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Wild Species *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides*—Biological Potential of Free Volatiles

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Abstract: Extracts from plants of the genus *Veronica* have been and continue to be used in traditional medicine to treat various diseases throughout the world. Although often considered a weed, many scientific reports demonstrate that these plants are a source of valuable biologically active compounds and their potential for horticulture should be investigated and considered. In this study, free volatile compounds of essential oils (EO) and hydrosols were extracted from two species: *Veronica officinalis*, which is most commonly used in traditional medicine, and *Veronica saturejoides*, an endemic plant that could be obtained by cultivation in horticulture. Volatiles were analyzed by gas chromatography coupled with mass spectrometry (GC, GC-MS). The most abundant compounds identified in the EOs were hexadecanoic acid in *V. officinalis* EO and caryophyllene oxide in *V. saturejoides* EO. The hydrosols were characterized by a high abundance of caryophyllene oxide in *V. saturejoides* hydrosol and of *p*-vinyl guaiacol for *V. officinalis* hydrosol. The sites where the volatile compounds are synthesized and stored were analyzed using SEM (Scanning Electron Microscopy); glandular and non-glandular trichomes were detected on stems, leaves and the calyx. Further, to investigate the activity of the free volatile compounds against pathogens, isolated volatile compounds were tested on the antiphytoviral activity against tobacco mosaic virus (TMV) infection. The hydrosols of both investigated species and EO of *V. officinalis* showed significant antiphytoviral activity. To further investigate the biological potential of these extracts they were also tested for their antiproliferative and antioxidant activities. The results indicate that these compounds are a valuable source of potential anticancerogenic agents that should be investigated in future studies. The presented results are the first report of hydrosol and EO activity against TMV infection, suggesting that these extracts from *Veronica* species may be useful as natural-based antiphytoviral agents.

Keywords: antioxidant activity; antiphytoviral activity; antiproliferative activity; essential oil; free volatile compounds; GC-MS; hydrosol; speedwell

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1. Introduction

The genus *Veronica* of the family Plantaginaceae grows predominantly in temperate Northern Hemisphere regions, with a smaller number of species growing in Southern Hemisphere regions and in Australia [1,2]. The many species of this plant family, about 450, show the great ecological adaptability of the genus *Veronica*. Species of this genus grow in wet and dry habitats, as well as in the marine belt and mountains [3].

The studied species *Veronica officinalis* L. (Figure 1b) (common speedwell) and *Veronica saturejoides Vis.* ssp. *saturejoids* (Figure 1a) (savory leafed speedwell) grow on Dinaric Massif (Republic of Croatia). Both species are perennial herbaceous plants with small

attractive violet flowers. *Veronica officinalis* is slightly taller (10–50 cm) and has longer leaves (1.5–5 cm) than *V. saturejoides* ssp. *saturejoides* (stem length 10–30 cm, leaf length 1–3 cm). The latter species is a plant endemic to Croatia [4].

(**a**) (**b**)

Figure 1. Investigated plants in their natural habitat: (**a**) *Veronica saturejoides* ssp. *saturejoides* on Dinara Mountain; (**b**) *Veronica officinalis* L. on Kamešnica Mountain.

In general, the study of chemical compounds produced by wild plants is extremely important because these compounds ultimately affect not only the plant in which they are found, but also indirectly other plants in the vicinity as well as the environment as a whole [5]. These compounds are important factors in plant adaptation to abiotic stresses. Moreover, neighboring plants detect other plant volatiles as 'messages' about herbivore or pathogen attacks, and consequently adapt their metabolism responses [6]. Plants of the genus *Veronica* are used in traditional medicines in countries around the world, which sparked interest in the studying these plants in terms of their chemical composition and biological activity. Many different biological activities of the various extracts have been reported in recent studies [7,8]. For example, methanolic and ethyl-acetate extracts of *V. spicata* were tested for antimicrobial activity and MIC values were between 1.25 and 5.00 mg/mL. This plant extract has also shown substantial antioxidant activity, especially the methanol extracts of flowers and leaves with IC_{50} and DPPH values of 8.21 µg/mL and 8.69 µg/mL, respectively [9]. Ertas et al. reported antimicrobial activity for phenolic extracts of *Veronica thymoides* subsp. *pseudocinerea* and the MIC value determined was 31.25 mg/mL for methanol extract against *Escherichia coli* [10].

Veronica officinalis, which is the subject of this study, is traditionally used in the medicine of Balkan peoples. Mocan et al. reported antioxidant activity for ethanol extracts of phenolic compounds for *V. officinalis* to be 157.99 ± 6.58 mg Trolox equivalents/g d.w [11]. Valyova et al. also confirmed antioxidant activity of phenolic extracts for the *V. officinalis* in their study [12]. The aerial parts of speedwells are used to treat liver, spleen, kidney, and bladder diseases, as well as snakebites wound healing, skin lesions, eczema and ulcers [9,10,13].

Green prevention strategies and means of virus control of natural origin are particularly important today to support organic production and the replacement of synthetic chemicals with biologically based antivirals. This approach to modern agriculture is still

under development, and one of our goals is to find harmless and environmentally friendly antiphytoviral agents. Tobacco mosaic virus (TMV) is a model virus in plant virology and a very important pathogen for agricultural crops, causing significant yield losses. TMV belongs to the positive-strand RNA viruses and encodes two proteins that function as replicases (molecular weights 126 kDa and 183 kDa), a movement protein (30 kDa) that facilitates virus movement between host cells, and a coat protein (17.5 kDa) that plays an important role in virion formation [14]. Various plant products such as essential oils, flavonoids, polyphenols and organic, alcoholic and aqueous extracts from plants and natural compounds from other organisms such as fungal metabolites, have been used against a number of plant diseases caused by viruses, phytopathogenic bacteria, fungi, plant parasitic nematodes and parasitic and non-parasitic weeds [15–19] with the aim of finding natural-based products useful for plant protection against pathogens. Some of our previous studies and studies by other authors describe the activity of plant volatiles as natural antiphytoviral compounds [19–27]. Since this activity of Plantaginaceae family volatiles has not been tested so far, we investigated the antiphytoviral activity of both essential oil and hydrosol of *V. officinalis* and *V. saturejoides*, with the aim of increasing the knowledge about the antiphytoviral activity of essential oils and especially hydrosols, which have been very little studied in this regard. To further investigate the biological potential, the antiproliferative and antioxidant activity of these extracts was tested.

Thus, the aim of this study was to investigate the composition of the volatile compounds of these two species from the EOs and the water residues (hydrosols) and to discuss differences and similarities in composition. In addition, the biological potential of these extracts for possible use in horticulture were evaluated either to cultivate and thus preserve the endemic species *V. saturejoides* or to promote the cultivation of the wild plant *V. officinalis*. To our knowledge, this is the first report on the EO and hydrosol composition of *V. officinalis* and *V. saturejoides* from this site, and the first report on their biological activity, as well as on the micromorphology of the trichomes of *V. officinalis*.

2. Materials and Methods

2.1. Plant Material

Plant material for *V. officinalis* and *V. saturejoides* was collected from the sites at Dinara Massif (Table 1, Figure 1). The voucher specimens were deposited in the Faculty of Science Herbarium (PMFST-HR), University of Split, Croatia. For GC and GC-MS analyses, the samples were air dried in a single layer and protected from direct sunlight for three weeks. The dried plant material was kept in the dark in double paper bags labeled with the sample number and stored in a dry place.

Table 1. Locations of the plant material collection (for volatile compounds extraction).

	Locality	Coordinates	Altitude a.s.l. (m)	Date of Collection
Veronica officinalis L.	Kamešnica Mountain	43°43′03.1″ N 16°50′34.1″ E	1445 m	July 2021
Veronica saturejoides Vis. ssp. *saturejoides*	Dinara Mountain	44°3′2.6″ N; 16°22′52.9″ E	1650 m	July 2021

Above ground plant parts (stems, leaves, and flowers) of seven plants of *V. officinalis* were fixed in solution made from formalin, 96% ethanol, acetic acid and water in a volume ratio of 5:70:5:20. After three days, the samples were transferred to 70% ethanol and stored in the refrigerator. The study of the trichomes for *V. saturejoides* was carried out in the previous work [28].

2.2. GC and GC-MS Analyses

Dried above ground parts (50 g) for each sample were hydrodistilled for 3 h in a Clevenger-type apparatus. Three separate extractions were performed for each species and volatile compounds were collected from the lipophilic layer (pentane/diethyl ether) and water residues (hydrosols). Both phases were analyzed by gas chromatography (GC), and gas chromatography and mass spectrometry (GC-MS). GC was performed by gas chromatograph (model 3900, Varian Inc., Lake Forest, CA, USA) that is supplied with a flame ionization detector (FID), mass spectrometer (model 2100T; Varian Inc., Palo Alto, CA, USA), non-polar capillary column VF-5ms (30 m × 0.25 mm inside diameter, coating thickness 0.25 µm, Palo Alto, CA, USA) and polar capillary column CP-Wax 52 CB (30 m × 0.25 mm i.d., coating thickness 0.25 µm, Palo Alto, CA, USA). The chromatographic conditions for the analysis of lipophilic fraction (essential oils) were: FID detector temperature 300 °C, injector temperature 250 °C. The gas carrier was helium at 1 mL min^{-1}. The conditions for the VF-5ms column were: temperature 60 °C isothermal for 3 min, and then increased to 246 °C at a rate of 3 °C min^{-1}, and held isothermal for 25 min. Conditions for the CP Wax 52 column were: temperature 70 °C isothermal for 5 min, and then increased to 240 °C at a rate of 3 °C min^{-1} and held isothermal for 25 min. The injected volume was 2 µL and the split ratio was 1:20. The MS conditions were: ion source temperature 200 °C, ionization voltage 70 eV, mass scan range 40–350 mass units [9,28]. The individual peaks for all samples were identified by comparison of their retention indices of *n*-alkanes to those of authentic samples and literature [29,30], comparing it to our libraries from previous work [9,28] and to other previously published material for *Veronica* species [10,12,31,32]. The results are given as the mean of three extractions with standard deviation.

2.3. Micromorphological Traits

For micromorphological investigations internodes of stem, central parts of leaves (left or right from the main axis), and the whole calyx were used. Before the scanning electron microscopic (SEM) analysis, the samples were transferred from 70% (*v*/*v*) ethanol to 70% (*v*/*v*) acetone. Thereafter, the samples were subjected to a dehydration procedure, i.e., they were transferred from 70% (*v*/*v*) to 90% (*v*/*v*), and 100% (*v*/*v*) acetone. Each transfer from one to another concentration of acetone was repeated twice and lasted 15 min each at room temperature. With the use of CO_2 as the drying medium, the dehydrated samples were subjected to a process of critical point drying in CPD030 device (Bal-tec, Balzers, Liechtenstein). The samples thus prepared were sputter coated with gold in Sputter Coater device (Agar Scientific Ltd., Essex, UK) and researched under the scanning electron microscope XL30 ESEM (FEI Company, Eindhoven, Netherlands) with an acceleration voltage of 20 kV in high vacuum mode [33]. Common terminology [34] was used to describe the trichomes.

2.4. Antiphytoviral Activity

2.4.1. Virus and Plant Hosts

Leaves of *Nicotiana tabacum* L. cv. Samsun systemically infected with tobacco mosaic virus were used to prepare the virus inoculum as described by Vuko et al. [25]. Leaves of the native host *Datura stramonium* L. were pollinated with carborundum (Sigma-Aldrich, St. Louis, MO, USA) before virus inoculation, and the inoculum was diluted with inoculation buffer to obtain 5–30 lesions per inoculated leaf. Experiments were carried out when the plants had reached the 5–6 leaf stage. Care was taken to ensure that the experimental plants were as uniform in size as possible.

2.4.2. Antiphytoviral Activity Assay

Essential oil (final concentration adjusted to 500 ppm) or hydrosol (undiluted) were applied as a spray solution to the leaves of local host plants for three consecutive days prior to virus inoculation. Plants were then rubbed with the virus inoculum and treated once with the same spray solutions immediately after inoculation. The antiviral activity of

the essential oil and hydrosol was evaluated by the percentage inhibition of the number of local lesions on the leaves of the treated and control plants as described by Vuko et al. [25].

2.5. Cell Culture

Three cancer cell lines, Cervical Cancer Cell Line (HeLa), Human Colon Cancer Cell Line (HCT116) and Human Osteosarcoma Cell Line (U2OS), were grown in an incubator under humidified conditions with 5% CO_2 and 37 °C, in a Dulbecco's modified Eagle's medium (DMEM, EuroClone, Milan, Italy) containing 4.5 g/L glucose, 10% fetal bovine serum (FBS), and 1% antibiotics (penicillin and streptomycin, EuroClone).

2.6. Cell Proliferation Assay

The antiproliferative capacity of EO of *V. officinalis* L. and *V. saturejoides* Vis. ssp. *saturejoides* was determined on HeLa, HCT116 and U2OS cancer cells using the MTS-based CellTiter 96® Aqueous Assay (Promega). Cells were grown in an incubator at 37 °C and 5% CO_2 until they reached 80% confluence. They were counted using a handheld automated cell counter (Sceptre, Merck), and 5000 cells/well were seeded in 96-well plates with a serial dilution of EOs and hydrosols. The cells were then cultured for an additional 48 h. Thereafter, 20 µL MTS tetrazolium reagent (Promega) was added to each well and left in the incubator for an additional 3 h. Then, absorbance was measured at 490 nm using a microplate reader (Bio-Tek, EL808). IC_{50} values were calculated from three independent experiments using GraFit 6 data analysis software (Erithacus, East Grinstead, UK).

2.7. Antioxidant Activity of Essential Oils and Hydrosols

2.7.1. ORAC

The assay was performed in a Perkin–Elmer LS55 spectrofluorimeter, using 96-well white polystyrene microtiter plates (Porvair Sciences, Leatherhead, UK) following a method described by Fredotović et al. [35], with some adjustments based on different extracts. Hydrophilic assay was performed for hydrosols and lipophilic assay for EOs. Adjustments were made for the EO antioxidant assay. EOs were dissolved in acetone (10 mg in 1 mL acetone). The EO acetone dilutions were further dissolved 40× and 80× in the phosphate buffer for the experiments. For the hydrosol assays total undiluted hydrosols were used. All measurements were performed in triplicate according to the method described in Nazlić et al. [28].

2.7.2. DPPH

The antioxidant capacity of the extracts was determined using the DPPH method already described by Mensor et al. and Payet et al. [36,37] and adapted to tested plant extracts. Plant extracts as described in the ORAC method were used (acetone-dissolved essential oils and absolute hydrosols) for the assay. An amount of 100 µL of methanol (Kemika, Zagreb, Croatia) and 200 µL of sample was pipetted into each well. Serial dilutions of samples were prepared by pipetting 100 µL from the first row with a multichannel pipette into the wells in the second row and so on to the last row, where 100 µL of the solution was ejected after mixing. In the first column, in 96-well plates, a blank sample was always added. For EOs, the acetone and methanolic solution were used as blank and for hydrosols, water and methanolic solution were used as blank. The calculation and presentation of the results were performed according to the method described in the previous research by Nazlić et al. [28].

2.8. Statistical Analyses

Statistical analysis was performed in GraphPad Prism Version 9. All data are presented as mean ± SD ($n \geq 3$). Statistical significance for free volatile compounds and antioxidant activity was assessed by multiple *t*-test, $p < 0.05$. Statistical tests were performed separately for lipophilic (essential oils) and hydrophilic fractions (hydrosols).

3. Results and Discussion

3.1. Composition of Free Volatile Components

The chemical composition of the free volatile compounds of *Veronica officinalis* L. and *Veronica saturejoides* Vis. ssp. *saturejoides* is shown in Table 2. The free volatile compounds were analyzed from the lipophilic-essential oil and aqueous-hydrosol fractions.

Table 2. Chemical composition of the essential oil (EO) and hydrosol (H) from the aerial parts of *Veronica officinalis* and *Veronica saturejoides* Vis. ssp. *saturejoides* (Plantaginaceae).

			V. officinalis V. saturejoides		***V. officinalis V. saturejoides***	
			Essential Oils		**Hydrosols**	
Component	**RI[1]**	**RI[2]**	**Mean ± SD (%)**		**Mean ± SD (%)**	
Monoterpene hydrocarbons			NI	NI	0.15	0.89
α-Thujene	924	1012	NI	NI	NI	0.23 ± 0.01
α-Pinene *	935	1017	NI	NI	NI	0.66 ± 0.03
β -Phellandrene	1002	1195	NI	NI	0.15 ± 0.03	-
Oxygenated monoterpenes			1.36	12.39	13.48	15.93
1,8-Cineole	1026	1210	NI	NI	0.76 ± 0.01	NI
γ-Terpinene	1057	1225	NI	NI	2.61 ± 0.01	NI
Linalool	1095	1506	0.52 ± 0.01 ^{b}	0.89 ± 0.05 ^{a}	4.72 ± 0.01 ^{a}	1.39 ± 0.01 ^{b}
allo-Ocimene	1128	1390	0.22 ± 0.15	NI	NI	NI
Camphor	1151	1499	NI	NI	0.72 ± 0.01	NI
Borneol	1176	1719	NI	NI	1.59 ± 0.01	NI
α-Terpineol	1184	1660	NI	0.88 ± 0.01	3.08 ± 0.03 ^{a}	2.79 ± 0.01 ^{b}
trans-1(7),8-p-Mentadien-2-ol	1187	1803	0.62 ± 0.01	10.62 ± 0.02	NI	11.75 ± 0.01
Sesquiterpene hydrocarbons			13.32	12.93	7.76	7.6
α-Copaene	1377	1484	0.78 ± 0.01	NI	NI	NI
E-Caryophyllene *	1424	1585	6.78 ± 0.04 ^{b}	7.63 ± 0.01 ^{a}	0.56 ± 0.01 ^{b}	1.39 ± 0.01 ^{a}
allo-Aromadendrene	1465	1662	1.32 ± 0.01 ^{a}	0.87 ± 0.01 ^{b}	2.59 ± 0.01 ^{b}	3.87 ± 0.01 ^{a}
β-Chamigrene	1478	1724	NI	NI	0.27 ± 0.12	NI
Germacrene D	1481	1692	NI	2.61 ± 0.01	NI	2.34 ± 0.01
δ-Selinene	1492	1756	3.32 ± 0.01	NI	4.34 ± 0.01	NI
δ-Cadinene	1517	1745	1.12 ± 0.01 ^{b}	1.82 ± 0.01 ^{a}	NI	NI
Oxygenated sesquiterpenes			6.54	34.04	15.74	25.82
Spathulenol	1577	2101	NI	1.8 ± 0.01	5.25 ± 0.01	NI
Caryophyllene oxide *	1581	1955	1.42 ± 0.01 ^{b}	23.65 ± 0.01 ^{a}	7.52 ± 0.01 ^{b}	21.28 ± 0.01 ^{a}
Viridiflorol	1592	2099	NI	NI	NI	1.53 ± 0.01
γ-Eudesmol	1632	2175	1.82 ± 0.01 ^{a}	0.2 ± 0.03 ^{b}	NI	0.23 ± 0.01
α-Muurolol	1645	2181	3.30 ± 0.01 ^{b}	7.86 ± 0.01 ^{a}	2.38 ± 0.01	2.37 ± 0.01
α-Cadinol	1655	2208	NI	NI	NI	0.41 ± 0.01
α-Bisabolol	1685	2210	NI	NI	0.59 ± 0.03	NI
α-Bisabolol oxide	1748	2511	NI	0.53 ± 0.01	NI	NI
Phenolic compounds			0.27	10.23	11.59	23.31
p-Vinyl guaiacol	1313	2156	0.27 ± 0.03	NI	11.59 ± 0.01	NI
Methyl eugenol	1403	2005	NI	10.23 ± 0.01	NI	23.31 ± 0.01
Phenylpropanoids			6.02	1.41	1.75	2.61
Z-Methyl isoeugenol	1451	2045	1.46 ± 0.03 ^{a}	1.41 ± 0.01 ^{b}	1.75 ± 0.01 ^{b}	2.16 ± 0.03 ^{a}
Benzyl benzoate	1760	2613	4.56 ± 0.01	NI	NI	NI

Table 2. *Cont.*

			V. officinalis V. saturejoides		*V. officinalis V. saturejoides*	
			Essential Oils		**Hydrosols**	
Component	**RI[1]**	**RI[2]**	**Mean ± SD (%)**		**Mean ± SD (%)**	
Fatty aldehyde, acids, alcohol, esters and ketones			56.56	22.33	36.84	14.58
Isopentyl acetate	863	1127	NI	6.24 ± 0.01	0.24 ± 0.09 ^{b}	0.59 ± 0.01 ^{a}
Benzaldehyde	952	1508	0.98 ± 0.01 ^{b}	4.29 ± 0.01 ^{a}	9.25 ± 0.01 ^{a}	8.87 ± 0.01 ^{b}
Benzene acetaldehyde	1036	1633	0.48 ± 0.01	NI	4.75 ± 0.01 ^{a}	3.68 ± 0.01 ^{b}
n-Nonanal	1100	1389	0.89 ± 0.02	NI	1.68 ± 0.01 ^{a}	0.28 ± 0.01 ^{b}
Hexyl 2-methyl butanoate	1233	1425	NI	NI	0.21 ± 0.01 ^{a}	0.15 ± 0.03 ^{b}
Menthyl acetate	1294	1550	NI	NI	1.58 ± 0.02	NI
(E)-*β*-Damascone	1384	1819	NI	NI	6.69 ± 0.01	NI
β-Ionone	1487	1935	17.88 ± 0.01	NI	10.74 ± 0.01	NI
Hexahydrofarnesyl acetone	1839	2113	13.92 ± 0.01 ^{a}	6.18 ± 0.01 ^{b}	0.25 ± 0.03 ^{b}	0.39 ± 0.03 ^{a}
1-Hexadecanol	1874	2371	1.79 ± 0.01 ^{a}	1.51 ± 0.03 ^{b}	NI	NI
Hexadecanoic acid	1959	2912	20.62 ± 0.01 ^{a}	4.11 ± 0.01 ^{b}	1.45 ± 0.01 ^{a}	0.62 ± 0.02 ^{b}
Hydrocarbons			10.86	1.12	1.78	0.49
Eicosane *	2000	2000	4.21 ± 0.01 ^{a}	1.12 ± 0.01 ^{b}	1.51 ± 0.04	NI
Heneicosane *	2100	2100	0.98 ± 0.17	NI	0.27 ± 0.01	NI
Docosane *	2200	2200	2.13 ± 0.01	NI	NI	NI
Tricosane *	2300	2300	NI	NI	NI	NI
Tetracosane *	2400	2400	0.83 ± 0.01	NI	NI	0.49 ± 0.01
Pentacosane *	2500	2500	2.71 ± 0.04	NI	NI	NI
Total identification (%)			**94.93**	**95.45**	**89.09**	**91.23**

Retention indices (RIs) were determined relative to a series of *n*-alkanes (C8–C40) on capillary columns VF5-ms (RI^1) and CPWax 52 (RI^2). Identification method: RI, comparison of RIs with those in a self-generated library reported in the literature [29] and/or with authentic samples; comparison of mass spectra with those in the NIST02 and Wiley 9 mass spectral libraries; * co-injection with reference compounds; NI, not identified; SD, standard deviation of triplicate analysis. Significant differences for every volatile compound present in both species were determined using multiple *t*-test. ^{a,b} Mean values in the same row with different superscript letters indicate a statistically significant difference between data from two species ($p < 0.05$); ^{a} for the higher abundance of the compound, ^{b} for the lesser abundance of the compound.

The major compounds in the lipophilic fraction of *V. officinalis* are: hexadecanoic acid (20.62%), β-ionone (17.88%), hexahydrofarnesyl acetone (13.92%) and *E*-caryophyllene (6.78%). These compounds were also identified in the hydrosol fraction of *V. officinalis* but with much lower proportions, except for *β*-ionone, which has a high proportion of 10.74%. Moreover, the phenolic compound *p*-vinyl guaiacol (11.59%) is the most represented compound in the hydrosol fraction of *V. officinalis*, followed by benzaldehyde (9.25%), caryophyllene oxide (7.52%) and *(E)*-*β*-damascone (6.69%).

The oxygenated sesquiterpenes are the major group in both fractions of *V. saturejoides* ssp. *saturejoids* (Table 1, Figure 2) with a predominant caryophyllene oxide compound (23.65% in EO and 21.28% in H). Moreover, among the phenolic compounds, only methyl eugenol was identified as the most abundant compound, in the EO-fraction with 20.23% and in the hydrosol fraction it with 23.31%. Among the oxygenated monoterpenes in *Veronica saturejoides* ssp. *saturejoids* in both fractions, *trans*-1(7),8-p-mentadien-2-ol was the most abundant compound (10.62% in EO and 11.75% in H).

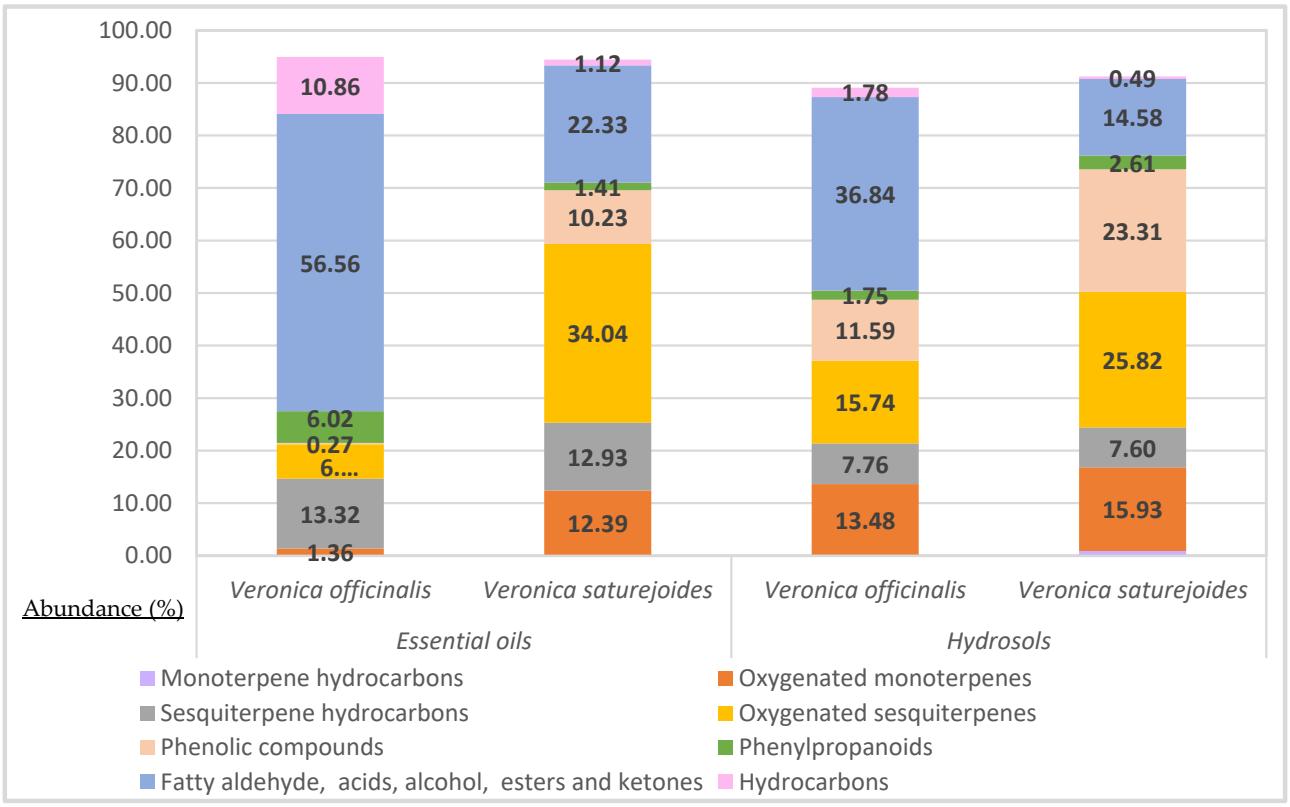

Figure 2. Volatile compounds distribution by categories for all samples of essential oils and hydrosols for the investigated species *V. officinalis* and *V. saturejoides* ssp. *saturejoides*.

In the previous studies, composition of EO and hydrosol for the species *V. saturejoides* ssp. *saturejoides* was analyzed but from two different sites (Kamešnica and Prenj Mountain) [28]. In hydrosols from these two locations *trans*-1(7),8-p-mentadien-2-ol was the most abundant compound, with a percentage of 31.75% for Prenj Sample and 36.63% for the Kamešnica sample. Caryophyllene oxide was also present in both fractions of *V. saturejoides* (essential oil and hydrosol) which is consistent with the results of this current research. The composition of the EO of *V. spicata* was also previously analyzed and the study found that the most abundant compound was phytol (21.13%) [9]. Not many studies have been carried out on the composition of the volatile compounds of other *Veronica* species. Valyova et al. studied extracts of the Bulgarian species *V. officinalis* by GC-MS and identified in ethanol extracts terpinen-4-ol, neophytadiene, hexahydrofarnesyl acetone, vitamin E, phytol and squalene for the first time in the genus *Veronica* [12]. Other research on the EOs of the genus *Veronica* were carried on *V. thymoides* subsp. *pseudocinerea* [10], *V. linariifolia* [31] and *Veronica* sp. [32]. Ertas et al. found that the most abundant constituent of the essential oil from *Veronica thymoides* subsp. *pseudocinerea* was hexatriacontene (21%) which belongs to the hydrocarbon compound group [10]. In another research of the essential oil composition of *Veronica linariifolia* Pall. ex Link the major constituents were cyclohexene (25.83%), *β*-pinene (11.61%), 1S-*α*-pinene (10.65%), *β*-phellandrene (10.49%), *β*-myrcene (10.42%), and germacrene D (4.99%) (monoterpene and sesquiterpene hydrocarbons) [31]. Çelik et al. studied the essential oils extracted from *Veronica* sp. and found that the main components were mainly linalool (4.18%) and carvacrol (7.28%) [32].

3.2. Glandular and Non-Glandular Trichomes

Glandular trichomes are 'bio-factories' of volatile compounds that are part of EOs. They play an important role in protecting plants from herbivores and pathogens and in attracting pollinators [38]. Two types of glandular trichomes can be seen on the surface of the studied plants of *V. officinalis*: peltate and capitate (Figure 3). Peltate glandular trichomes are composed of a basal cell, a very short stalk cell and a multicellular head with a fairly large subcuticular space (Figure 3c). These trichomes are very rare and they were noticed only on the calyx of *V. officinalis*. Nazlić et al. [28] did not describe peltate trichomes in *Veronica saturejoides* ssp. *saturejoides*. On the other hand, peltate trichomes are common in some other plant species, especially in Lamiaceae [39,40]. Capitate glandular trichomes could be further divided in two subtypes. Subtype 1 (C1) capitate trichomes consist of a stalk cell and two elliptically shaped head cells with a subcuticular space (Figure 3g). They are present on the adaxial and abaxial sides of the leaf, on the calyx and on the stem. Subtype 2 (C2) capitate trichomes are uniseriate, unbranched, multicellular, long and folded at different levels (Figure 3e,h). They consist of several (most often four to five) stalk cells and a head cell with a subcuticular space. They are present on the stem and calyx of *V. officinalis*. Only C1 trichomes are present on the leaf surface and they appear to be denser than on the stem and calyx. C1 trichomes were also observed on the surface of stem, leaves and calyx of *V. saturejoides* ssp. *saturejoides*. On the other hand, the C2 trichomes were not found in *V. saturejoides* ssp. *saturejoides* [28]. According to available data, C1 capitate trichomes were previously observed in *Veronica beccabunga* L. [41]. Comparable, but more or less upright capitate trichomes with a short stalk and two-celled head were observed in *Marrubium vulgare* L. [39,40] and in endemic *Salvia smyrnea* L. from Turkey [39,40].

Non-glandular (NG) trichomes also have a protective function in plants. They can protect plants from herbivores and prevent greater water loss through transpiration [38]. NG trichomes were observed on the calyxes, leaves and stems of *V. officinalis*. According to SEM investigation the NG trichomes are unbranched and uniseriate. It can also be observed that they are short (two-celled) or longer (multicellular) trichomes (Figure 3b,f). These trichomes are folded at different levels. The NG trichomes are denser on the stem surface than on leaves and calyxes (Figure 3). The presence of the same type of NG trichomes was noted before on aerial parts of *V. saturejoides* [28], on the flower parts of *Veronica* sp. [42] and for *V. persica* Poir. [43].

3.3. Antiphytoviral Activity

The numerous biological activities of essential oils known to date and their role in the interaction of plants with their biotic and abiotic environment suggest that these specialized plant metabolites are much more than just plant fragrances. Hydrosols are a by-product of essential oil distillation, making the usability of all products of this process ecologically and biologically desirable. Therefore, the antiphytoviral activity is imposed as a continuation of the study of the biological activities of this genus, especially since this activity of the volatiles of the genus *Veronica* has never been tested before.

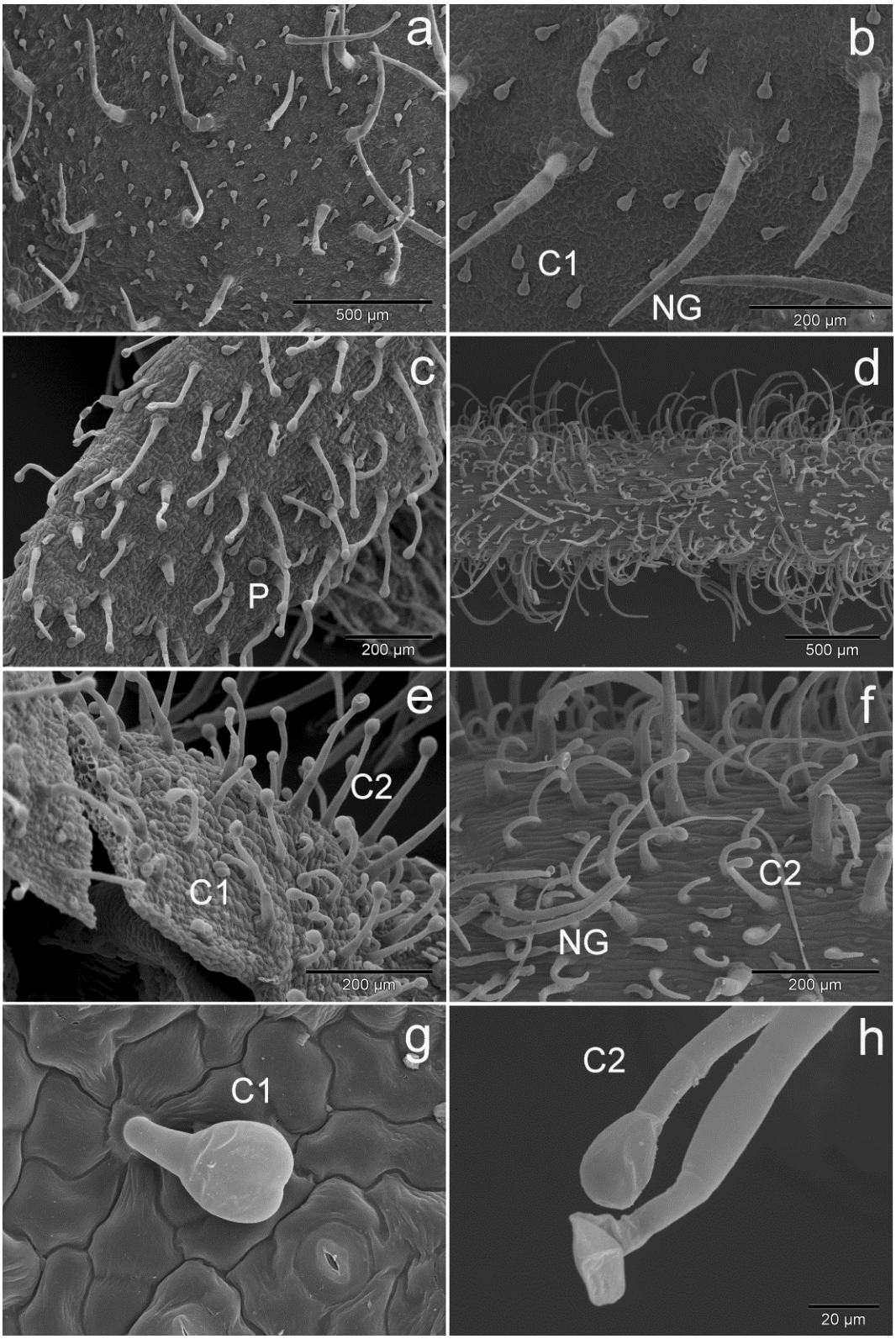

Figure 3. *V. officinalis* SEM micrographs showing different types of trichomes on adaxial (**a**,**g**) and abaxial (**b**) leaf surface, on the sepal (**c**,**e**) and stem (**d**,**f**,**h**). Non-glandular trichomes (NG), peltate trichomes (P), subtype 1 (C1) and subtype 2 (C2) of capitate trichomes.

The results show that plants treated with hydrosol (H) of *V. officinalis* and *V. saturejoides* prior to TMV infection significantly reduced the number of local lesions compared to control plants. In the essential oil (EO) treated plants, the number of local lesions decreased only on the leaves of the plants treated with *V. officinalis* EO (Table 3), while the plants treated with *V. saturejoides* EO in the preliminary experiment developed similar or even more severe infection than the control plants. On the third day post inoculation, the inhibition of lesions on the leaves of the plants treated with *V. officinalis* H and *V. saturejoides* H was 84.69% and 74.11%, respectively, while it was 59.43% on the leaves of the plants treated with *V. officinalis* EO (Figure 4). Until the seventh day after inoculation, the inhibition of local lesions was still pronounced in all treated groups, with 79.09% and 68.38% on the leaves of plants treated with *V. officinalis* H and *V. saturejoides* H, respectively, and 62.70% on the leaves of plants treated with *V. officinalis* EO. We compared these results with the antiphytoviral activity of plant volatiles reported in some of our previous studies and studies by other authors [19–27]. Thus, essential oils isolated from the aromatic species *Satureja montana* ssp. *variegata* and *Teucrium arduini* inhibited TMV infection by 29.2% and 25.7%, and cucumber mosaic virus (CMV) infection by 24.1% and 21.9%, respectively [20]. The essential oils of species of the genus *Micromeria* (*M. graeca*, *M. fruticulosa* and *M. croatica*) showed antiphytoviral activity on plants infected with satellite RNA associated cucumber mosaic virus (satCMV) with an activity of 59.3%, 43.6% and 34.5%, respectively [19,24,44]. The essential oils extracted from four species of the genus *Teucrium* (*T. polium*, *T. flavum*, *T. montanum* and *T. chamaedrys*) were shown to reduce the number of lesions in the host plants infected with CMV, with the essential oil of *T. polium* having the strongest effect with an activity of 41.4% [23]. The essential oils of *Hypericum perforatum* ssp. *veronense*, *Eryngium alpinum* and *E. amethystinum* showed promising antiviral activity rates of 88%, 77.8% and 80.5%, respectively [22,25]. In addition, among the essential oils extracted from 29 indigenous Chinese aromatic plants, the oils of ginger, lemon, tea tree, tangerine peel, artemisia, and lemongrass caused more than 50% inhibition of TMV at the concentrations tested [26,27]. In addition to plant extracts, inhibitors of plant viruses derived from metabolites of microbes are also considered as potential alternatives to chemical pesticides [45]. Ningnanmycin isolated from the fermentation broth of *Streptomyces noursei* var. *Xichangensis* exhibits comprehensive antiphytoviral activity and is characterized by increased resistance, excellent efficiency and low toxicity in host plants by acting through expression of pathogenesis-related proteins, increasing salicylic acid biosynthesis and inducing systemic resistance in host plants [46–48].

Table 3. Number of local lesions (LLN) on leaves of treated *Datura stramonium* plants inoculated with tobacco mosaic virus and on leaves of control plants (C) on the third, fifth, and seventh day post inoculation (dpi). Treated plants were sprayed with hydrosol (H) and essential oil (EO) of *V. officinalis* (V.off) or *V. saturejoides* (V.sat) for three consecutive days prior to and once immediately after inoculation.

		LLN		
dpi	**Mean ± SD**		**Mean ± SD**	
3rd	C	5.86 ± 2.39	C	6.55 ± 1.24
	V. off H	0.83 ± 0.31 *	V.off EO	2.57 ± 0.18 *
	V. sat H	1.50 ± 0.62 *	V.sat EO	n.a.
5th	C	8.95 ± 2.95	C	8.39 ± 2.20
	V. off H	1.36 ± 0.58 *	V.off EO	2.75 ± 0.58 *
	V. sat H	2.24 ± 0.63 *	V.sat EO	n.a.
7th	C	10.55 ± 3.62	C	9.67 ± 2.24
	V. off H	2.10 ± 0.87 *	V.off EO	3.39 ± 0.50 *
	V. satH	3.02 ± 0.89 *	V.sat EO	n.a.

SD, standard deviation of triplicate analysis; n.a., no activity; significant differences were determined by *t*-test; * statistically significant differences between control and EO/H treatment data ($p < 0.05$).

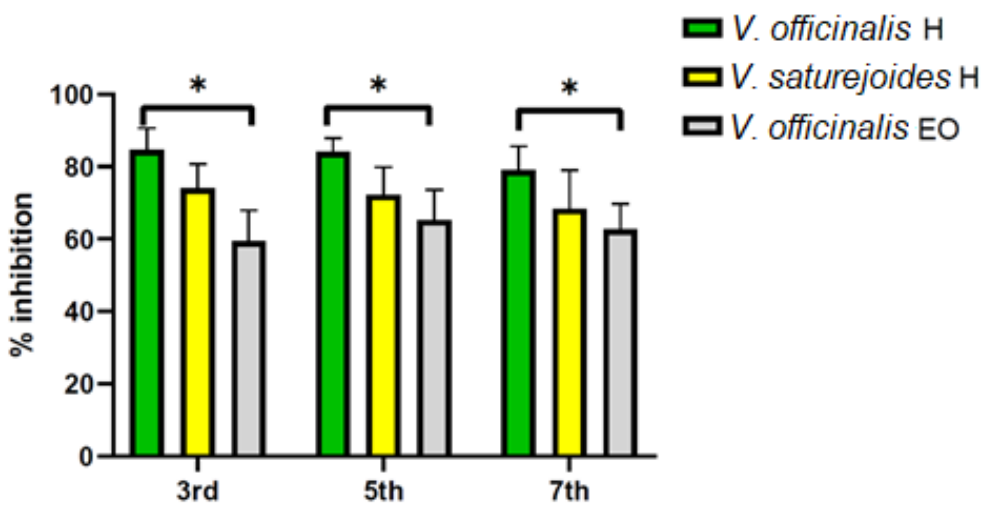

Figure 4. Percentage of inhibition of lesions on leaves of treated *Datura stramonium* plants inoculated with tobacco mosaic virus compared to control plants on the third, fifth and seventh day post inoculation. Treated plants were sprayed with hydrosol (H) and essential oil (EO) of *V. officinalis* or *V. saturejoides* for three consecutive days prior and once immediately after inoculation. Error bars show standard deviation of triplicate analyses; significant differences were determined by *t*- test and marked with * ($p < 0.05$).

This and previous studies dealing with essential oils and hydrosols of aromatic plant species [20–25] show that volatile plant compounds can increase the resistance of plants to viral pathogens. The activity of hydrosol of both *Veronica* species is even more promising than that of essential oils, and the presented results suggest the need for further studies on the antiviral activity of volatiles of *Veronica* species. The activity of the tested extracts is probably related to their chemical composition, where a synergistic effect of the volatiles could activate plant signaling pathways and lead to increased resistance to viral infections. Among the major constituents of the essential oil (Table 2), *β*-ionone and hexadecanoic acid are abundant in *V. officinalis* EO (17.88% and 20.62%, respectively), in contrast to *V. saturejoides* EO, where these constituents are less abundant (hexadecanoic acid) or absent (*β*-ionone). In addition, *δ*-selinene (3.32%), benzyl benzoate (4.56%), docosane (2.13%) and pentacosane (2.71%) are noteworthy, which are also detected only in *V. officinalis* EO, but not in *V. saturejoides* EO. Comparing the essential oil composition with that of hydrosol, *α*-terpineol, *allo*-aromadendrene, benzene acetaldehyde, *n*-nonanal and hexyl 2-methylbutanoate are listed in similar abundance in both *V. saturejoides* H and *V. officinalis* H, while they are less abundant or absent in *V. saturejoides* EO. The above similarities and differences in the composition of the tested extracts may be useful for future predictions of their efficacy in plant protection.

The reported antiphytoviral activity of *Veronica* species should be analyzed in more detail in the future, including further studies to evaluate their efficacy against viral diseases under field conditions.

3.4. Antiproliferative Activity

In the present study antiproliferative activity of the essential oil (EO) and hydrosol of two *Veronica* species was analyzed. This was of particular interest to us because there are no data in the literature on the antiproliferative activity of *V. saturejoides* and *V. officinalis*. The results of this study showed significant antiproliferative activity of the oil and hydrosol of these species. Essential oil and hydrosol of *V. saturejoides* collected from two different locations (Kamešnica sample (KS*) and Dinara sample (DS)) and *V. officinalis* (Kamešnica sample (KS)) were tested on three cancer cell lines: HeLa (human cervical cancer cell line), HCT116 (colon cancer cell line) and U2OS (osteosarcoma cell line). The best antiprolifera-

tive activity on all three cell lines tested was shown by the essential oil of *V. saturejoides* KS (Figure 5a). A particularly significant difference in the antiproliferative power of *V. saturejoides* KS compared to *V. saturejoides* DS and *V. officinalis* KS was observed in the HeLa and U2OS cell lines. Interestingly, the hydrosol showed slightly different results in contrast to the essential oil (Figure 5b). The hydrosol of *V. saturejoides* KS and DS showed similar antiproliferative effect on all the tested cancer cells. The hydrosol of *V. officinalis* DS showed the strongest antiproliferative activity on HeLa and HCT116 cell lines with IC_{50} values of 64.93% and 45.93%, respectively. *V. saturejoides* KS hydrosol had the best growth inhibition on the U2OS cell line with IC_{50} value of 48.23%.

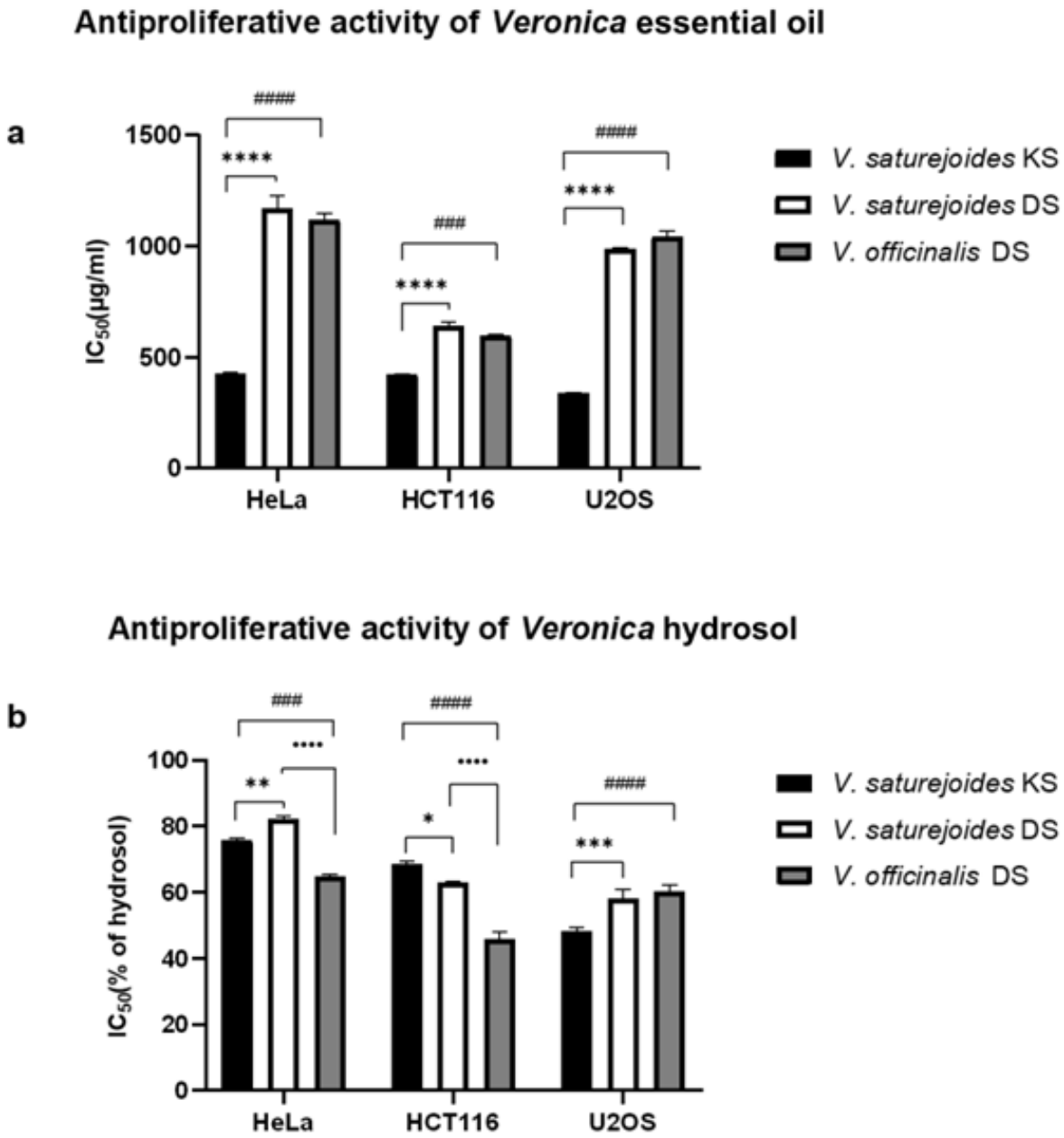

Figure 5. Antiproliferative activity of essential oil (**a**) and hydrosol (**b**) of *V. saturejoides* KS, DS and *V. officinalis* determined by MTS-based cell proliferation test. Results are expressed as the mean of three independent experiments ± SD (presented as error bars). Statistical analyzes were performed using the two-way ANOVA method followed by the Tukey's multiple comparisons test, and significant differences were labeled as * ($^{\#}$ or $^{\bullet}$) $p < 0.05$; ** ($^{\#\#}$ or $^{\bullet\bullet}$) $p < 0.01$; *** ($^{\#\#\#}$ or $^{\bullet\bullet\bullet}$) $p < 0.001$; and **** ($^{\#\#\#\#}$ or $^{\bullet\bullet\bullet\bullet}$) $p < 0.0001$.

Species of the genus *Veronica* have long been used in traditional medicine to treat a number of diseases including cancer, influenza, hernia, cough, respiratory diseases and many others [49–51]. Studies have shown that these plants are a source of secondary metabolites that are largely responsible for their excellent biological activity, such as antimicrobial, antioxidant and anti-inflammatory properties. Although various *Veronica* extracts have been used in folk medicine for the treatment of cancer, very few species have been studied for their cytotoxic and anticancer activity [7]. Previous research on various *Veronica* species has mainly led to the isolation of biologically active compounds such as iridoid glucosides, which have been found to be excellent natural anticancer agents [52–57]. Moreover, methanolic and aqueous extracts of several *Veronica* species have been tested on different cancer cell lines. The cytotoxic activity of methanolic extracts of five *Veronica* species (*V. cymbalaria, V. hederifolia, V. pectinata* var. *glandulosa, V. persica* and *V. polita*) was tested against KB (human epidermoid carcinoma) and B16 (mouse melanoma) cells. All species showed similar dose-dependent effects against tested cells [54]. Methanolic extract of *V. americana* managed to stop the division of the two cancer cells of HF-6 (colon) and PC -3 (prostate) cancer cell lines [58]. Feng et al. showed that flavonoids isolated from *V. sibirica* (Vtfs) induced dose-dependent apoptosis in breast cancer cells MCF-7 with IC_{50} of 42 µg/mL [59]. Aqueous extracts of *V. cuneifolia* subsp. *cuneifolia* and *V. cymbalaria* showed moderate cytotoxic activity against Hep-2 (human epidermoid carcinoma), RD (human rhabdomyosarcoma) and L-20B (transgenic murine L-cells) [60]. Water fractions of the methanolic extracts of *V. persica* and *V. crista-galli* effectively inhibited proliferation of HeLa and MCF-7 cells but showed no toxicity against normal fibroblast cell line [61]. These water fractions contained a high concentration of flavonoids and phenols, which is why they exhibited significant antioxidant activity in addition to their cytotoxic activity.

The results of this study show for the first time that both the essential oil and hydrosol of the species *V. saturejoides* KS, *V. saturejoides* DS and *V. officinalis* KS have significant antiproliferative activity and thus possible chemotherapeutic properties, which is why they deserve further investigation. It is necessary to conduct further studies with the essential oil and hydrosol of *V. saturejoides* and *V. officinalis* and their major constituents on different cancer cell lines to determine the mechanism of antiproliferative action and to evaluate the possibility of their use in medicine and pharmacology.

*Note: Chemical composition of the EO and hydrosol of *V. saturejoides* ssp. *saturejoides* from the Kamešnica location (KS) and plant material information was reported in the previous work by Nazlić et al. [28].

3.5. Antioxidant Activity

The antioxidant activity of the extracted volatile compound was analyzed by two methods, ORAC and DPPH, and the results are presented in the Table 4. The essential oil of *V. saturejoides* ssp. *saturejoides* showed higher antioxidant activity than the EO of *V. officinalis* by both methods. This could be due to a much higher content of oxygenated sesquiterpenes, especially the compound caryophyllene oxide. The hydrosols showed slightly different results. In the DPPH method, hydrosol of *V. saturejoides* showed higher activity, and in the ORAC method, hydrosol of *V. officinalis* showed higher activity (Table 4). Hydrosols of *V. saturejoides* also have higher content of oxygenated sesquiterpenes. Sesquiterpenes have been previously reported to possess numerous biological activities [62,63]. Comparing these results with those previously reported for *V. saturejoides* ssp. *saturejoides* from two other locations (Kamešnica and Prenj Mountain), it can be seen that the antioxidant activity of EO of *V. saturejoides* (Dinara sample) showed similar ORAC activity to the other two previously reported EOs, but the DPPH activity seems to be lower than the EOs of *V. saturejoides* from Kamešnica and Prenj Mountain [28]. The sample from Kamešnica seems to have the highest antioxidant activity, as was also shown for antiproliferative activity. *V. officinalis* EO has lower antioxidant activity than the *V. saturejoides* EO sample from this research report (Dinara sample) and also lower than the two previously reported EOs. Therefore, the conclusion can be drawn that *V. officinalis* EO has lower antioxidant activity

than *V. saturejoides* EO. Comparing the results for the hydrosols, it is evident from Table 4 and previous results for *V. saturejoides* [28] that both the hydrosols of *V. officinalis* and *V. saturejoides* have lower antioxidant activity than the previously reported hydrosols from the two locations of *V. saturejoides*. Comparing these results with reports on other plants, it appears that *V. officinalis* hydrosol has higher antioxidant ORAC activity than *Salvia officinalis* hydrosol [64]. Reports on the antioxidant activity of other extracts (phenolic compounds and iridoid glycosides) from *Veronica* species indicate that these plants have significant antioxidant potential. Valyova et al. studied phenolic extracts of *V. officinalis* and found that ethyl acetate extracts exhibited excellent antioxidant activity based on DPPH and ABTS assays [11]. Harput et al. investigated antioxidant activity for four *Veronica* species (*V. orientalis*, *V. baranetzkii*, *V. officinalis* and *V. peduncularis*) and reported the strongest antioxidant activity for aqueous extracts of *V. officinalis* (IC_{50} 54.19 µg/mL). This could be due to the highest reported total phenolic content being for this species (200.20 mg/g) [65]. In another study, Harput et al. reported antioxidant activity against SO for *V. chamaedrys* (IC_{50}) to be higher than the standards (BHA and quercetin) and the highest IC_{50} value for *V. officinalis* against DPPH radical to be 40.93 µg/mL [66]. Živković et al. investigated antioxidant activity of three *Veronica* species and reported highest antioxidant activity for *V. teucrium* 70% aqueous acetone extracts (IC_{50} 12.58 µg/mL) [67]. Dunkić et al. reported even higher antioxidant activity for methanol extracts of flowers of *V. spicata* with IC_{50} 8.21 µg/mL [9]. Sharifi-Rad et al. reported DPPH antioxidant activity for methanol extract of aboveground parts of *V. persica* to be IC_{50} 30 µg/mL. [68]. All these results show that speedwells should be further researched for their in vivo antioxidant activities or for potential usage in food preservations.

Table 4. Antioxidant potential of *V. officinalis* and *V. saturejoides* ssp. *saturejoides* of the essential oil and hydrosol determined by ORAC and DPPH method.

	Essential Oils (Mean ± SD)	
Antioxidant Assay	***V. officinalis***	***V. saturejoides* ssp. *saturejoides***
ORAC (Trolox eq)	58.75 ± 3.42 [b]	263.29 ± 4.89 [a]
DPPH (% inhibition)	15.51 ± 1.67	19.11 ± 4.09
DPPH (IC 50)	31.34 ± 2.91 [b]	15.99 ± 4.17 [a]
	Hydrosols (mean ± SD)	
Antioxidant Assay	*V. officinalis*	*V. saturejoides* ssp. *saturejoides*
ORAC (Trolox eq)	0.307 ± 0.011	0.258 ± 0.013
DPPH (% inhibition)	27.74 ± 0.77 [b]	38.39 ± 5.83 [a]

ORAC (oxygen radical absorbance capacity) results for EOs expressed as µmol of Trolox equivalents (TE) per g of EO (10 mg/mL) and for hydrosols as µmol of Trolox equivalents (TE) per g of the total (undiluted) tested hydrosol sample; DPPH, results are expressed in percentage of inhibition for the EO concentration of 10 mg per mL of acetone and for the hydrosols in percentage of inhibition for the absolute (undiluted) hydrosol; for the EOs, results are also expressed in IC50 value in mg/mL; SD = standard deviation of triplicate analysis; significant differences were determined using multiple *t*-test. [a, b] Mean values in the same row with different superscript letters indicate a statistically significant difference between data from four locations ($p < 0.05$), [a] for the higher activity, [b] for the lower activity.

4. Conclusions

In this study, the free volatile compounds and their biological activities of two speedwells, *V. officinalis* and *V. saturejoides* ssp. *saturejoides*, from the Dinaric Massif, were analyzed. The most abundant compound in the extracts was caryophyllene oxide for EO and hydrosol of *V. saturejoides*. Hexadecanoic acid and *p*-vinyl guaiacol were the most abundant compounds in the EO and hydrosols of *V. officinalis*, respectively. The volatile compounds extracted from the essential oils and hydrosols showed significant antiphytoviral activity, antiproliferative and antioxidant activity. The results for antiphytoviral activity show that

hydrosol of *V. officinalis* and *V. saturejoides* reduced the number of local lesions on the plants infected with the TMV virus compared to control plants. Essential oil of *V. officinalis* also showed results against TMV infection, while EO of *V. saturejoides* showed no antiphytoviral activity. The best antiproliferative activity on all three cell lines tested was shown by the essential oil of *V. saturejoides* from the Kamešnica location. However, for the hydrosols the results showed that *V. officinalis* had the strongest antiproliferative activity against HeLA and HCT116 cell lines. Antioxidant activity of the essential oil of *V. saturejoides* ssp. *saturejoides* appeared to be higher than the activity of the *V. officinalis* EO by both tested methods. Hydrosols gave different results, as hydrosols from the *V. officinalis* showed higher activity by the ORAC method and hydrosols from *V. saturejoides* showed higher activity by the DPPH method. These results show that plants of the genus *Veronica* are more than just a weed, as they are often considered, but a valuable source of biologically active compounds for human use and for neighboring plants if used in horticulture. Further research could be focused on in vivo studies. Furthermore, it would be valuable to grow these plants from seed in a controlled environment and compare chemical composition and biological activity to the species growing in the wild.

Author Contributions: Conceptualization, V.D. and M.N.; Methodology, V.D. and M.N.; Software, M.N.; Validation, V.D., D.K. and M.N.; Formal Analysis, M.N. V.D., Ž.F., D.K. and E.V.; Investigation, V.D., M.N., E.S., D.K., Ž.F., M.R., E.V. and L.F.; Data Curation, M.N. and V.D.; Writing—Original Draft Preparation, M.N.; Writing—Review and Editing, M.N. and V.D.; Visualization, M.N.; Supervision, V.D.; Funding Acquisition, V.D. All authors have read and agreed to the published version of the manuscript.

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