruskal Wallis and Dunn's Multiple comparison test.

Antioxidant activity

The results of the antioxidant activity of the plant extracts determined as DPPH, TEAC and FRAP scavenging activity, are shown in Fig. 1c, d and e, respectively.

The three different methods employed reveled different antioxidant capacity among the six WEPs. This variation is expected, as each method is based on the scavenging capacity against different reactive species. The FRAP method measures the extract capacity to reduce ferric ion (Fe^{3+}) to ferrous ion (Fe^{2+})[23] while TEAC and DPPH methods assay the extract antioxidant capacity against a cationic radical and a free radical, respectively[24]. The antioxidant activity measured by DPPH assay ranged from approximately 22 to 2 µmol TE/g DW with *S. alba*, *S. oleraceus*, *G. hederacea* and *S. vulgaris* characterized by having the highest ABTS values and *D. erucoides* and *C. album* by the lowest ABTS values (Fig. 1**.c**). We observed the same trend by measuring the TEAC scavenging activity (Fig. 1d). In this case, the antioxidant activity ranged from approximately 100 to 10 µmol TE/g DW. *S. alba*, *S. oleraceus*, *G. hederacea* and *S. vulgaris* were characterized by the extracts with the highest antioxidant values, as opposed to *C. album* and *D. erucoides* which showed very low antioxidant activity measured by this assay (Fig. 1d).

The FRAP scavenging activity varied significantly among the plant extracts, ranging from approximately 135 to 5 µmol TE/g DW. As reported in Fig. 1e, the extracts from *S. oleraceus* and *G. hederacea* exhibited similar FRAP values, which were significantly higher ($p < 0.0001$) than those of the other plant extracts, with values of 133.54 µmol TE/g DW and 109.05 µmol TE/g DW, respectively. In contrast to these plant species, *D. erucoides* showed the lowest FRAP activity, measuring 5.53 µmol TE/g DW. Similarly, low FRAP values were observed in *C. album*, and *S. vulgaris*, while *S. alba* represented the intermediate boundary between the extracts with the lowest and the highest capacity to reduce ferric to ferrous ions (Fig. 1e).

Polyphenolic profiles

We initially analyzed the polyphenolic profile of the six species by UPLC-PDA. To assess the possible presence of polyphenol families in the extracts, we examined in detail the UV spectra of the obtained peaks. As it is shown in the PDA chromatograms reported in Fig. 2, S. *oleraceus* and *G. hederacea* are the species with the highest number of peaks (Fig. 2b and c), while in *C. album* and *D. erucoides* chromatograms the number and the intensity of the peaks are very low (Fig. 2e and f). These results were found to be consistent with the total phenolic content and antioxidant activities reported in Fig. 1 for the aforementioned species. On the other hand, both *Silene* species revealed the presence in each extract of a predominant compound along with other minor peaks (Fig. 2a and d), in accordance with the low total phenolic content (TPC) of both extracts (Fig. 1a) but not in agreement with their high antioxidant activities (Fig. 1c, d and e).

Based on these results, we performed a more in deep characterization of the polyphenolic profile using LC-HRMS only in *S. alba, S.oleraceus, G. hederacea* and *S. vulgaris* (Table 2). However, by carefully studying the UV spectra of each peak in the chromatograms of *C.album* and *D.erucoides*, it can be assumed the presence in these extracts of compounds belonging to the class of phenolic acids, flavanols and flavones, but in significantly lower concentrations as compared to the other four species (Fig. 1a, Fig. 2e and f).

LC-HRMS analysis highlighted that in *S. alba* and *S. vulgaris* the most intense peaks were isovitexin 2"-O-glucoside and vitexin, respectively (Table 2a and d). Most of the other compounds identified in both *Silene* extracts were apigenin derivatives (as isovitexin 2"-O-glucoside, vitexin, etc.) or luteolin derivatives (as isoorientin, etc.), all belonging to the flavonoid class, in accordance with the recent findings of Jakimiuk et al. (2022)[25], who reported the flavonoids as the main bioactive compounds contained in *Silene* species[14,25]. According to these authors, mainly flavones were identified in the examined *Silene* extracts, although a small quantity of flavanols were also found in *S. alba* extract (Table 2a).

Concerning *Sonchus oleraceus*, we identified 18 compounds, including phenolic acids and flavonoids. Among flavonoids, we detected quercetin and kaempferol derivatives (flavanols) as well as luteolin and apigenin related compounds (flavones) (Table 2b). These results are in line with the literature data[26,27], although we found differences in the amounts of the individual compounds. Actually, quantitative analysis revealed the presence of large amount of chicoric acid, quercetin-3-glucoside and neo-chlorogenic acid in the extract under investigation. The reason for these differences is likely to be that the occurrence of secondary metabolites in plants is influenced not only by the plant species but also by the harvesting season, geographical area of the sampling, differences in climatic conditions and in the nature of soil where the plants have grown. Moreover, the extraction procedure, the drying process and the storage of plants also play a key role in their phytochemical profile.

In the *Glechoma hederacea* extract, we identified 12 phenolic compounds (Table 2c), all belonging to the phenolic acids and flavanols families. Quantitative data highlighted the presence in the extract of significant quantities of chlorogenic acid and its isomers whereas rutin alongside quercetin and kaempferol derivatives were some of the detected compounds among the flavanols family. As in the case of *S. oleraceus*, the reported data is in line with the literature data but with some differences due to the reasons stated above[28].

Biofilm inhibiting capacity

We then wanted to ascertain whether the hydroalcoholic extracts of the six WEP species displayed anti-biofilm properties on *S. aureus* sessile growth, being the biofilm formation the crucial step to give raise to AMR. We determined the Minimal Biofilm Inhibiting Concentration (MBIC) of the six extracts on *S. aureus* wild type attached culture, as shown in Fig. 3.

We allowed the biofilm to develop for 96 h to obtain a mature biofilm, which is the most difficult form of bacterial growth to eradicate[29], given the self-produced matrix composed of extra-cellular polymeric substances stratified over time.

For all the six extracts, the MBIC inducing the highest biofilm reduction was 0.25 mg of dry extract/mL of solvent (left axis label reports the biofilm fold-reduction values *vs* the solvent (Ethanol 60%), to subtract the

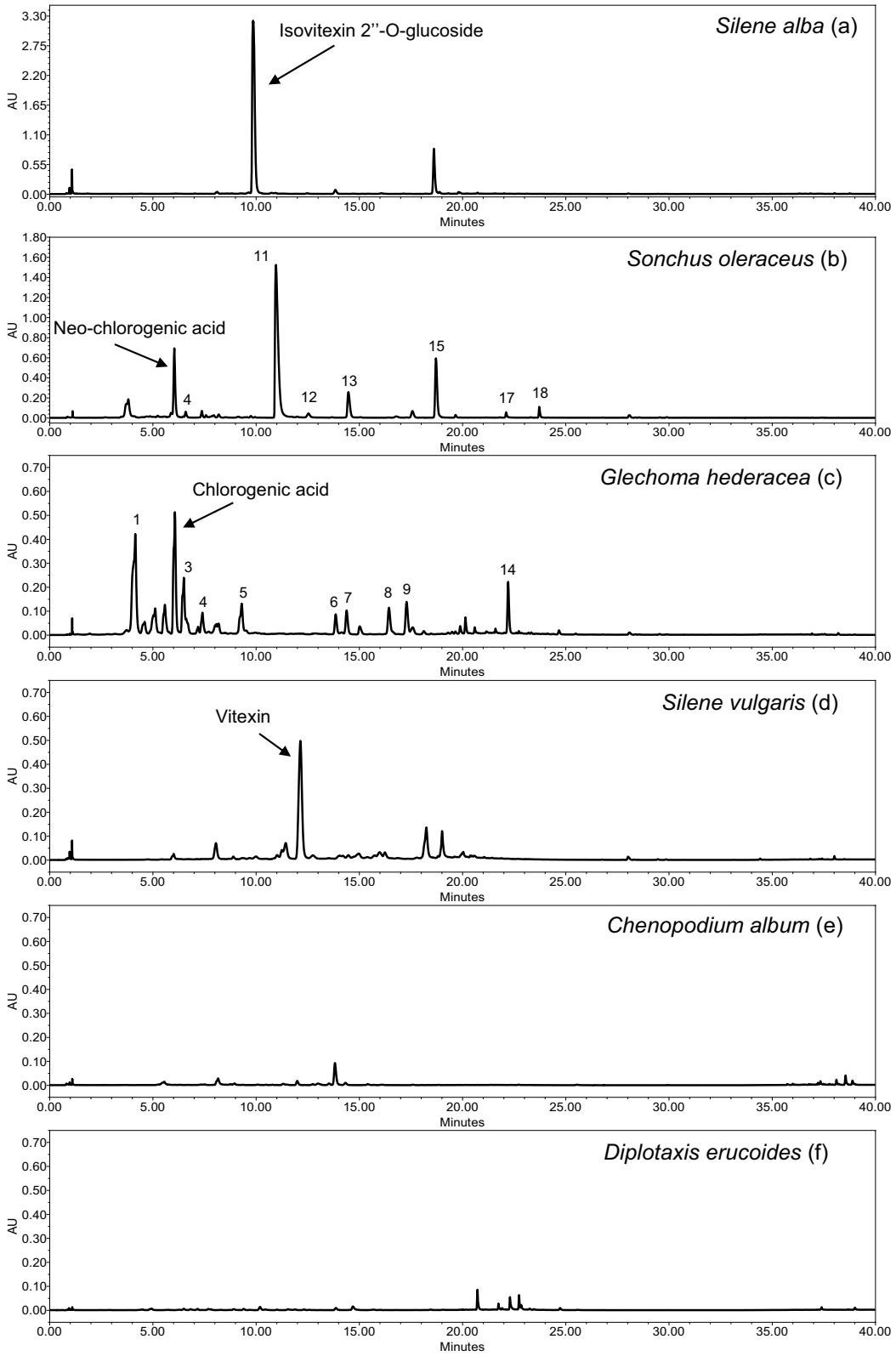


Fig. 2. UPLC-PDA chromatograms of the examined extracts monitored at 320 nm. Quantified peaks are numbered as listed in Table 2. The chromatograms are shown with different labels on the y-axis to highlight both the more abundant compounds present in *Silene alba* and *Sonchus oleraceus* and the lower amount of polyphenols present in the *Glechoma hederacea*, *Silene vulgaris*, *Chenopodium album* and *Diplotaxis erucoides* extracts compared to the two species described above.

(a) *Silene alba*							
Peak	**Compounds**	**$t_{R\ (min)}$**	**Formula**	**Δppm**	**$[M-H]^-$**	**HRMS/MS**	**(mg/g_{DW} ± SD)**
1	Quercetin-6,8-dihexoside[2]	9.12	$C_{27}H_{29}O_{16}$	2.12	609.1451	175.0390, 299.0557, 327.0511, 357.0618	
2	Isovitexin 2"-O-glucoside[2]	11.12	$C_{27}H_{30}O_{15}$	2.25	593.1512	311.0563, 431.0980, 473.1086, 503.1183	10.41 ± 0.125
3	Isovitexin 2"-O-glucoside chloride[2]	11.18	$C_{26}H_{30}O_{15}Cl$	0.96	629.1274	593.1514, 311.0563, 431.0980, 473.1086, 503.1183	
4	Quercetin-8-hexoside[2]	12.18	$C_{21}H_{19}O_{11}$	1.99	447.0931	175.0390, 299.0557, 327.0511, 357.0618	
5	Isovitexin 7-O-[Feruloyl]-Glucoside[2]	19.24	$C_{37}H_{38}O_{18}$	0.33	769.197	311.0563, 341.0667, 413.0889, 431.0993	
6	Isovitexin 7-O-[Feruloyl]-Glucoside chloride[2]	19.24	$C_{37}H_{38}O_{18}Cl$	0.17	805.174	311.0563, 341.0667, 413.0889, 431.0993, 769.1984	
7	Luteolin[1]	21.39	$C_{15}H_{10}O_6$	4.19	285.0404	133.0283, 151.0026, 175.0389, 199.0392	

(b) *Sonchus oleraceus*								
Peak	**Compounds**	**t_R (min)**	**Formula**	**Δppm**	**$[M-H]^+$**	**$[M-H]^-$**	**HRMS/MS**	**(mg/g_{DW} ± SD)**
1	Caftaric acid[2]	2.58	$C_{13}H_{12}O_9$	−2.50	313.0546		163.0386, 135.0438, 117.0334, 89.0389	
2	Caffeic Acid 3-Glucoside[2]	3.39	$C_{15}H_{18}O_9$	−2.68	343.1015		163.0386, 135.0439, 117.0335, 89.0390	
3	Neo-chlorogenic acid[2]	4.08	$C_{16}H_{18}O_9$	−3.31	355.1024		163.0385, 145.0280, 117.0334, 89.0388	1.16 ± 0.047
4	Caffeic acid[1]	4.33	$C_9H_8O_4$	−2.3	181.0491		163.0387, 145.0284, 117.0334, 105.0488, 89.0389	0.07 ± 0.003
5	Quercetin 3-Glucoside-7-Glucuronide[2]	5.30	$C_{27}H_{28}O_{18}$	1.83	641.1324		479.0810, 345.0598, 303.0491	
6	3-O-Coumaroylquinic Acid[2]	5.64	$C_{16}H_{18}O_8$	−2.67	339.1065		255.9325, 189.2199, 147.0437, 119.0490	
7	Vitamin B2[2]	6.37	$C_{17}H_{20}N_4O_6$	0.41	377.1446		243.0878, 216.0760, 198.0659, 172.0863	
8	3-O-Feruloylquinic Acid[2]	6.83	$C_{17}H_{20}O_9$	−2.89	369.1170		326.4700, 232.6290, 177.0543, 145.0281, 117.0334	
9	Caftaric acid isomer[2]	7.92	$C_{13}H_{12}O_9$	3.08	313.0545		163.0386, 135.0438, 117.0334, 89.0389	
10	Kaempferol 3-Gentiobioside[2]	8.20	$C_{27}H_{30}O_{16}$	2.39	611.1583		449.1070, 378.9859, 287.0542, 252.9781, 234.9682	
11	Cichoric acid[2]	8.52	$C_{22}H_{18}O_{12}$	1.98		473.0714	311.0411, 293.0305, 179.0341, 135.0439	3.05 ± 0.510
12	Quercetin 3-Glucoside[1]	9.25	$C_{21}H_{20}O_{12}$	−2.10	465.1017		409.6684, 303.0491, 289.0698, 153.0178	0.19 ± 0.010
13	Quercetin 3-O-Malonylglucoside[2]	11.31	$C_{24}H_{22}O_{15}$	2.89	551.1017		445.5625, 348.0588, 303.0490, 231.0497, 159.0283	1.80 ± 0.088
14	4,5-Di-O-Caffeoylquinic Acid[1]	12.20	$C_{25}H_{24}O_{12}$	−2.40	517.133		499.1222, 398.5441, 337.0981, 319.0982, 163.0385, 145.0280	
15	Apigenin 7-Glucuronide[2]	13.59	$C_{21}H_{18}O_{11}$	2.66		445.0777	269.0456, 175.0241, 113.0231	1.40 ± 0.092
16	Kaempferol 3-(6"-Malonylglucoside)[2]	15.10	$C_{24}H_{22}O_{14}$	−2.65	535.1072		378.0738, 314.3563, 287.0543, 243.8732, 145.0489, 127.0385, 109.0280	
17	Luteolin[1]	19.26	$C_{15}H_{10}O_6$	−2.73	287.0542		269.0439, 241.0492, 213.0535, 153.0179, 147.0436, 135.0437	0.070 ± 0.002
18	Apigenin[1]	21.38	$C_{15}H_{10}O_5$	−3.08	271.0594		187.4386, 171.0286, 163.0383, 153.0179, 145.0282, 119.0491, 91.0545	0.14 ± 0.007

(c) *Glechoma hederacea*								
Peak	**Compounds**	**t_R (min)**	**Formula**	**Δppm**	**$[M-H]^-$**	**$[M-H]^+$**	**HRMS/MS**	**(mg/g_{DW} ± SD)**
1	Neo-chlorogenic acid[2]	4.52	$C_{16}H_{18}O_9$	3.43	353.0878		135.0439, 173.0445, 170.0340, 191.0553	1.77 ± 0.015
2	Chlorogenic acid[1]	6.34	$C_{16}H_{18}O_9$	3.09	353.0878		191.0553, 173.0445	1.28 ± 0.048
3	Cripto-chlorogenic acid[2]	6.81	$C_{16}H_{18}O_9$	3.18	353.0878		135.0439, 173.0445, 170.0340, 191.0553	0.76 ± 0.018
4	Quercetin-x,x-di-glucoside[2]	7.82	$C_{27}H_{29}O_{17}$	2.00	625.1412		255.0290, 271.0240, 301.0355, 343.0459, 463.0878	0.08 ± 0.001
5	"Quercetin 3-(6"-Malonylglucoside)−7-Glucoside"[2]	9.57	$C_{30}H_{32}O_{20}$	2.49	711.1409		667,1519, 463,0867, 462,0807, 299,0197, 301,0354	
6	Rutin[1]	14.74	$C_{27}H_{29}O_{16}$	0.82	609.1455		300.0277, 301.0345, 302.038	0.53 ± 0.022
7	Quercetin-3-hexoside[2]	15.07	$C_{21}H_{19}O_{12}$	2.56	463.0883		255.0300, 271.0251, 300.0277, 301.0345	0.72 ± 0.027
8	Quercetin 3-(6"-Acetylglucoside)[2]	17.11	$C_{23}H_{22}O_{13}$	2.70	505,0983		463,0882, 300,0376, 301,034 271,0248, 255,0298	
9	Quercetin 3-[6"-(3-Hydroxy-3-Methylglutaryl)Galactoside][2]	17.99	$C_{27}H_{28}O_{16}$	1.97	607.1306		505,0992, 463,0887, 300,0276, 301,0345, 271,0248, 255,0298	0.61 ± 0.002
10	Kaempferol-3-hexoside[2]	18.38	$C_{21}H_{19}O_{11}$	2.47	447.0929		227.0345, 255.0299, 284.0338, 285.0339	0.06 ± 0.002
11	Rosmarinic acid[1]	19.30	$C_9H_5O_3$	0.54	359.0774		135.0440, 161.0234, 179.0340, 197.0448	
12	4,5 dicaffeoylquinic acid[1]	19.49	$C_{25}H_{23}O_{12}$	0.72	515.1188		173.0446, 179.0341, 353.088	0.24 ± 0.001
13	Kaempferol 3-[6"-(3-hydroxy-3-methylglutaryl)glucoside][2]	19.91	$C_{27}H_{27}O_{15}$	1.57	159.1352		284.0328, 285.0402, 447.0934, 498.1040	
14	Luteolin[2]	21.23	$C_{15}H_{10}O_6$	0.35		287.0551	269,0445, 241,0494, 213,0541, 153,0183	
15	Kaempferol[1]	22.99	$C_{15}H_9O_6$	3.76	285.0404		117.0330, 135.0069, 151.0022, 257.0430	

(d) *Silene vulgaris*							
Peak	Compounds	t_R (min)	Formula	Δppm	$[M-H]^+$	HRMS/MS	(mg/g_{DW} ± SD)
1	Isoorientin 2"-Glucoside [2]	6.02	$C_{27}H_{30}O_{16}$	−3.42	611.1586	449.1061, 413.0845, 353.0540, 329.0644, 299.0538	
2	Vitamin B2[2]	6.38	$C_{17}H_{20}N_4O_6$	4.02	377.1440	243.0878, 216.0760, 198.0659, 172.0863	
3	Luteolin 6-C-glucoside-8-C-arabinoside[2]	6.86	$C_{26}H_{28}O_{15}$	−4.32	581.1477	563.1381, 509.1088, 461.0884, 425.0885, 395.0748, 365.0640, 341.0642, 311.0539	
4	Isoorientin 2"-Glucoside (isomer)[2]	7.25	$C_{27}H_{30}O_{16}$	−3.42	611.1586	449.1061, 413.0845, 353.0540, 329.0644, 299.0538	
5	Luteolin 8-C-Glucoside[2]	7.82	$C_{21}H_{20}O_{11}$	−3.64	449.1062	431.0957, 413.0954, 353.0632, 339.0849, 329.0645, 299.0542	
6	Apigenin 6-C-Alpha-L-Arabinopyranoside-8-C-Beta-D-Glucopyranoside[2]	7.91	$C_{26}H_{28}O_{14}$	−4.91	565.1527	511.1229, 481.1126, 409.0902, 379.0796, 337.0694, 325.0696, 295.0589	
7	Luteolin-8-glucoside-2-O-arabinosylpyranoside[2]	8.02	$C_{27}H_{30}O_{15}$	−3.96	595.1631	449.1060, 431.0957, 413.0954, 353.0632, 339.0849, 329.0645, 299.0542	
8	Luteolin-8-hexityl monoxyloside (adonivenith)[2]	8.14	$C_{26}H_{28}O_{15}$	−3.59	581.1477	449.1060, 431.0957, 413.0954, 353.0632, 329.0645, 299.0542	
9	Vitexin[1]	9.29	$C_{21}H_{20}O_{10}$	−4.46	433.1113	397.0908, 337.0681, 323.0981, 313.0694, 297.0753, 283.0587	1.01 ± 0.001
10	Vitexin 2"-O-Xyloside[2]	9.56	$C_{26}H_{28}O_{14}$	−2.75	565.1528	433.1118, 397.0908, 337.0681, 313.0694, 295.0508, 283.0587	
11	Vitexin 2"-O-Rhamnoside[2]	10.09	$C_{27}H_{30}O_{14}$	−1.99	579.1682	433.1118, 397.0908, 367.0805, 337.0681, 313.0694, 283.0587	
12	Luteolin 7-O-(6-O-Malonyl-Beta-D-Glucoside)[2]	10.54	$C_{24}H_{22}O_{14}$	−2.31	535.1070	449.1060, 431.0957, 413.0954, 353.0632, 329.0645, 299.0542	
13	Vitexin 2"-O-Rhamnoside-4""-Acetate[2]	15.56	$C_{29}H_{32}O_{15}$	−2.26	621.1783	433.1118, 397.0908, 367.0805, 337.0681, 313.0694, 283.0587	

Table 2. Plant extract characterization. **(a)***Silene alba*; **(b)***Sonchus oleraceus*; **(c)***Glechoma hederacea*; **(d)***Silene vulgaris*. Compounds identified in the four antimicrobial extracts by UHPLC-HRMS and content of the predominant (2.a, 2.d), or the more abundant (2.b, 2.c), phenolic compounds assessed by UPLC-PDA. Quantitative data are the mean values of three measurements and are reported as mg of compound per gram of plant material (DW). t_R = retention time. 1 MS, MS^2 = Compounds identified by the reference standards injection. 2 MS, MS^2 = Compounds identified based on Experimental Data, Library MS^2.

effect, even if minimal, of the solvent used for the extracts). This MBIC induced roughly a fourfold biofilm reduction for all the species but *G. hederacea*, which induced a twofold biofilm reduction.

To exclude any antimicrobial activity of the solvent, we calculated the biofilm fold-change induced by WEPs extracts *vs* the solvent, which did not induce any change when used alone (1.0-fold *vs* untreated bacteria, right axis label (blue color) reports the biofilm fold-reduction values *vs* the untreated bacteria). The effect of Gentamicin and Rifampicin (biofilm fold-reduction values of 0.8- and 0.7-fold reduction *vs* the untreated bacteria, used at MIC of 5 µg/ml) was much less pronounced in comparison with that induced by the WEPs extracts (Fig. 3).

MIC and MBC

We then carried out the MIC and MBC determination of the six WEPs hydroalcoholic extracts on *S. aureus* wild type and MRSA strains in planktonic growth, to ascertain their efficacy in bacteria growing in liquid culture media. We analyzed a range of concentrations from 0.5 to 2.8 mg of dry extracts/mL of solvent.

The achieved results (Table 3) highlight that S. *alba* and *S. vulgaris* were, respectively, the most and the least effective extract, among the four species that exhibited inhibiting and bactericidal power.

The other two WEP species, *C. album* and *D. erucoides*, did not display antimicrobial properties over the range of concentrations tested. In particular, the highest concentration tested (2.8 mg of dry extracts/mL of solvent) was equivalent to that of the undiluted extracts of *C. album* and *D. erucoides*.

Cell wall damage evaluation

In order to assess whether the extracts antibiofilm and bactericidal effects could be due to a damage to the bacterial cell wall, we used the Propidium Iodide (PI) staining of the WEP-treated bacteria. PI staining is not a vital one, since it is not able to enter in viable bacterial cells, with an intact cell wall[30]. On the contrary, in killed bacteria, it passes through injured cell membranes and stains the nuclei[31]. According to numerous studies, PI positive staining indicated damaged plasma membranes[31,32]. In Fig. 4b. we show, again, that the three extracts with the greater antibacterial activity (*S. alba*, *S. oleraceus* and *G. hederacea*), used at the MIC concentration, caused the higher cell wall damage, whereas there was no impact on the cell wall of untreated bacteria, or bacteria treated with the solvent (Fig. 4a). The other three WEPs extracts (*S. vulgaris*, *C. album* and *D. erucoides*,

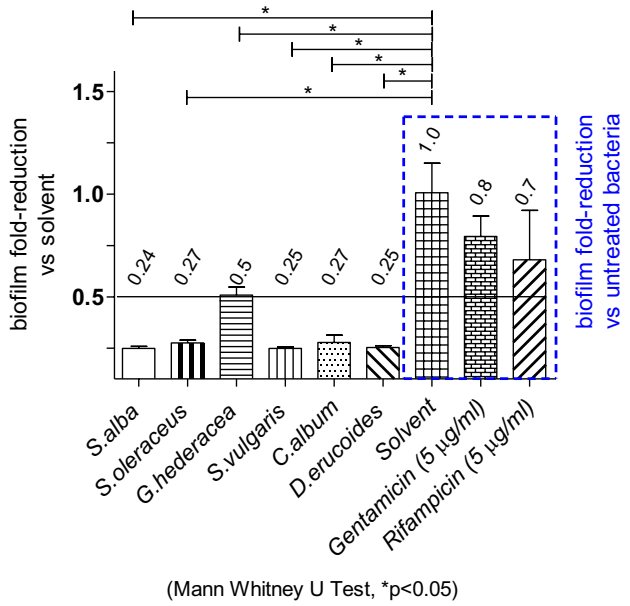


Fig. 3. Mature biofilm inhibiting capacity of the six WEPs extracts *vs S. aureus* wild type, ATCC 25923. For all the six extracts, the MBIC inducing the highest biofilm reduction was 0.25 mg/mL. This MBIC induced roughly a fourfold biofilm reduction for all the species but *G. hederacea*, which induced a 2.0-fold biofilm reduction. n = 5. Effect of the extracts: Left axis label (black color) reports the biofilm fold-reduction values *vs* the solvent, to subtract the effect, even if minimal, of the solvent used for the extracts. Effect of Solvent, Gentamicin and Rifampicin: right axis label (blue color) reports biofilm fold-reduction, respective values of 1.0-, 0.8- and 0.7-fold, *vs* the untreated bacteria, inside the dashed blue frame. We used Gentamicin and Rifampicin at MIC values of 5 µg/ml. Different statistical difference ($p < 0.05$) are based on Mann Whytney U test.

	Planktonic bacteria			
	S. aureus		MRSA	
	MIC	MBC	MIC	MBC
Plant Species	[mg/mL]			
Silene alba	0,6	0,61	0,60	0,61
Sonchus oleraceus	1,1	1,13	1,13	1,26
Glechoma hederacea	1,6	1,89	1,70	1,89
Silene vulgaris	1,98	2,64	1,98	2,64
Chenopodium album	> 2,8	> 2,8	> 2,8	> 2,8
Diplotaxis erucoides	> 2,8	> 2,8	> 2,8	> 2,8

Table 3. MIC-MBC determination. MIC and MBC of the six WEPs extracts *vs S. aureus* wild type and MRSA strains. We assayed each extract in a concentration range between 2.8 and 0.5 mg dry extract/mL of solvent in a microdilution assay.

Fig. 4c) caused a much smaller cell wall damage. The analysis of mean fluorescence confirms a strong differences between the most and lowest active species, by setting a cut off the mean fluorescence value at 10,000 (Fig. 4d).

Anti-adherence properties of the six WEPs hydroalcoholic extracts

We performed a Zone Of Inhibition (ZOI) agar-based assay[33] in order to assess whether the observed anti-biofilm effect could be due to the anti-adherence effect of the WEPs extracts. This assay was performed to ascertain whether the WEPs extracts administration, at a reduced MIC (1/50 of the MIC value), might be able to inhibit the initial *S. aureus* and MRSA bacterial adhesion to a substrate, which is the very early step for the following biofilm formation[34]. The analyses performed on the six species confirmed that *S. vulgaris, C. album and D. erucoides* did not induce an anti-adherence effect (data not shown), while *G. hederacea*, *S. alba* and *S. oleraceus* induced a clear anti-adherence effects.

In Fig. 5, we show that the extracts of *G. hederacea*, *S. alba* and *S. oleraceus* induced the higher ZOI in the growth of both *S. aureus* and MRSA. The mean inhibition halos diameter in the three experiments performed

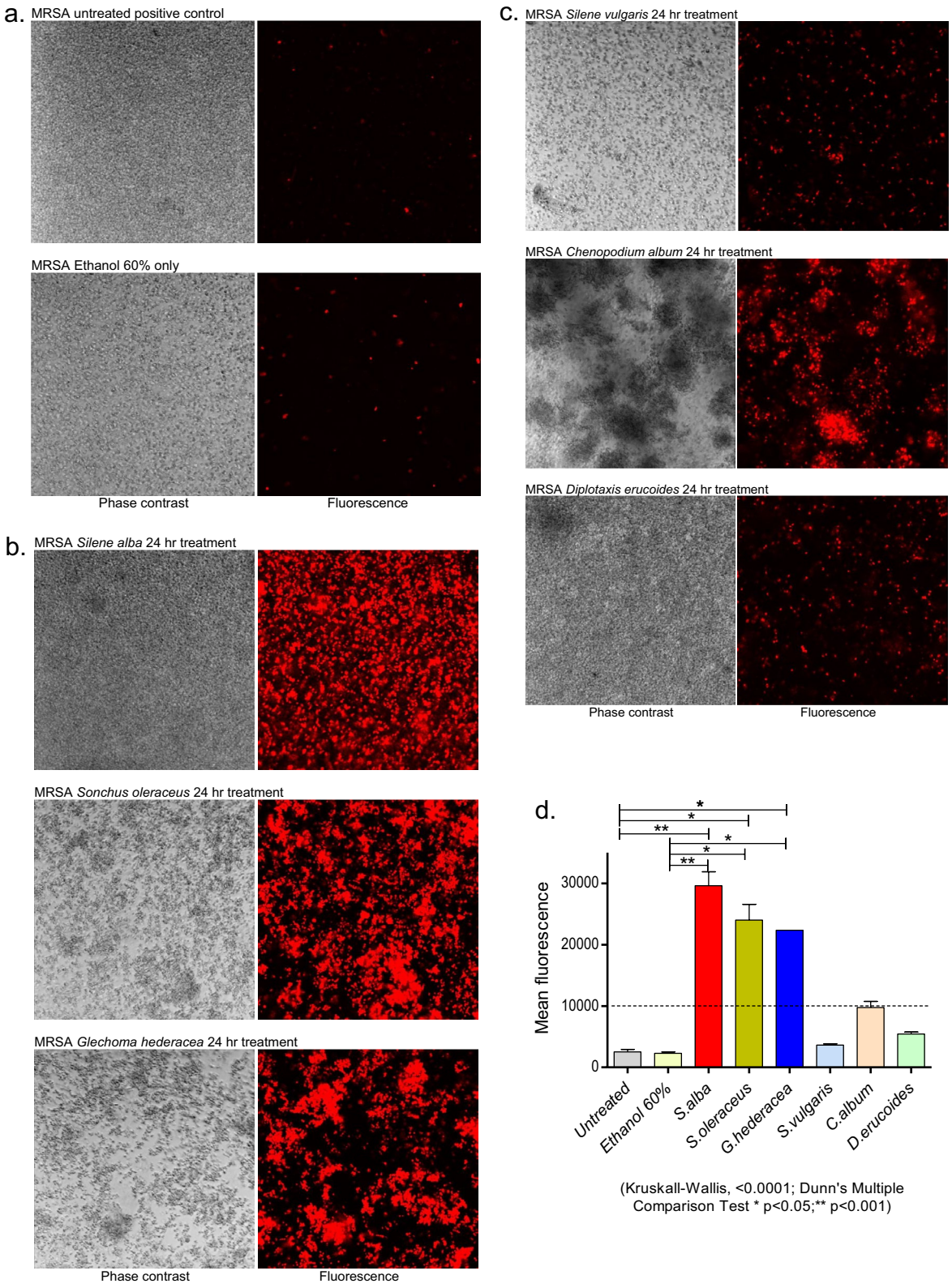


Fig. 4. MRSA Bacterial Cell wall damage evaluation by Propidium Iodide staining. The phase contrast of the wells is on the left panel, and the fluorescence signal is on the right panel for each line. **a.** untreated bacteria (upper panel) and bacteria treated with solvent (lower panel); **b.** bacteria treated with *S. alba* (upper panel), with *S. oleraceus* (middle panel) and *G. hederacea* (lower panel) extracts; **c.** bacteria treated with *S. vulgaris* (upper panel), *C. album* (middle panel) and *D. erucoides* (lower panel) extracts; **d.** mean fluorescence values of the samples. n = 6 for Solvent and Untreated bacteria, and n = 4 for all the others datasets. Different statistical difference ($p < 0.05$) are based on Kruskal Wallis and Dunn's Multiple comparison test.

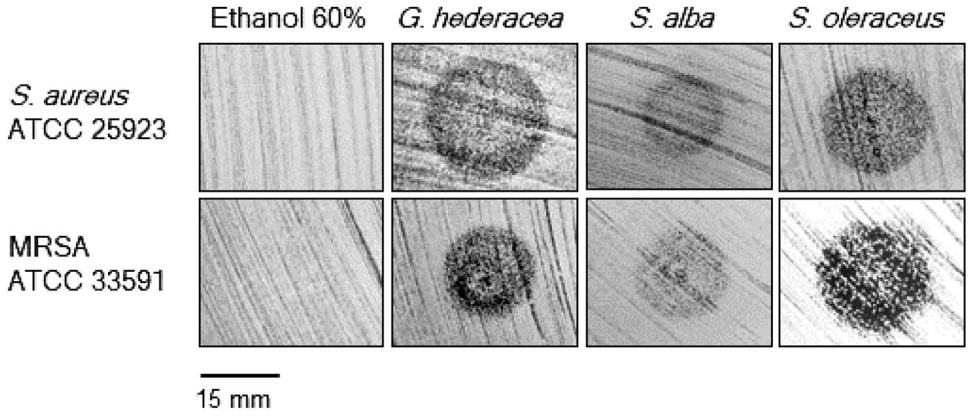


Fig. 5. Anti-adherence properties of the three most powerful WEPs extracts *vs S. aureus* wild type and MRSA in terms of halos diameter of adhesion inhibiting activity, displaying one representative experiment, out of three. The concentration of all the extracts was 1/50 of the respective MIC value.

was, for *G. hederacea,* 19 mm *vs S.aureus* and 15 mm *vs* MRSA; for *S. alba,* 15 mm *vs S. aureus* and MRSA and, for *S. oleraceus,* 18 mm vs *S. aureus* and 20 mm *vs* MRSA.

It is interesting to note that this result is coherent with that of the MIC-MBC assessment, in which the very same three species displayed the most powerful effect on the bacterial planktonic growth (Table 3). It is also worth noting that, in this case, the effective extract concentration is rather low, being it 1/50 of the MIC value, suggesting that a smaller amount of plant extract is needed to induce an adherence inhibition zone on MH agar plate, as compared to the inhibition of planktonic growth at 24 h in liquid culture.

Disc-diffusion assay and antibiotic bacterial resistant colonies occurrence

We then used the disc-diffusion assay to assess the growth inhibition diameter induced by the three most bactericidal and adhesion-inhibiting WEPs extracts (Fig. 6a). To gain a better understanding of the results of co-administration with the selected antibiotics, we decided to reduce the MIC value of the extracts by half (1/2 MIC).

We selected the antibiotics by searching The Antimicrobial index Site (https://antibiotics.toku-e.com/#:~:text=The%20Antimicrobial%20Index%20is%20a,worldwide%20scientific%20community%20at%20large) for drugs with a known MIC and MBC on *S. aureus*. We then used Antimicrobial Susceptibility Test Discs with Ampicillin (30 µg/disc) and Amoxicillin (10 µg/disc) at their respective MIC value for *S. aureus* 25923, the two antibiotics to which the two *S.aureus* strains resulted resistant (see Figure S1).

For each WEP extract, on the same agar plate, we compared the growth inhibition diameter induced by the extract (blue line), by the Antibiotic (grey line), and by the co-administration of the extract on the antibiotic disc (red line). The graph in Fig. 6b shows the values of the subtraction of the blue circles (only extract) from the red ones (extract plus antibiotic).As shown in Fig. 6a, all the three extracts co-administration with antibiotics increased the growth inhibition diameter (ranging from 21 to 54% increase in mm), as compared with the extract, or the antibiotic, when administered alone (Table 4a). Although we cannot define this result a proper synergy, it might suggest that the extracts co-treatment with the antibiotics could have made *S.aureus* and MRSA bacteria more susceptible to these drugs, as shown by the statistical analysis in Table 4a.

We then wondered also whether the inhibition zones contained bacteria that were either inhibited or killed. To this end, we plated the agar, collected inside the inhibition zones (Figure S2b), on a fresh MH agar plate, without any compound.

Concerning *S. alba* and *G. hederacea,* the bactericidal effect prevailed in the co-administration (no bacterial growth was already evident with the extract alone, after 24 h on a fresh plate), whereas, regarding *S. oleraceus,* the co-administration strengthened both the extract (with which few CFU were present in extract alone fresh plate) and the Antibiotics (where bacterial growth was evident when they were administered alone, after 24 h on a fresh plate, Table 4b).

Finally, after 120 h culture, we determined the possible occurrence of ABR colonies in MRSA, in Antibiotic *vs* Extract-Antibiotic treated bacteria. As it is shown (Fig. 6c), in the case of Ampicillin, after 120 h of culture, the growth inhibition zone was recolonized by Amp-Resistant MRSA bacteria. Differently, no bacterial colonies were found in the combination of the extracts (green drop) with Amp on the very same disc, thus confirming that death of the bacterial cells treated with the hydro-alcoholic WEPs' extracts.

Discussion

Plant specialized metabolites, commonly referred to as phytochemicals, are emerging for their effectiveness in combating antibiotic-resistant bacterial pathogens[35]. Recent research has shifted focus from the health properties of individual bioactive metabolites to the potential of phytocomplexes, where diverse secondary metabolites act synergistically to exert a therapeutic effect, including antioxidant or anti-inflammatory activities[36]. Our study embraced the latter type of approach, aiming to reveal the healthy features of these WEPs, inspired by the

concept of food synergy[37]. By providing a comprehensive profile of these WEPs, we intended to encourage the increase of their consumption in the daily diet and to promote their potential as adjuvant to antibiotics in the treatment of bacterial infections.

Medicinal plants have been reported to inhibit both Gram-negative and Gram-positive bacteria. Furthermore, their composition, consisting of hundreds of different molecules makes it challenging for bacteria to develop resistance to these natural compounds. In addition, their antioxidant properties have a positive effect on the human immune system, enhancing its ability to combat infections[38].

In this scenario, we outlined the whole antimicrobial and phytochemical profile of six WEPs hydro-alcoholic extracts, among which the ethanolic extracts of *S. alba*, *S. oleraceus* and *G. hederacea* showed remarkable antimicrobial properties against *S. aureus* and MRSA, both in planktonic culture and in mature biofilm. This is what makes them so interesting, because bacteria do not always respond equally to antimicrobials in both growth modes. All six WEPs hydro-alcoholic extracts demonstrate a higher efficacy in reducing biofilm formation compared to the antibiotics Gentamicin and Rifampicin (Fig. 3). The limited effectiveness of Gentamicin and Rifampicin in reducing biofilm formation is unsurprising, given the significantly higher level of antibiotic resistance in biofilm than in younger sessile cultures[39]. A more detailed investigation into the effects of the hydro-alcoholic extracts of the six WEPs on *S. aureus* and MRSA revealed that *S. alba*, *S. oleraceus* and *G. hederacea* exhibit stronger antimicrobial effects than the other three species. Specifically, these extracts induce a higher MRSA cell wall damage than the other species (Fig. 4) and display clear anti-adherence properties (Fig. 5), whereas the other three species do not alter the bacterial adherence capacity (not shown). Interestingly, preventing bacterial adhesion to surfaces is the critical first step in avoiding biomaterial-associated infections[34]. Moreover, *S. alba*, *S. oleraceus* and *G. hederacea* also demonstrate a clear bactericidal effects, with bacterial colonies treated with their hydro-alcoholic exhibiting reduced antibiotic resistance (Fig. 6c).

For three out of the six species (*S. alba*, *S. vulgaris* and *D. erucoides*) this is, to the best of our knowledge, the first study analyzing their antimicrobial properties *vs* MRSA.

Glechoma hederacea has been previously studied, but only against *S. aureus* wild type strains, where it was found to be either inactive as industrial tincture[40] or bacteriostatic as a fractionated aqueous extract[41].

This underlines the complexity of comparing extracts obtained through different extraction methods.

S. oleraceus was only tested against *S. aureus*[42], while only one study[43] employed a fractionated extract against a clinical isolate of MRSA.

An apparent paradox occurs for *C. album,* a widely studied WEP species, compared to the other five species examined in our study[44]. In the literature, *C. album* was described as antibacterial only against *S. aureus*[16].

Differently, in our experiment, *C. album* did not show similarly strong antibacterial activity. However, previous studies on *C. album* utilized a different solvent and used plants harvested from diverse countries, including South Africa, India and the inland area of Kilis in Turkey. Otherwise, our *C. album* samples were harvested in the Mediterranean region of central Italy, characterized by a more temperate climate. It is known that climatic conditions during plant growth, as well as the timing of harvest, strongly influence the phytochemical composition and the content of antioxidant molecules, which in turn influence the antimicrobial properties.

The comparison of antioxidant properties, total phenolic content and phenolic profiles of the six WEPs provides insight into their antimicrobial capacity. Phenolic compounds in *S. oleraceus* and *G. hederacea*, two of the three most effective WEPs, were significantly higher, both in quantity and diversity, compared to the other examined species (Table 2a,b,c,d). These species were characterized by the presence of various polyphenols classes like phenolic acids, flavonoids and flavones.

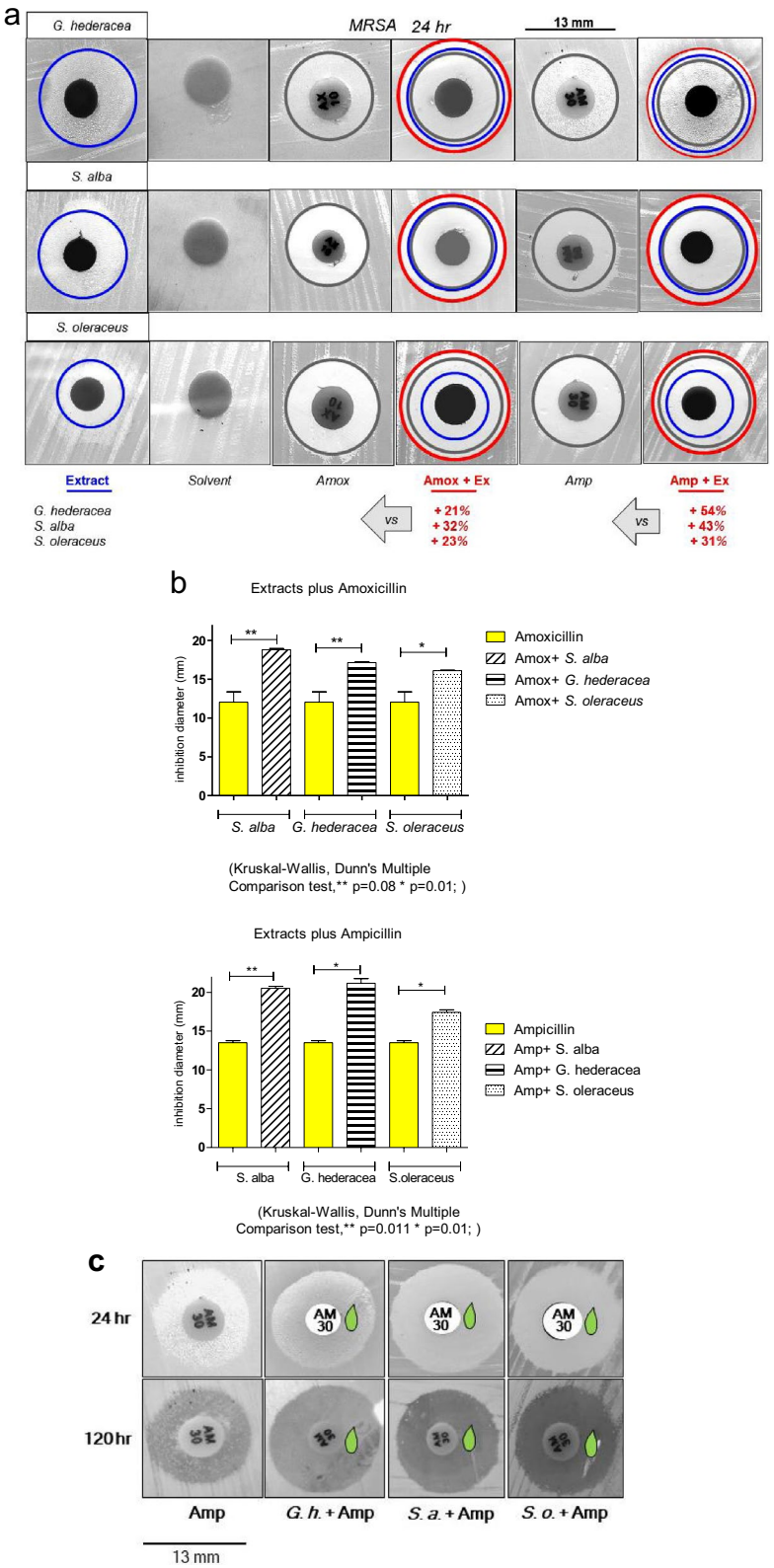
These findings are consistent with the high antioxidant and antimicrobial properties showed by these plants (Fig. 1c-e, Figs. 3,4,5,6). Notably, both extracts contained chlorogenic acid, a polyphenol that has recently been shown to disrupt the *S. aureus* outer membrane and increase both outer and plasma membrane permeability[45]. This increased permeability could have easily played a role in the cell wall damage induced by the extracts of these two species (Fig. 4).

On the other hand, the low TPC observed in both the analyzed *Silene* species is not in accordance with their high antioxidant and antimicrobial activities, particularly in the case of *S. alba*, one of the three species exhibiting the strongest antimicrobial properties. It is important to note that there are significant differences in both the specific phenolic compounds present and their concentrations between the two *Silene* species (see the different scale in Fig. 2 and Table 2).

In these species the most active phenols might be apigenin and luteolin derivatives (as isovitexin 2"-O-glucoside, vitexin, isoorientin, etc.) which are known to have very high biological activities (anticancer, anti-inflammatory, antioxidant and antimicrobial properties)[46,47]. In particular, Luteolin[48] was reported able to destroy the integrity of the cell membrane and to possess a strong inhibitory effect on the biofilm formation. A recent study on the properties of a *Cajanus cajan* extract[49], found that the presence of various isovitexin derivatives in the extract was associated with the death of *S. aureus* bacteria, as evidenced by PI staining analysis. Thus, isovitexin-associated PI positive staining confirms cell wall damage and subsequent permeabilization. Interestingly, isovitexin is the main peek present in *S. alba* (Fig. 2, Table 2).

In *Silene*, as well as in the other WEPs species, it cannot be excluded that other specialized metabolites contribute to the observed antimicrobial properties.

Notably, the tree most active WEPs are characterized by higher level of ASC and a greater capacity to reduce ferric to ferrous ions compared to the other three species (Fig. 1b and e). In the last years, several papers have highlighted the antibacterial effects of ASC, which can modify the antimicrobial activity of various antibiotics[50]. In addition to its antioxidant properties, ASC can also act as pro-oxidant. Ferric ions are involved in the Fenton reaction, one of the most dangerous spontaneous reaction occurring in aerobic environment that produces toxic oxygen-derived radical species[51]. Therefore, the elevated levels of ASC and ferric ion production may contribute to the antimicrobial properties of the three most effective WEPs.



Finally, as mentioned earlier, *Chenopodium album* and *Diplotaxis erucoides* did not display a great biological activity, except for bacterial biofilm inhibition. According to this outcome, both extracts showed low TPC and lower antioxidant activities compared to the other analyzed extracts (Fig. 1). Moreover, Figs. 1 and 2 clearly shows that these two extracts have a very low content of phenolic compounds.

As suggested by the concept of food synergy[37,52,53], we suppose that there is a relationship between the different phytochemical groups identified and the antimicrobial activity of the analyzed WEPs extracts.

◀**Fig. 6**. MRSA ZOI pattern after administration of *S. alba*, *G. hederacea* and *S. oleraceus* WEPs Extracts, both alone or in combination with the two Antibiotics to which the *S.aureus* wild type and MRSA resulted resistant (Ampicillin and Amoxicillin, see Figure S1). **a.** picture of a representative experiment, out of three, of the growth inhibition diameter induced by the three extracts alone at 1/2 of their MIC (blue line), or in combination with Ampicillin and, respectively, Amoxicillin (red line); the administration of each antibiotic alone is indicated by a grey line. **b.** different statistical differences are based on Kruskal Wallis, $p < 0.05$, with particular attention to Antibiotics (n = 9) *vs* Antibiotics plus Extracts (n = 3) inhibition diameter lengths; **c.** appearance of ABR colonies, after 120 h, between Antibiotic alone (Ampicillin), and Antibiotic co-administration with Extracts. The green drop drawing indicates the extract administration on the Antibiotic disc.

a					
Amoxicillin					
	Inhibition halos diameter (mm)			**Delta vs Amox**	
Species	**Extract**	**Amox**	**Ex + Amox**	**mm**	**%**
Silene alba	15	14	**18,5**	4,5	**32**
Glechoma hederacea	18	14	**17**	3	**21**
Sonchus oleraceus	10	13	**16**	3	**23**
Ampicillin					
	Inhibition halos diameter (mm)			**Delta vs Amp**	
Species	**Extract**	**Amp**	**Ex + Amp**	**mm**	**%**
Silene alba	15	14	**20**	6	**43**
Glechoma hederacea	18	13	**20**	7	**54**
Sonchus oleraceus	10	13	**17**	4	**31**

b					
	Extract	**Amox**	**Ex + Amox**	**Amp**	**Ex + Amp**
Species	**CFU at 24 h**				
Silene alba	no CFU	few CFU	no CFU	CFU	no CFU
Glechoma hederacea	no CFU	few CFU	no CFU	CFU	no CFU
Sonchus oleraceus	few CFU	few CFU	no CFU	CFU	no CFU

Table 4. a. Measures of the growth inhibition diameter induced by the three extracts alone, or in combination with Ampicillin and Amoxicillin (one representative experiment, out of three, as shown in Fig. 6a, Antibiotic plus extracts; **b.** comparison of inhibiting (few CFU) and bactericidal (no CFU) effects by plating the agar inside the inhibition zone after 24 h (see also figure S2b).

Conclusion

In Fig. 7, we grouped all the findings of this study, to identify a common trait that could potentially classify an antimicrobial plant extract. At a first glance, the presence of active polyphenols, the ascorbate content and the iron reducing capacity seems to be related to the ability of causing adhesion inhibition and cell wall damage to *S. a*. and MRSA. This trait characterizes the three most antimicrobial WEPs species *S. alba*, *S. oleraceus* and *G. hederacea*. The bactericidal effects of the hydro-alcoholic extracts and their capacity of reducing antibiotic resistance make these WEPs of particular interest.

It is also worth noting that all the six extracts inhibited the *S. aureus* mature biofilm, supporting the established knowledge that the bacterial cell wall composition of the sessile growth is very dissimilar in respect with the planktonic one[39]. To cite some studies, Williams[39]describes significant physiological adaptation occurring the early phase of the attached growth, like bacterial colony size, protein content, which are smaller in attached *vs* planktonic *S. aureus* cultures. Belley reported that the lipoglycopeptide Oritavancin sterilized biofilms of MSSA, MRSA, at minimal biofilm eradication concentrations (MBEC). Importantly, MBECs for Oritavancin were within 1 doubling dilution of their respective planktonic broth MICs. The study also showed that the attached culture displayed a lower membrane potential as compared with the planktonic one[54]. Another previous study characterized the composition of attached and planktonic bacterial culture composition, showing that the first is composed by 10% of viable cells, and the second is close to 100% viable cells[55].

These differences, among others, might explain the reported diverse response of biofilm and liquid culture of bacteria to the very same extract of *Chenopodium album* and *Diplotaxis erucoides*. We might figure out that these two species, that we found poorer of phenolic compounds than the other four species, evidently contained enough specialized metabolites to affect, at a low concentration (1/20 MIC), the mature bacterial biofilm (96 h), mostly composed of non-viable cells.

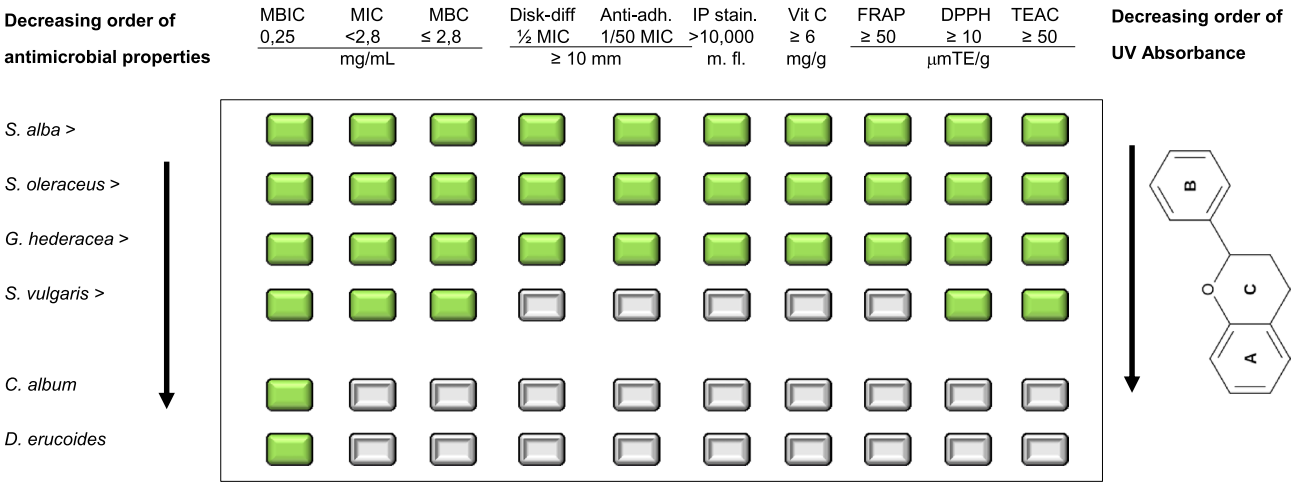


Fig. 7. Summary picture of the properties displayed by the six WEPs species, in decreasing order of bactericidal power, determined by MIC-MBC assay and polyphenols UV absorbance at 320 nm. In green, we annotated the extracts displaying: a) an MBIC = 0.25 mg/mL; b) a MIC < 2.8 mg/mL; c) an MBC ≤ 2.8 mg/mL; d) a disc-diffusion and e) an anti-adherence inhibition halos ≥ 10 mm; f) an IP stain with the mean fluorescence signal > 10,000; g) a total Ascorbate ≥ 6 nmol/g DW; h) a FRAP and a i) TEAC assay ≥ 50 μmTE/g DW and a l) DPPH assay ≥ 10 μmTE/g DW.

However, in future studies such a finding will deserve further examination of the bacterial cell wall integrity and the cell membrane permeability upon treatment with our WEPs extracts. Furthermore, it will be useful to analyze whether these WEPs extract induce ROS generation in treated bacteria.

Finally, the three most efficacious species are still largely used in Italian regional recipes, hence our results might well encourage their further consumption, thus improving dietary plant biodiversity and contributing to the maintenance of human health and the contrast of bacterial ABR insurgence.

This work could actually help promoting the valorization of WEPs, an essential activity to preserve edible plant biodiversity. In addition, these data might help improving the western eating habits and propose these species as, both, a nutraceutical for the daily diet and a valuable phytotherapeutic tool to counteract the onset of antibiotic-resistance in pathogenic bacteria.

Methods

Plant materials

S. alba, S. vulgaris, C. album, S. oleraceus, and *G. hederacea* were kindly provided by the Phytoalimurgic Garden in Veneto Region, Padua University. *Diplotaxis erucoides* was harvested in the Garden of Simples (http://www.isb.cnr.it/orto-dei-semplici/) and identified according to EuroMed PlantBase (https://europlusmed.org). The six WEPs species analyzed in this study are listed in Table 1. All of them are edible and known as medicinal wild herbs, as annotated by the Acta Plantarum botanical website (https://www.actaplantarum.org/forum/viewtopic.php?t=14081; 47,922; 83,179; 3300; 7645; 5732). The aerial part of each species is shown in Suppl. Mat. Figure S1.

Preparation of plant extracts

Stems and leaves of the plants were chopped under liquid N_2 and then macerated in 60% ethanol–water at a 1:5 plant to solvent ratio for 15 days at room temperature in the dark, according to the method reported in European Pharmacopeia in 2006[56] (EMA, 2006).

Notably, plants material (20 g) was macerated with 100 mL of the hydroalcoholic solvent.

The extract was then filtered using a sterile cotton tissue, sterilized using a 0.22 μm Polyethersulfone (PES) filter and then stored at – 80 °C until use. Aimed at determining the extract amount per mL of solvent, a precise aliquot (1 mL) of each one was freeze-dried after ethanol removal under a gentle stream of nitrogen.

Total phenolic content and ascorbate quantification

Total phenolic content was assayed according to the Folin–Ciocalteau method[57], with minor modification, using gallic acid as standard. A volume of 1580 μL of a mixture of methanol/water (50:50) and 100μL of Folin–Ciocalteau reagent were added to 20 μL of sample extract (or standard solution) and incubated at room temperature for 8 min. Then, 300 μL of Na_2CO_3 (20%w/v), were added. The solution mixture was incubated for 2 h in the dark at room temperature. The absorbance at 765 nm, was measured in a 96-well plate (Greiner Bio-one, Frickenhausen, Germany) using a multifunctional microplate reader (Tecan Infinite M200PRO multiplate reader, Männedorf, Switzerland). The results are expressed as mg of gallic acid equivalents (GAE) per g of dry weight (DW). Total ascorbate content (reduced and oxidized forms) was measured according to Cimini et al.[58]. In both cases, three biological replicas were carried out, and for each biological replicate, at least three technical replicates were made.

Antioxidant capacity assays

For all antioxidant assays, the extract was centrifuged at 14,000 ×g (rcf) for 10 min at 4 °C, and the resulting supernatant was collected. For the experiments, three biological replicas were carried out, and for each biological replicate, at least three technical replicates were made. Results are expressed as μmol Trolox equivalent (TE) per g of dry weight.

DPPH (1,1-Diphenyl-2-Picrylhydrazyl) radical scavenging assay was analyzed as previously reported by Tonto et al. (Tonto et al., 2023)[59]. Trolox was used as a standard for calibration curve construction in the range of 10–200 mg L^{-1}.

FRAP (Ferric-Reducing Antioxidant Power) assay was based upon the methodology previously described by Tonto et al.[59]. Trolox was used as a standard for calibration curve construction in the range 20–200 mg L^{-1}.

TEAC (Trolox Equivalent Antioxidant Capacity) assay was evaluated as previously described by Tonto et al.[59] (Tonto et al., 2023). Trolox was used as a standard for calibration curve construction in the range of 10–700 μM.

LC-HRMS analysis

An Agilent 1290 UHPLC system coupled to a Thermo Fisher Q-Exactive Focus Quadrupole-Orbitrap Mass Spectrometer equipped with an electrospray ionization (HESI) interface was employed for the plant extracts analysis. Samples were injected (2 μL) and chromatographically separated using a reversed-phase C18 HSS T3 ACQUITY column 2.1 ×100 mm, 1.8 μm particle size (Waters, Milford, MA, USA). A gradient profile was applied using water (eluent A) and acetonitrile (eluent B), both acidified with 0.1% formic acid as mobile phases. A multi-step elution dual-mode gradient was developed as reported in Supp. Mat. The column was maintained at 60∘C and a flow rate of 0.40 mL/min was used.

The extracts were analyzed both in positive and negative ionization mode with a DDA approach: positive and negative scan spectra were acquired in a range from 66,7 to 1000 m/z, the full MS resolution was 70,000 FWHM, AGC target 3e6, maximum IT 200 ms and with a profile spectrum data type. Additional information on the HESI source settings, LC-HRMS data analysis and quantification of the main phenolic compounds can be found in Supplementary material.

Bacterial strains and mature biofilm formation

We determined the WEPs extracts antibiofilm activity on mature biofilm of collection strains of *Staphylococcus aureus* (*S.aureus* or *S. a.*) wild type, ATCC N°25923, and Methicillin-resistant *S. a.* (MRSA), ATCC N° 33591, in a BSL2 laboratory, as previously shown[60], see Supp. Mat. Briefly, *S. a.* and MRSA biofilms were left to grow for 4 days at 37 ∘C. On the fifth day, the wells were emptied, washed with PBS and the WEPs extracts were added at concentrations ranging from 2.8 to 0.25 mg dry extract/ml. The plates were then left at 37 ∘C overnight and the biofilm formation was observed the following day. Each plate contained untreated bacteria, as positive control, and Mueller–Hinton broth without bacteria, as negative one. We analyzed each experimental condition in triplicate.

Crystal violet and demolition assay on S. aureus biofilm

We measured the biofilm demolition on 96 wells/plate (flat bottom) by Crystal Violet (CV) assay, as previously shown[60] (see Supp. Mat.). On the fifth day of culture, we proceeded as previously shown. Briefly, the wells were washed again with PBS before 100 μl of 0.1% CV solution were added. After 45 min at room temperature, the plates were emptied and washed with distilled water to remove excess CV. For biofilm quantification, 50 μl of 95% ethanol were added to the wells to solubilize all biofilm-associated dye and the absorbance at 630 nm was determined using a microplate reader. We analyzed each experimental condition in triplicate.

Minimal inhibiting concentration (MIC)-Minimal bactericidal concentration (MBC) determination

To assess the MIC and MBC values of the six WEPs extracts, we used the microdilution assay[61]. The six hydroalcoholic extracts were used in a concentration range between 0.5 and 2.8 mg of dry extracts/mL of solvent (see Supp. Mat.). Briefly, the supernatant from transparent wells, cultured for 20 h in MH broth, was collected on a plastic tube and used for the MIC-MBC assay. Samples were serially diluted in PBS/0.05% Tween 80 and 20 μL drops were applied to MH agar plates and incubated for 24 h at 37 °C to determine CFU number. Each plate contained untreated bacteria, as positive control, and Mueller–Hinton broth without bacteria, as negative one. We analyzed each experimental condition in triplicate.

Bacterial cell wall damage assay by propidium iodide uptake

We put the *S. aureus* and MRSA strains in culture for 16 h in a Chamber Slides (1 μ-Slide 8 Well IbITreat, IBIDI GmbH, Germany) with the extracts at the respective MIC value. The following day, a Propidium Iodide solution (1 mg/mL, Invitrogen) was added in each Chamber slide well, at the final concentration of 1 μg/mL. After 5 min staining, we analyzed the samples by a fluorescent microscope with an Olympus FV1200 confocal laser scanning microscope, as previously shown[60] (see Supp. Mat.). Each plate contained untreated bacteria, as positive control, and Mueller–Hinton broth without bacteria, as negative one. We analyzed each experimental condition in triplicate.

Anti-adherence and disc-diffusion assay

We spread on Muller-Hinton (MH) agar plates 500 μL of *S. aureus* and MRSA cultures, adjusted to an approximate cell density 1.5 ×10^8 CFU/mL, and dried them under the sterile flow bench for 15 min. We then dropped 10 μL of the WEP extracts (at 1/50 of the MIC value identified) on the inoculated agar surface and dried them as previously described[33]. To exclude the solvent contribution to the inhibiting effect, we also dropped 10 μL of the

solvent on a control plate (negative control). Finally, we incubated the plates for 16 h at 37 °C. The anti-adherence activity was expressed as the mean of the diameter of inhibition halos (mm) produced by each extract. We did not used a positive control, given that an empty sterile disc could not induce any growth inhibition diameter. Blue circle indicates the growth inhibition diameter induced by each extract; grey indicates the growth inhibition diameter induced by the Antibiotic, and red circle the one induced by the co-administration of the extract on the antibiotic disc. We then measured the values of the subtraction of the blue circles (only extract) from the red ones (extract plus antibiotic).

The disc-diffusion assay was performed following Kirby-Bauer protocol[62], using the Antimicrobial Susceptibility Test Discs (ASTD) Kairo-Safe (TS, Italy). We analyzed each experimental condition in triplicate.

Statistical analysis

All the statistical analyses were performed using the software GraphPad Prism version 5.0 and Stata software. Data were analyzed by Kruskal Wallis test, followed by Dunn's Multiple Comparison test and Mann Whytney U comparison test ($p < 0.05$).

Data availability

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

Received: 3 February 2025; Accepted: 19 May 2025
Published online: 06 August 2025

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Acknowledgements
We are in debt with Dr. M.C. Zuin, responsible of the Phytoalimurgic Garden in Veneto Region, Padua University (https://www.research.unipd.it/handle/11577/2432488), for having provided us with plant material and seeds of the six WEPs species analyzed in the present study. We are also grateful with Dr. Giuliana Papoff for her generous assistance with the confocal microscope.

Author contributions
Conceptualization: FM, LDG; Data curation. ED, IN, SC, LR; Formal analysis: ED, IN, SC, FM, LR, VR; Funding acquisition: FM, LDG; Investigation: VR, ED, LR, SC, FM; Methodology: IN, ED, SC, VR, FM; Roles/Writing—original draft: FM, ED, SC, LDG; and Writing—review & editing: all authors.

Funding
CNR Biomemory (https://biomemory.cnr.it/); CNR FOE-2021 DBA.AD005.225; NRRP, Next Generation EU project: i) Project code PE00000003, "ON Foods—Research and innovation network on food and nutrition Sustainability, Safety and Security – Working ON Foods"; ii) Project code PE00000007: "One Health Basic andTranslation Research Actions addressing unmet Needs on Emerging Infectious Diseases, INF-ACT". This research was also partially funded by the European Union—NextGenerationEU – under the National Biodiversity Future Center. This project belongs to the National Recovery and Resilience Plan (NRRP), Mission 4 Component 2 Investment 1.4—Call for tender No. 3138 of 16 December 2021, rectified by Decree n.3175 of 18 December 2021 of Italian Ministry of University and Research funded by the European Union – NextGenerationEU; Award Number: Project code CN_00000033, Concession Decree No. 1034 of 17 June 2022 adopted by the Italian Ministry of University and Research, CUP C83 C22000530001, Project title "National Biodiversity Future Center—NBFC".

Declarations

Competing interests
The authors declare no competing interests.

Additional information
Supplementary Information The online version contains supplementary material available at https://doi.org/10.1038/s41598-025-03166-6.

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