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Evaluation of Antiphytoviral and Antibacterial Activity of Essential Oil and Hydrosol Extracts from Five *Veronica* Species

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Abstract: Agricultural production without pollution is possible using bioactive plant compounds, which include essential oils as important substances of plant origin. The aim of this study was to evaluate the antiphytoviral and antibacterial potentials of lipid (essential oil, EO) and water (hydrosol, HY) extracts from five *Veronica* species (Plantaginaceae) obtained by Clevenger hydrodistillation (HD) and microwave-assisted extraction (MAE), with analysis by gas chromatography coupled with mass spectrometry. The antiphytoviral activities of both extracts were tested on local host plants infected with tobacco mosaic virus (TMV). The antibacterial potential was tested against ten strains of opportunistic pathogens using the broth microdilution test. Species *V. chamaedrys* EO-MAE extract, *V. arvensis* EO from both extractions and *V. montana*, *V. serpyllifolia*, and *V. persica* EO-HD extracts were more effective in inhibiting TMV infection. Furthermore, HY- HD extracts of *V. arvensis*, *V. chamaedrys* and *V. persica* showed significant antiphytoviral activity. HY fractions had no effect on bacterial growth, regardless of the *Veronica* species tested, likely due to the fact that the maximum concentrations of the HY fractions tested in this study were low (1.83 and 2.91 mg/mL). EOs showed significant antibacterial activity independent of the extraction method. Notably, *V. chamaedrys* EO-MAE fraction, showed significantly better activity against *Listeria monocytogenes* and *Enterococcus faecalis*. Also, the EO-HD fraction of *V. arvensis* showed slightly better antibacterial activity. By combining extracts and using different extraction methods, valuable bioproducts can be obtained from the investigated *Veronica* species for safe use in agricultural production and food conservation.

Keywords: *Veronica*; essential oil; hydrosol; antiphytoviral activity; tobacco mosaic virus; antibacterial activity

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Citation: Nazlić, M.; Dunkić, V.; Dželalija, M.; Maravić, A.; Mandić, M.; Srečec, S.; Vrca, I.; Vuko, E.; Kremer, D. Evaluation of Antiphytoviral and Antibacterial Activity of Essential Oil and Hydrosol Extracts from Five *Veronica* Species. *Agriculture* **2023**, *13*, 1517. https://doi.org/10.3390/agriculture13081517

Academic Editors: Khalid Haddi and Geraldo Andrade Carvalho

Received: 30 June 2023
Revised: 25 July 2023
Accepted: 26 July 2023
Published: 29 July 2023

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1. Introduction

The results of phytochemical studies on a number of higher plants indicate that plants are endowed with pesticidal properties that can be exploited for use in agriculture and related fields. The need to use plant-based products arises from the fact that synthetic pesticides can be harmful to humans and to the entire ecosystem due to their high toxicity and persistence [1–3].

According to previous studies, most plant species with biopesticide activity belong to the Fabaceae and Lamiaceae families, followed by Apocynaceae, Cucurbitaceae, Euphorbiaceae, and Plantaginaceae [4]. The subjects of this study are representatives of the family Plantaginaceae, i.e., five species of the genus *Veronica*: *V. arvensis* L. (Corn speedwell), *V. chamaedrys* L. (Germander speedwell), *V. montana* L. (Wood speedwell), *V. serpyllifolia* L. (Thyme-leaved speedwell) and *V. persica* Poir. (Persian speedwell). These plants were

selected for this study because the genus *Veronica* is the largest in this family and it is widely distributed [5–7], making it an easily accessible plant material for analysis and use. All five species studied belong to the medicinal plants, and previous studies on their pharmacological activity focused on iridoids, flavonoids, and saponins [7–9]. The activity of other specialized metabolites in the *Veronica* species, such as the free volatile compounds (FVCs), has been much less studied and has not been systematically investigated [10–12]. Our previous research on five *Veronica* species that are the subject of this study focused on the extraction and identification of FVCs isolated by two techniques, hydrodistillation in a Clevenger apparatus (HD) and microwave-assisted extraction (MAE). Each extract obtained consists of two phases: a lipophilic phase (essential oil, EO) and an aqueous phase (hydrosols, HY) [13–15]. The hydrosol part of the extract is often wrongly classified as wastewater [16,17], although it contains very valuable bioactive components. These isolates are natural substances of plant origin and are therefore among the potential biocontrol products. This was confirmed by Raveau et al. in a review article that classifies biocontrol products into four main classes: macroorganisms, microorganisms, semiochemical products, and natural products derived from plants, algae, microorganisms, animals, or minerals [18]. Substances of plant origin were classified as non-living biocontrol natural products by Stenberg et al. [19]. They emphasized that the division into living and non-living biological substances is important to maintain scientific clarity and the integrity of biological control. These authors define biological control as the use of living organisms, including viruses, to control pests and pathogens that directly or indirectly affect human welfare [19]. It is precisely because of the impact on human health and the risk to the environment that biocontrol products, especially EOs, are of great scientific interest, as they are products of biological origin that are considered more ecological and alternative solutions compared to synthetic pesticides [20]. Among plant diseases, viral infections are of particular concern because of the lack of effective means of virus control in crop protection. Treatments with EOs and HYs of five selected *Veronica* species were conducted to evaluate the activity of their volatiles against tobacco mosaic virus (TMV) infections. TMV is a positive-strand RNA virus and one of the viral pathogens of agricultural crops. The interest in the antiphytoviral activity of HYs and EOs of species of the genus *Veronica* is a continuation of research on the biological activities of species of this genus. Our hypothesis of antiphytoviral activity of FVCs of *Veronica* species is supported by the fact that volatile components of aromatic plant species can stimulate plant defense responses against infections by various pathogens, including viruses [21,22]. We found that most published data refer to the effects of EOs on animal viruses rather than plant viruses [23,24]. Valuable effects of EOs have been documented, such as antiproliferative, antimicrobial, antioxidant, insecticidal, and other effects [25–27], and new findings, such as antiphytoviral activity, have been recently documented [21,28,29].

EOs and their active natural compounds have antibacterial properties against food-borne bacteria and their applications in food preservation could provide alternatives to conventional bactericides and fungicides. Several research studies discovered that shelf-life of fruits and vegetables can be substantially extended when applying specific essential oils [30]. Consequently, the antibacterial effectiveness of five *Veronica* species was assessed against emerging opportunistic pathogens, among which were clinically isolated multidrug-resistant ESKAPE strains as well as those commonly associated with skin and wound infections and food poisoning outbreaks. Notably, the most recent data on the evidence of the global burden of antimicrobial resistance show that multidrug-resistant bacterial infections are among the major causes of death for people of all ages, resulting in a total of 4.95 million deaths in 2019 [31]. Thus, clinical strains of high-priority pathogen methicillin-resistant *Staphylococcus aureus* (MRSA) [32] as well as the most common food-borne pathogens *Listeria monocytogenes* and *Bacillus cereus* [33] were included in this study.

The aim of this study was to establish a comparative chemical characterization and biological activity potential of FVCs from five *Veronica* species. To achieve this goal, three objectives were defined: (1) comparison of the volatile profiles of the total extraction

products contained in the EO and HY fractions, (2) screening of antiphytoviral activity against TMV, and (3) antibacterial activity against common foodborne pathogens.

2. Materials and Methods

2.1. Plant Material and Extracts—Review Protocol

Free volatile compounds (FVCs) were extracted from five *Veronica* species listed in Table 1 (herbarium specimens are presented in the Supplemental Material, Figures S1–S5), and information on their composition has been published in previous articles [13–15]. Extracts were obtained by hydrodistillation in a Clevenger apparatus (HD) (Šurlan, Medulin, Croatia) and by microwave-assisted extraction (MAE) (Milestone 'ETHOS X' microwave laboratory oven, 1900 W maximum, Sorisole, Italy). The distillate consists of two layers: essential oil (EO) collected in a side tube using a pentane/diethyl ether trap and a water layer (hydrosol). Identification of FVCs was performed by gas chromatography-mass spectrometry (model 3900; 2100 T; Varian Inc. Lake Forest, CA, USA). Method for FVC analyses is presented in detail in a paper by Dunkić et al. [14].

Table 1. Details on origin and collection data of investigated plant material.

Taxa	Locality	Latitude	Longitude	Altitude a.s.l. (m)	Voucher No.
V. arvensis	Hvar Island	43°10′42.3″ N	16°36′43.6″ E	38	CROVeS-12-2021
V. chamaedrys	Radoboj	46°09′49.4″ N	15°55′36.1″ E	260	CROVeS-13-2021
V. montana	Papuk Mt	45°30′38.1″ N	17°39′57.2″ E	761	CROVeS-15-2021
V. serpyllifolia	Zagreb	45°49′40.3″ N	15°58′59.5″ E	192	CROVeS-20-2021
V. persica	Samoborsko gorje	45°49′41.6″ N	15°40′32.9″ E	301	CROVeS-18-2021

2.2. Antiphytoviral Activity Assay

The above-mentioned extracts were tested on local host plants infected with tobacco mosaic virus (TMV). The species *D. stramonium* L. was used as a local host for TMV because it develops clearly visible lesions 3–4 days after inoculation. *Nicotiana tabacum* L. cv. Samsun, which was systemically infected with TMV, was used for virus propagation and virus inoculum production. The experiments were conducted when the local hosts reached the 5–6 leaf stage. Care was taken to ensure that the experimental plants were as uniform in size as possible. The inoculum prepared from systemically infected leaves was diluted with phosphate buffer to obtain 10 to 40 lesions per inoculated leaf. The concentrations (mg/mL) of FVCs from the EO and HY fractions of *Veronica* species are listed in Table 2 (stock solutions). The HY was applied as an undiluted spray solution to the leaves of *D. stramonium* plants for two consecutive days. Leaves were dusted with silicon carbide (Sigma-Aldrich, St. Louis, MO, USA) and inoculated with a prepared inoculum. Antiphytoviral activity of HYs was evaluated by the percentage inhibition of the number of local lesions on the leaves of the treated and control plants. The stock solutions of EOs were added to the virus inoculum (500 ppm) and simultaneously inoculated to the leaf halves of the experimental plants. The control leaf halves were inoculated with the same concentration of virus inoculum. The percentage of inhibition was calculated by comparing the number of lesions on the EO-treated and control leaf halves [21].

Table 2. Concentrations (mg/mL) of free volatile compounds from *Veronica* species essential oil (EO) and hydrosol (HY) fractions used for antiphytoviral activity and antibacterial susceptibility testing.

Species	Isolation Method	EO Fraction		HY Fraction	
		Stock Conc.	Max. Tested Conc.	Stock Conc.	Max. Tested Conc.
V. arvensis	HD	58.03	17.43	8.16	2.45
V. arvensis	MAE	56.45	16.95	6.21	1.86
V. chamaedrys	HD	81.6	24.5	5.03	1.51
V. chamaedrys	MAE	67.9	20.39	9.7	2.91
V. montana	MAE	75.3	22.61	7.39	2.22
V. montana	HD	114.45	34.37	6.17	1.85
V. serpyllifolia	MAE	65.18	19.57	5.9	1.77
V. serpyllifolia	HD	90.88	27.29	8.31	2.49
V. persica	MAE	75.18	22.55	6.32	1.89
V. persica	HD	142.83	42.89	6.11	1.83

2.3. Antibacterial Activity

2.3.1. Microbial Strains and Culture Conditions

To investigate the antibacterial potential, FVCs of *Veronica* species were tested against ten strains of emerging opportunistic pathogens. Antibacterial susceptibility testing included Gram-negative *Escherichia coli* ATCC (American Type Culture Collections, Rockville, USA) 25922 and *Acinetobacter baumannii* ATCC 19606, and the following seven Gram-positive species: *Staphylococcus aureus* (including ATCC 29213 and a methicillin-resistant *S. aureus* (MRSA) clinical strain MRSA-1, *Staphylococcus epidermidis* human isolate, *Streptococcus agalactiae* clinical isolate, *Streptococcus pyogenes* ATCC 19615, *Enterococcus faecalis* ATCC 29212, *Listeria monocytogenes* ATCC 19111 (1/2a), and a foodborne isolate of *Bacillus cereus* [34,35]. Strains were tested for antibiotic susceptibility using Etest (AB Biodisk, Sweden) and the VITEK 2 system (bioMérieux, France). Bacterial cells were stored at −80 °C and subcultured on tryptic soy agar (Biolife, Milano, Italy) before testing.

2.3.2. Broth Microdilution Testing

Antibacterial activity was tested using the broth microdilution assay following the European Committee on Antimicrobial Susceptibility Testing (EUCAST) guidelines for bacteria [36].

EO fractions were diluted in 5% dimethyl sulfoxide (DMSO). Two-fold dilutions of EO- and HY-derived FVC were tested at the maximal concentrations listed in Table 2. Assays were performed in 96-well microtiter plates as previously described [34,35]. In brief, exponentially grown bacterial cultures in Mueller–Hinton broth (Biolife, Milano, Italy) were spectrophotometrically adjusted to a density of 10^6 CFU/mL, added to serial twofold dilutions of FVC in a final volume of 100 µL per well, and incubated at 37 °C for 18 h. The minimum inhibitory concentration (MIC) was recorded as the lowest concentration showing no visually detectable bacterial growth (e.g., turbidity in the wells) and was the consensus value of the experiment performed in triplicate. To determine the minimum bactericidal concentration (MBC), aliquots from the wells corresponding to the MIC, 2× MIC and 4× MIC were plated on Mueller–Hinton agar (Biolife, Milano, Italy) plates. After incubation at 37 °C for 18 h, MBC was determined as the lowest concentration resulting in 99.9% killing of the initial inoculum.

Data on the susceptibility of the microbial strains used in this study were published previously [37,38].

2.4. Statistical Analysis

Statistical analysis was performed using GraphPad Prism version 9. All data were expressed as mean ± SD (standard deviation); $n = 3$. statistical significance was determined by *t*-test. Differences were considered significant at * $p < 0.05$.

3. Results and Discussion

3.1. Free Volatile Compounds of Five Veronica Species

Free volatile compounds (FVC) were extracted from five *Veronica* species (family Plantaginaceae): *V. arvensis* L., *V. chamaedrys* L., *V. montana* L., *V. serpyllifolia* L., and *V. persica* (Table 1, Figures S1–S5) as explained in the Section 2. A total of 20 samples were analyzed, which means that four samples were made for each of the five *Veronica* spp. studied, and all extracts were analyzed by gas chromatography coupled with mass spectrometry (GC-MS). Complete information on the composition of EOs and HYs for all five *Veronica* species studied can be found in previous articles [13–15]. Tables 3 and 4 show the main constituents with the largest relative proportions for all selected species. The major compounds extracted in EOs with HD/MAE are listed in Table 3, and the compounds extracted in HYs with HD/MAE are listed in Table 4. The compounds (*E*)-caryophyllene, caryophyllene oxide, hexadecanoic acid, hexahydrofarnesyl acetone, and phytol are the most abundant components in EO. In the composition of HY, the common components of all *Veronica* species studied are caryophyllene oxide, benzene acetaldehyde, and (*E*)-*β*-damascenone. In Tables 3 and 4, the main constituents of the extract composition are highlighted because FVCs, as secondary metabolites, have a wide chemical diversity and are represented differently in each taxa, so it is important to highlight them [39]. In this study, we tested the antiphytoviral and antibacterial activity of the total extracts EO and HY from five *Veronica* species. When extracts that are a mixture of compounds have a greater biological effect than the sum of the individual effects of the compounds, a synergistic effect occurs [40]. In this study, we investigated the biological potential of the natural bioactive components that make up the mixture, i.e., their synergistic effect.

Table 3. Main constituents (%) of essential oils (EOs) of five *Veronica* species studied. HD, Clevenger hydrodistillation; MAE, microwave-assisted extraction [14].

Species		(*E*)-Caryophyllene	Caryophyllene Oxide	Hexadecanoic Acid	Hexahydrofarnesyl Acetone	Phytol	Pentacosane
V. arvensis	HD	6.21	14.11	3.17	6.35	7.54	0.71
V. arvensis	MAE	3.25	7.11	17.42	17.55	22.57	-
V. chamaedrys	HD	2.43	6.25	5.73	10.82	31.66	0.56
V. chamaedrys	MAE	1.05	1.22	15.83	16.69	18.88	8.36
V. montana	HD	0.13	7.28	9.24	6.86	18.53	10.47
V. montana	MAE	0.44	2.61	5.81	9.17	37.03	14.90
V. serpyllifolia	HD	2.11	4.19	12.28	7.92	39.79	0.98
V. serpyllifolia	MAE	6.83	14.74	7.71	6.54	18.72	0.18
V. persica	HD	9.29	10.11	7.35	10.31	20.21	-
V. persica	MAE	2.62	3.14	5.31	18.47	23.71	5.27

Table 4. Main constituents (%) of hydrosols (HYs) of five *Veronica* species studied. HD, Clevenger hydrodistillation; MAE, microwave-assisted extraction [13,15].

Species		Linalool	(*E*)-Caryophyllene	Caryophyllene Oxide	*α*-Muurolol	Benzene Acetaldehyde	(*E*)-*β*-Damascenone	β-Ionone
V. arvensis	HD	-	1.63	7.92	9.76	13.52	11.21	8.94
V. arvensis	MAE	7.53	-	13.66	2.02	10.32	8.85	13.71
V. chamaedrys	HD	1.03	2.04	21.11	23.16	8.64	5.01	9.37
V. chamaedrys	MAE	3.15	3.31	18.16	22.45	5.43	3.35	7.16
V. montana	HD	5.45	5.82	4.88	-	25.33	4.43	10.55
V. montana	MAE	4.64	6.24	8.14	-	19.52	36.04	-
V. serpyllifolia	HD	3.52	5.12	37.03	1.24	16.44	3.73	5.32
V. serpyllifolia	MAE	4.84	5.27	18.83	10.36	4.33	4.95	11.46
V. persica	HD	-	1.44	22.73	7.21	11.03	9.32	11.73
V. persica	MAE	6.68	3.72	10.17	-	20.05	2.05	16.49

3.2. Antiphytoviral Activity of Veronica Species

The antiphytoviral activity of the extracts containing volatiles of *Veronica* species confirmed our hypothesis, as the results showed a significant reduction of viral infection

on the leaves of local host plants. Local lesions on leaf halves inoculated simultaneously with EO and virus were compared with control leaf halves. Results showed that among the EOs, *V. chamaedrys* EO obtained by MAE was the most effective in reducing viral infection, with an inhibition rate of 68.89% (Figure 1a).

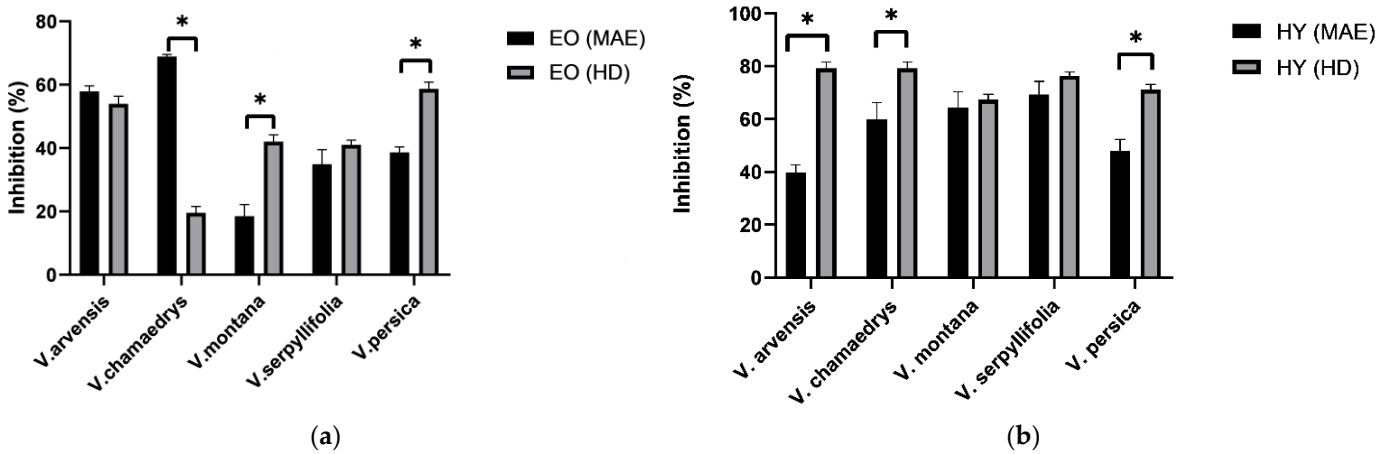


Figure 1. Comparison of antiphytoviral activity of investigated Veronica species extracts: (**a**) essential oils (EOs) extracted by hydrodistillation (HD) and microwave-assisted extraction (MAE); (**b**) hydrosols (HYs) extracted by HD and MAE. Statistical significance was determined by *t*-test. Differences were considered significant at * $p < 0.05$.

Regardless of the higher concentration of *V. chamaedrys* EO obtained by HD compared to MAE (Table 2), the EO of this species was significantly less effective when extracted by HD, suggesting that the choice of extraction method should be targeted in the context of further use of the natural extract. This activity could be explained with the qualitative as well as quantitative composition of extracts obtained by different methods. Differences in the composition of volatiles obtained by different extraction methods have already been described [11,41]. The compounds (*E*)-caryophyllene, caryophyllene oxide, hexadecanoic acid, hexahydrofarnesyl acetone, and phytol (Table 3) are the same in composition of the EOs of all *Veronica* species studied, obtained by both extraction methods. However, the percentages of these components differ significantly depending on the extraction method. For example, in the species *V. arvensis*, phytol was identified with threefold higher percentage with MAE (22.57%) than with HD (7.54%). On the other hand, in the species *V. serpyllifolia* twice as much phytol was identified with the extraction method HD (39.79%) compared to MAE, where 18.72% was identified (Table 3). In the composition of HY, the common components of all *Veronica* species studied are caryophyllene oxide, benzene acetaldehyde, and (*E*)-*β*-damascenone (Table 4). Again, we did not observe any regularities in the percentages of identification of these components depending on the extraction method. The most similar percentage of identification in the composition of HY in the species *V. chamaedrys* is for the compound α-muurolol (23.16% in HD and 22.45% in MAE) (Table 4) [13–15]. The described differences in volatile composition are reflected in the antiphytoviral activity. The present results suggest that MAE is the preferred extraction method for antiphytoviral use of *V. chamaedrys* EO. Out of the other *Veronica* species tested, the EO of *V. arvensis* extracted by both methods was effective in reducing viral infection, with inhibition rates of 57.89% with MAE and 53.97% with HD, respectively, but there was no statistical difference between the activity of the two extracts, suggesting that both methods are useful in terms of the antiphytoviral activity of the EO of this species. The EOs of the remaining three species tested were more effective in inhibiting TMV infection when extracted by the HD method. Among these oils, the oil of *V. persica* showed an inhibition rate of 58.67% on HD (Figure 1a) and was significantly more effective than the same oil extracted using MAE. A similar difference between the activity of the EOs extracted by the

two methods was observed for the oil of *V. montana*, which showed an inhibition rate of 42.00% and 18.49% on HD and MAE, respectively. The EO of *V. serpyllifolia* showed no such difference between the activity of the oils extracted by MAE and HD.

The antiphytoviral activity was confirmed and was even more pronounced when the HYs of the tested *Veronica* species were applied as a spray solution to the local host plants two days before virus inoculation (Figure 1b). HYs as aqueous solutions are harmless to the plants and were applied without dilution after both distillation processes. Comparison of antiviral activity of the HYs obtained by MAE and HD showed that the latter method yielded more effective hydrosols, as promising antiviral activities of 79.21% (*V. arvensis* and *V. chamaedrys*) and 71.13% (*V. persica*) were observed. From the data obtained, it can be concluded that HD is the preferred extraction method when HYs of these three *Veronica* species are used as possible antiviral agents. MAE and HD showed no significant difference in the antiviral activity of the extract when the hydrosols of *V. montana* and *V. serpyllifolia* were tested, as promising activities ranging from 64.42% to 76.38% were observed.

Aside from quantitative and qualitative composition that reflects activity, HYs and EOs also have a different mechanism of action, as already reported in the literature. In the study on the antiviral activity of EOs from 29 autochthonous Chinese aromatic plant species, inhibitory activity on TMV infection was described for 13 species, with inhibition rates ranging from 21.0% to 77.9% [42]. Authors described that the EOs of all species showed weaker protective effect than inactivating effect. EOs extracted from *Melaleuca alternifolia* and *Plectranthus tenuiflorus* species and applied as spray solution to *Nicotiana glutinosa* plants before inoculation also showed antiviral activity against TMV [29,43]. Several studies have found that plants are able to initiate a defense response to viral infection using a wide range of regulatory mechanisms and the phenylpropanoid pathway plays a role in such defense strategies [44,45]. Phenylalanine ammonia lyase (PAL) is involved in the initial steps of this metabolic pathway, and the associated gene is known to be involved in plant responses to biotic stresses via transcriptional regulation, leading to the synthesis of several specialized metabolites that play a role in protecting plants from pathogens [46,47]. Taglienti at al. [44] described a significant upregulation of PAL transcription level during treatment when EO or HY of *Origanum vulgare*, *Thymus vulgaris*, and *Rosmarinus officinalis* were applied to *Cucurbita pepo* plants simultaneously or after inoculation of tomato leaf curl New Delhi virus. An induction of PAL gene expression was recorded 12 days post inoculation and then was restored to the level of the untreated control, indicating an early defense response of the plant to the virus infection that may have been enhanced by the treatments.

Based on the above literature findings and supported by the results of our research group, we present that EOs and HYs of *Veronica* species can reduce viral infection of plants. We assume that EOs, when inoculated simultaneously with the virus, are likely to cause direct inactivation of virus particles and thus are likely to interrupt an early event in the virus replication cycle, as opposed to stimulating the host defense response as hypothesized for the activity of HYs during pretreatment. Our focus on finding volatile compounds with beneficial effects aims to find EOs and HYs that can be used as natural phytotherapeutics. This approach leads us to an ecologically desirable application of all components of the distillation process and, furthermore, to a practical use of extracts that are completely natural and harmless to the environment. As previously reported, different extraction methods affect the composition of volatile components of EOs and HYs [14,48,49] and our results show for the first time that the extraction method also affects the biological activity of the extract. Therefore, the extraction method should be targeted to achieve maximum potential of biological activity of the natural extract. In this study, we demonstrated the possibility of developing natural resources from the genus *Veronica* for crop protection. Based on that mentioned above, we highlight five species of the genus *Veronica* with their volatiles as a valuable source of natural products with beneficial effects and point out their antiphytoviral activity here for the first time.

3.3. Antibacterial Activity of Five Veronica Species

The antimicrobial potential of various plant extracts from a considerable number of species from the genus *Veronica* was previously explored and summarized [7,8]. In this study, we aimed to evaluate the antibacterial activity of FVCs from EO and HY fractions obtained by two extraction methods from five *Veronica* species using a broth microdilution assay against a wide range of human opportunistic pathogens associated with skin and wound infections, as well as foodborne pathogens. Bacterial growth was not affected by HY fractions, regardless of the *Veronica* species. However, it should be noted that the maximum concentrations of the fractions tested in this study were low and ranged between 1.83 and 2.91 mg/mL, respectively. Therefore, these results agree with Dunkić et al. who found that aqueous extracts of *Veronica spicata* (at a concentration of 10 mg/mL) were ineffective against several bacterial and fungal pathogens [50]. In addition, Stojković et al. reported *Veronica montana* water extract to be active at ≥7.5 mg/mL [51].

On the other hand, the EO-derived FVCs exhibited an antibacterial effect which varied depending on the *Veronica* species and mostly showed similar activities depending on the isolation method used (Table 5). In general, EOs were uniformly more effective against Gram-positive bacteria compared to Gram-negative ones, which in most cases were not successfully inhibited at the maximum concentrations tested. This was expected, as several authors reported elevated MIC values of various extracts of *Veronica* species against Gram-negative bacteria [7]. Notably, Dunkić et al. reported better activity against Gram-positive species for methanolic and ethyl acetate extracts of *Veronica spicata*, and Hassan and Ullah for ethyl acetate extracts of *V. biloba* [50,52]. Similarly, Živković et al. documented that Gram-positive bacteria, especially *Staphylococcus aureus*, were most sensitive to methanolic extracts of *Veronica urticifolia* [53]. In addition, ethanolic extracts of *Veronica officinalis*, *V. teucrium*, and *V. orchidea* showed stronger antibacterial activity against Gram-positive bacterial pathogens, including several *Listeria* spp. and *Bacillus cereus* [10].

Table 5. Antibacterial activity of *Veronica* species essential oils (EOs)—fractions obtained by microdilution assay.

Bacterial Species	Strain Origin	MIC/MBC of EO-Derived FVC (mg/mL)									
		V. persica		*V. montana*		*V. serpyllifolia*		*V. chamaedrys*		*V. arvensis*	
		MAE	HD	MAE	HD	MAE	HD	HD	MAE	HD	MAE
Gram-positive bacteria											
Staphylococcus aureus	ATCC 29213	22.55/ >22.55	42.89/ 42.89	22.61/ >22.61	17.18/ 17.18	4.89/ 9.78	6.82/ 13.64	24.5/ 24.5	20.39/ 20.39	8.71/ 8.71	16.95/ >16.95
Staphylococcus aureus	Clinical/ MRSA	22.5/ >22.5	42.89/ >42.89	22.61/ >22.61	17.18/ 17.18	9.78/ 9.78	13.64/ 13.64	24.5/ >24.5	20.39/ 20.39	8.71/ 8.71	16.95/ >16.95
Staphylococcus epidermidis	Human	22.55/ >22.55	42.89/ >42.89	>22.61/ >22.61	17.18/ 17.18	9.78/ 9.78	13.64/ 13.64	24.5/ 24.5	20.39/ 20.39	8.71/ 8.71	16.95/ >16.95
Streptococcus pyogenes	ATCC 19615	5.64/ 11.28	5.36/ 5.36	2.83/ 2.83	8.59/ 8.59	0.61/ 1.22	1.71/ 1.71	6.13/ 12.25	10.19/ 10.19	4.36/ 4.36	4.24/ 8.47
Streptococcus agalactiae	Clinical	5.64/ 11.28	5.36/ 10.72	2.83/ 5.65	8.59/ 8.59	1.22/ 1.22	1.71/ 1.71	12.25/ 12.25	10.19/ 10.19	4.36/ 4.36	4.24/ 8.47
Enterococcusfaecalis	ATCC 29212	1.41/ 5.64	2.68/ 10.72	5.65/ 22.61	4.29/ 4.29	1.22/ 1.22	1.71/ 1.71	24.5/ 24.5	1.28/ 5.09	1.08/ 2.17	2.12/ 8.47
Listeriamonocytogenes	ATCC 19111	1.41/ 2.82	2.68/ 21.45	2.83/ 11.31	2.15/ 2.15	1.22/ 1.22	0.43/ 0.85	6.13/ 24.5	0.32/ 1.28	2.17/ 4.36	0.53/ 2.12
Bacillus cereus	Food	5.64/ 11.28	10.72/ 21.45	11.31/ >22.61	4.29/ 4.29	2.45/ 2.45	1.71/ 1.71	12.25/ 24.50	20.39/ 20.39	4.36/ 4.36	8.47/ 8.47
Gram-negative bacteria											
Escherichia coli	ATCC 25922	>22.55/ >22.55	>42.89/ >42.89	>34.37/ >34.37	>34.37/ >34.37	19.57/ 19.57	27.29/ >27.29	>24.50/ >24.50	>20.39/ >20.39	17.43/ 17.43	>16.95/ >16.95
Acinetobacter baumannii	ATCC 19606	>22.55/ >22.55	>42.89/ >42.89	>34.37/ >34.37	>34.37/ >34.37	19.57/ 19.57	27.29/ >27.29	>24.50/ >24.50	>20.39/ >20.39	17.43/ >17.43	>16.95/ >16.95

This pattern of susceptibility has been associated with the different structure of the cell wall in Gram-positive bacteria and the presence of a complex outer membrane in Gram-negative bacteria, which reduces the uptake of compounds into the cells while at

the same time supporting their multifactorial efflux pump system [52,54,55]. Nevertheless, it is important to note that different extraction methods were used in the aforementioned studies, and this paper represents the first report of the antibacterial activity of FVCs from EO fractions obtained by HD and MAE. Notably, among the tested EO fractions, those from *V. chamaedrys*, *V. serpyllifolia*, and *V. arvensis* exhibited the strongest antibacterial effect (Table 5). To the best of our knowledge, an overview of the scientific literature did not reveal any available data on the antibacterial activity of EO-associated FVCs from *V. chamaedrys*, although this species is a long-known traditional medicinal plant [56], whose aqueous and alcoholic extracts were shown to have anti-inflammatory and antioxidant effects [8]. More importantly, nothing has been found in the literature about any type of *V. arvensis* fractions investigated in the context of biological activity, including antibacterial. As for *V. serpyllifolia*, only one study reported the radical scavenging activity of its water extract [57].

In this study, *Listeria monocytogenes* was the most sensitive bacterium to EO fractions from the five *Veronica* species (Table 5). This emerging foodborne pathogen, which has been associated with outbreaks worldwide and mortality rates as high as 20% [58], was successfully inhibited at concentrations ranging from 0.32 to 6.13 mg/mL, with *V. chamaedrys*, *V. serpyllifolia*, and *V. arvensis* active at concentrations below 1 mg/mL. On the other hand, *S. aureus* was the least sensitive among the Gram-positive bacteria with MIC values ranging from 4.89 to 42.89 mg/mL. However, it is noteworthy that the clinical MRSA strain was inhibited at the same MIC values as the antibiotic-sensitive ATCC strain in most cases. Notably, *S. aureus* is one of the most common hospital and community-acquired opportunistic pathogens worldwide [59]. It frequently causes skin, soft tissue, and bloodstream infections; 70% of which are due to MRSA strains that are resistant to most β-lactam antibiotics as well as those from other classes [60].

Moreover, the results of the susceptibility testing did not provide conclusive evidence of uniformly enhanced antibacterial activity when a particular isolation method was used. Although the stock solutions of the fractions differed depending on the isolation method employed (Table 2), the fractions of *V. serpyllifolia* exhibited very similar MIC and MBC values (Table 5), successfully killing *S. pyogenes*, *S. agalactiae*, *E. faecalis*, and *L. monocytogenes* within a range of 0.85 to 1.77 mg/mL. In the case of *V. chamaedrys*, the fraction derived by MAE showed considerably better activity only against *L. monocytogenes* and *E. faecalis* at MIC values of 0.32 mg/mL and 1.28 mg/mL, respectively. On the other hand, the EO fractions of *V. arvensis* exhibited somewhat better activity when the HD method was used, except in the case of *L. monocytogenes*, whose growth was inhibited at 0.53 mg/mL. Nevertheless, the overall range of observed MIC values was within 2-fold for the majority of bacteria tested.

Previously, studies investigating *Veronica* extracts showed that the presence of various biologically active compounds is species-specific and dose-dependent, but those exhibiting antibacterial effect mainly include glycosides, flavonoids, iridoids, and saponins [7,9]. Moreover, it was hypothesized that phenolic compounds in particular may contribute to antibacterial activity by producing a synergistic effect with other bioactive compounds [10,50,51]. In Tables 3 and 4 the main constituents of EOs and HYs, previously analyzed and published, are reviewed [14]. These compounds were found to exert an antibacterial effect. In fact, phytol exhibits various biological activities, including antimicrobial, cytotoxic, antioxidant, autophagy- and apoptosis-inducing, anti-inflammatory, and immunomodulatory [61]. Compound (*E*)-caryophyllene is known for its anti-inflammatory and anesthetic properties, while Montanari et al. also pointed out that a high content of sesquiterpenes (up to 35%) in EOs from Verbenaceae species corresponds with their moderate antibacterial activity [62].

Although the exact mechanism of action of terpenes has not yet been elucidated, there is increasing evidence that these compounds, including eugenol [63], act rapidly against bacterial cells via highly reactive hydroxyl groups that form hydrogen bonds with the active sites of target enzymes, leading to their inactivation [64,65], as well as acting via changes in membrane ion channels (Na^+, K^+, Ca^{2+}, or Cl^-) that cause cell rupture and a leakage of the cellular contents [66]. Considering the chemical composition of the EO

fractions [14], we speculate that the high amounts of oxygenated sesquiterpenes (13.2 to 40.44%), especially caryophyllene oxide and eudesmol (14.11% and 19.98% in the HD-EO fraction of *V. arvensis*, respectively), play an important role in their antimicrobial activity (Table 5). Moreover, these fractions were rich in diterpene phytol, of which the HD-EO fractions of *V. serpyllifolia* and *V. chamaedrys* accounted for 39.79% and 31.66%, respectively. It is also important to note that the most active EO fractions uniformly contained >2% phenolic compounds, of which the HD fraction from *V. arvensis* contained a total of 6.99% with 2.16% eugenol [14]. However, as we have already noted, there is no clear pattern of enhancement of their antibacterial activity with respect to the isolation method used, as the choice of method does not uniformly enrich or reduce the amount of a particular biologically active volatile compound in EO fractions [14].

4. Conclusions

The Plantaginaceae family is one of the most important families with biopesticidal activity. In our previous study, the free volatile compounds (FVCs) of five *Veronica* species contained in essential oil (EO) and hydrosol (HY) were obtained by classical (Clevenger apparatus, HD) and green hydrodistillation (microwave-assisted extraction, MAE) and the compositions of the extracts were compared. Continuing on this research, in the present study we investigated the antiphytoviral and antibacterial activities of FVCs. The results showed that among the EOs, EO-MAE extracted from *V. chamaedrys* was the most effective in reducing viral infection. More effective antiphytoviral activity was shown by the extracts of HY-HD, especially in *V. arvensis*, *V. chamaedrys*, and *V. persica*. In general, for the majority of tested extracts, hydrosols showed better antiphytoviral activity than EOs. Extracts from the five *Veronica* species were also tested by broth microdilution against a wide range of human opportunistic pathogens associated with skin and wound infections and foodborne pathogens. HY fractions had no effect on bacterial growth, regardless of the *Veronica* species tested, likely due to the fact that the maximum concentrations of the HY fractions tested in this study were low. EOs showed significant antibacterial activity independent of the extraction method. Only *V. chamaedrys*, the EO-MAE fraction, showed significantly better activity against *Listeria monocytogenes* and *Enterococcus faecalis* than EO-HD. The EO-HD fraction of *V. arvensis* also showed slightly better antibacterial activity. We can conclude that by combining extracts and using different extraction methods, valuable bioproducts can be obtained for safe use in agricultural production, which have great benefits for human health and the environment as a whole. The results of this study indicate the potential use of all parts of the extracts of the studied *Veronica* species as natural phytotherapeutics relevant to biological control of pests, with significant ecological benefits.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture13081517/s1, Figure S1: Herbarium specimen of the species *Veronica arvensis* L.; Figure S2: Herbarium specimen of the species *Veronica chamaedrys* L.; Figure S3: Herbarium specimen of the species *Veronica montana* L.; Figure S4: Herbarium specimen of the species *Veronica serpyllifolia* L.; Figure S5: Herbarium specimen of the species *Veronica persica* Poir.

Author Contributions: Conceptualization, V.D., A.M. and E.V.; methodology, A.M., E.V., V.D., M.N. and M.D.; validation, A.M., M.M. and E.V.; formal analysis, A.M., M.D., E.V., M.M., V.D., M.N., I.V., D.K. and S.S.; investigation, A.M., M.D., E.V., M.M., V.D., M.N., I.V., D.K. and S.S.; resources, V.D.; data curation, E.V., M.M., A.M., M.D., M.N. and V.D.; writing—original draft preparation, E.V., A.M. and V.D.; writing—review and editing, V.D., M.N., D.K. and I.V.; visualization, V.D., E.V. and A.M.; supervision, V.D.; funding acquisition, V.D. and S.S. All authors have read and agreed to the published version of the manuscript.

Funding: This research was supported by the project "Croatian *Veronica* species: Phytotaxonomy and Biological Activity", CROVeS-PhyBA, funded by the Croatian Science Foundation. Project number IP-2020-02-8425.

Institutional Review Board Statement: Not applicable.

Data Availability Statement: Not applicable.

Conflicts of Interest: The authors declare no conflict of interest.

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