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Suppressive Activity of *Glechoma hederacea* Extracts against the Phytopathogenic Oomycete *Plasmopara viticola*, and First Screening of the Active Metabolites

Jesús G. Zorrilla [1,2], Oscar Giovannini [3], Stefano Nadalini [3,4], Alberto Zanini [4], Maria Teresa Russo [1], Marco Masi [1,*], Gerardo Puopolo [3,4,*] and Alessio Cimmino [1]

[1] Department of Chemical Sciences, University of Naples Federico II, Complesso Universitario Monte S. Angelo, Via Cintia, 80126 Naples, Italy; jesus.zorrilla@uca.es (J.G.Z.); mariateresa.russo2@unina.it (M.T.R.); alessio.cimmino@unina.it (A.C.)
[2] Allelopathy Group, Department of Organic Chemistry, Facultad de Ciencias, Institute of Biomolecules (INBIO), University of Cadiz, C/Avenida República Saharaui, s/n, 11510 Puerto Real, Spain
[3] Research and Innovation Centre, Fondazione Edmund Mach, Via E. Mach 1, 38098 San Michele all'Adige, Italy; oscar.giovannini@fmach.it (O.G.); stefano.nadalini@unitn.it (S.N.)
[4] Center Agriculture Food Environment (C3A), University of Trento, Via E. Mach 1, 38098 San Michele all'Adige, Italy; alberto.zanini@studenti.unitn.it
* Correspondence: marco.masi@unina.it (M.M.); gerardo.puopolo@unitn.it (G.P.)

Abstract: *Plasmopara viticola* is a destructive oomycete that affects grapevines, causing significant economic losses worldwide. This study highlights how the plant *Glechoma hederacea* might be at the basis for the development of biofungicides to control *P. viticola*. The aqueous extract obtained from *G. hederacea* aerial parts showed strong inhibition activity against *P. viticola*, comparable to that of copper hydroxide. The bioguided purification of the extract by chromatographic techniques led to the isolation of six pure metabolites, identified as the aromatic compounds carvacrol, caffeic acid and methyl caffeate, the flavonoids cirsimaritin and apigenin and the polyphenolic acid rosmarinic acid by spectroscopic methods. This is the first report about the isolation of methyl caffeate and cirsimaritin from *G. hederacea*. Caffeic acid and methyl caffeate showed the highest disease severity reduction, while carvacrol, cirsimaritin and apigenin also showed moderate activity against *P. viticola*. The inhibitory activity of the aqueous extract could suggest synergetic or additive action of caffeic acid and methyl caffeate together with other compounds contained in the extract. This study provides insights into the potential of *G. hederacea* as an allelopathic tool for developing control methods against *P. viticola*, revealing the combined action of different metabolites involved in the mechanism of action of the active compounds.

Keywords: *Glechoma hederacea*; *Plasmopora viticola*; allelopathy; plant extracts; caffeic acid; methyl caffeate

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1. Introduction

Plasmopara viticola, the causal agent of grapevine (*Vitis vinifera* L.) downy mildew, is a plant pathogenic oomycete that affects grapevines, causing significant losses in grapevine production worldwide, and is considered as one of the most devastating diseases in viticulture. *P. viticola* infections lead to severe yield losses and decrease of the quality of grapes and wines while having an indirect negative impact on the environment caused by massive use of fungicides for its control [1–3]. The pathogen can spread rapidly under favorable environmental conditions, such as high humidity and moderate temperatures, through repeated infection cycles [4]. Infections occur in the leaves, showing distinctive yellow discoloration lesions (oil spots) on the upper side and a white visible sporulation on the undersides, impairing the photosynthetic potential of the plant [5]. Moreover, *P. viticola* can infect inflorescences before and during blooming, while clusters and berries

in the early stages after fruit set, leading to tissue necrosis and hence to potentially heavy yield losses [6–8]. Thus, effective disease management is crucial to protect grapevine plants and ensure the production of high-quality grapes and wines.

In organic viticulture, copper-based fungicides are the oldest plant protection products used against *P. viticola* and they still play a crucial role in disease management. However, copper, being a heavy metal, has a negative impact on the environment and on human health, since it accumulates in agricultural soils and has been linked to liver and neurological diseases [9,10]. On the other hand, growers have to face the outburst of *P. viticola* populations resistant to single-site chemical fungicides, which have been used widely in integrated pest management [5].

For these reasons, the scientific community is focusing on research for alternative plant protection products, based on plant extracts or biocontrol agents, in order to reduce copper inputs in agriculture and to develop sustainable disease management strategies [11].

In past decades, several studies have investigated the potential anti-oomycete activity of different plant extracts in germination tests, leaf disc bioassays and on greenhouse-cultivated grapevine seedlings, obtaining significant inhibitory activity against *P. viticola*. For instance, *Pinus pinaster* knot and *V. vinifera* canes extracts guarantee complete zoospore mobility and development inhibition [12,13]. Other studies highlighted disease severity reduction above 90% on leaf discs with plant extracts and essential oils obtained from different species such as *Artemisia absinthium*, *Equisetum arvense*, *Frangula alnus*, *Glycyrrhiza glabra*, *Melaleuca alternifolia*, *Picea abies*, *Quillaia saponaria*, *Rheum palmatum*, *Salix alba* and *Yucca schidigera* [14–20]. Moreover, *Inga sapindoides*, *Juncus effusus*, *Larix sibirica*, *Oreganum vulgare*, *Pinus sylvestris*, *Solidago canadensis* and *Verbesina lanata* plant extracts have been successfully tested in greenhouse-cultivated seedlings, with plant protection efficacy above 80% [21–25].

However, only a limited number of candidates have displayed satisfying results during field trials, with plant protection efficacies ranging from around 70%, after the application of *Larix decidua* and *Magnolia officinalis* plant extracts [11,26], to 94% on grapevine plants treated with a *Salvia officinalis* extract [27].

Glechoma spp., flowering plants of the *Lamiaceae* family with some traditional uses in herbal medicine and culinary applications [28,29], stand as a potential source to be explored for bioactive extracts or metabolites of interest for the control of *P. viticola*. Recently, ground ivy (*G. hederacea*) extracts showed relevant antifungal activity against different fungi (i.e., *Candida albicans* and *Sclerotinia sclerotiorum*) [29]. Thus, further studies on *G. hederacea* could provide novel results regarding its applicability. As applied in classical allelopathic studies, the evaluation of extracts and their metabolites in bioassays could provide new sources to be proposed for the development of new tools for more sustainable disease management [30,31]. This study covers the first evaluation of *G. hederacea* extracts against *P. viticola*, followed by a screening of the metabolites responsible for the biological activity.

2. Materials and Methods

2.1. Experimental Procedures

Proton nuclear magnetic resonance (^{1}H NMR) spectra were recorded at 400 MHz on a Bruker 400 Anova Advance (Bruker, Karlsruhe, Germany) spectrometer. The spectra were recorded using deuterated chloroform ($CDCl_3$) or deuterated methanol (CD_3OD), and the same solvents were used as internal standards. Electrospray ionization mass spectra (ESIMS) were performed using a liquid chromatography/mass spectrometry time of flight (LC/MS TOF) system Agilent 6230B (Agilent Technologies, Milan, Italy), high-performance liquid chromatography (HPLC) 1260 Infinity in positive mode. Column chromatography (CC) was performed using a silica gel (Kieselgel 60, 0.063–0.200 mm, Merck, Darmstadt, Germany). Thin-layer chromatography (TLC) was performed on analytical and preparative silica-gel plates (Kieselgel 60, F_{254}, 0.25 and 0.5 mm, respectively, Merck, Darmstadt, Germany). Spots were visualized by exposure to ultraviolet (UV) light (254 nm) and/or iodine vapors, and/or by spraying with 10% sulphuric acid (H_2SO_4) in methanol (MeOH)

and 5% phosphomolybdic acid in ethanol (EtOH), followed by heating at 110 °C for 10 min. Sigma-Aldrich Co. (Milan, Italy) supplied all the solvents.

C log*P* values of the isolated compounds were calculated using ChemOffice v20.1 (Perkin Elmer, Waltham, MA, USA) by means of the appropriate tool in ChemDraw Professional [32].

2.2. Plant Material and Microorganisms

G. hederacea plants were collected in May 2022 from a field at San Michele all'Adige (Italy) and maintained under controlled conditions (25 ± 0.5 °C; 70 ± 10% relative humidity, RH) in 0.7 L pots containing a mixture of peat and pumice (3:1) for six months. The aerial part of the plants, consisting of stems and leaves, was collected each month to have sufficient material for the evaluation of aqueous extracts and the subsequent chemical analysis.

P. viticola was isolated from an untreated vineyard in San Michele all'Adige (Italy) in 2022 and maintained on grapevine plants (*V. vinifera* cv. Pinot Noir, grafted onto Kober 5BB.) by subsequent weekly inoculations using a hand sprayer according to Puopolo et al. [33]. Briefly, grapevine plants showing oil spot symptoms were kept overnight in the dark at 20–21 °C and 100% RH to induce the sporulation of *P. viticola*. The sporangia of *P. viticola* were collected by washing the abaxial leaf surface of grapevine leaves covered with sporulating lesions with cold (4–5 °C) distilled water. The final concentration of the sporangia suspension was adjusted to 2.5×10^4 sporangia/mL by counting with a hemocytometer under a light microscope.

2.3. Production of G. hederacea Aqueous Extracts and Activity Evaluation against P. viticola

Using a scissor, 40 g of the aerial parts (stems and leaves) of *G. hederacea* was cut into small pieces and macerated in a 1 L box with 200 mL of sterile distilled water at 20 °C in the dark. After 14 days, the aqueous extract was collected into sterile 50 mL tubes, centrifuged (4000 rpm, 20 min) to remove plant debris and filter-sterilized (0.22 µm; Sigma-Aldrich, Milan, Italy). A 1 L box containing only sterile distilled water (200 mL) was treated similarly and used as the untreated control.

For efficacy tests against *P. viticola*, leaf discs (18 mm in diameter) were obtained from the third and fourth apical leaves from the shoots of grapevine plants (*V. vinifera* cv. Pinot Noir, grafted onto Kober 5BB) grown in greenhouse conditions at 25 °C with relative humidity of 60 ± 10% and a photoperiod of 16 h of light for two months, resulting in a light intensity of 1050 mmol/m^2/s. Leaf discs were transferred (lower surface uppermost) onto sterilized moist filter paper (three foils) contained in Petri dishes (five grapevine leaf discs for each dish). The aqueous extract of *G. hederacea* was sprayed on grapevine leaf discs using a hand sprayer (7 mL for each Petri dish). Sterile distilled water and copper hydroxide (Coprantol Hi Bio, Syngenta, Basel, Switzerland; 2 g/L) were used as controls. Once treated, Petri dishes were stored at 25 °C in the dark. After 24 h, a *P. viticola* sporangia suspension (2.5×10^4 sporangia/mL) was sprayed on the grapevine leaf discs (7 mL for each Petri dish) using a hand sprayer. Petri dishes were incubated in the dark at 25 °C overnight and then maintained under controlled greenhouse conditions (25 °C, 60–80% RH with a 16/8-h day/nightlight regime). After seven days, the severity of downy mildew disease was assessed visually as the percentage of abaxial leaf disc area covered by white sporulation of *P. viticola*, according to the standard guidelines of the European and Mediterranean Plant Protection Organization [34]. Five replicates (Petri dishes) were used for each treatment, and the experiment was repeated.

2.4. Obtaining G. hederacea Extracts, and Isolation and Identification of Secondary Metabolites

In 300 mL of distilled H_2O/MeOH (1/1, *v*/*v*), 40 g of dried aerial parts (leaves and stems) of *G. hederacea* were blended and macerated at room temperature for 48 h. The resulting hydroalcoholic suspension (225 mL) was centrifuged (7000 rpm; 25 min) and extracted with *n*-hexane (3 × 150 mL) and methylene chloride (CH_2Cl_2, 3 × 150 mL). The residue aqueous phase was concentrated under reduced pressure to remove the methanol

and was then extracted with ethyl acetate (EtOAc, 3 × 150 mL). Each extract was dried over anhydrous sodium sulphate (Na_2SO_4), filtered and concentrated to dryness under reduced pressure. The extraction process was performed for a second time. The total amount obtained for each extract was 98.6 mg (*n*-hexane), 105.5 mg (CH_2Cl_2) and 170.3 mg (EtOAc).

The *n*-hexane extract (98.6 mg) was purified by column chromatography on Si-gel eluted with $CHCl_3$/*i*-propanol (9/1, *v*/*v*), yielding seven homogeneous fractions (FH1-FH7). The residue of FH4 (27.7 mg) was purified by preparative TLC eluted with $CHCl_3$/*i*-propanol (99/1, *v*/*v*), yielding a pure compound identified as carvacrol (26.2 mg).

The CH_2Cl_2 extract (105.5 mg) was purified by column chromatography on Si-gel eluted with $CHCl_3$/*i*-propanol (gradient from 9/1 to 7/3, *v*/*v*), yielding nine homogeneous fractions (FC1-FC9). The residues of FC2 (8.2 mg), FC3 (7.9 mg) and FC4 (16.1) were individually purified by two successive steps of analytical TLC eluted with $CHCl_3$/*i*-propanol (95/5, *v*/*v*) and with *n*-hexane/EtOAc (1/1, *v*/*v*), obtaining three pure compounds identified as cirsimaritin (6.4 mg), apigenin (3.6 mg) and methyl caffeate (5.9 mg).

The EtOAc extract (170.3 mg) was purified by column chromatography on Si-gel eluted with EtOAc/MeOH/H_2O (85/10/5, *v*/*v*), yielding seven homogeneous fractions (FE1-FE7). The residue of FE2 (8.7 mg) was purified by reversed-phase TLC eluted with H_2O/MeOH (1/1, *v*/*v*), yielding a pure compound identified as caffeic acid (3.3 mg). The residue of FE5 (31.7 mg) was purified by preparative TLC eluted with EtOAc/MeOH/H_2O (85/10/5, *v*/*v*), yielding a pure compound identified as rosmarinic acid (4.9 mg).

The structural characterization of the six compounds isolated was performed by comparison of their spectroscopic nuclear magnetic resonance (NMR) and mass data with those previously reported for the identified compounds as summarized below. The NMR spectra are provided in the Supplementary Materials.

Carvacrol: ^{1}H NMR spectrum (Figure S1) in agreement with data previously reported [35]; ESIMS (+) *m*/*z*: 151 $[M + H]^+$, consistent with the molecular formula $C_{10}H_{14}O$.

Cirsimaritin: ^{1}H NMR spectrum (Figure S2) in agreement with data previously reported [36]; ESIMS (+) *m*/*z*: 315 $[M + H]^+$, consistent with the molecular formula $C_{17}H_{14}O_6$.

Apigenin: ^{1}H NMR spectrum (Figure S3) in agreement with data previously reported [37]; ESIMS (+) *m*/*z*: 271 $[M + H]^+$, consistent with the molecular formula $C_{15}H_{10}O_5$.

Methyl caffeate: ^{1}H NMR spectrum (Figure S4) in agreement with data previously reported [38]; ESIMS (+) *m*/*z*: 195 $[M + H]^+$, consistent with the molecular formula $C_{10}H_{10}O_4$.

Caffeic acid: ^{1}H NMR spectrum (Figure S5) in agreement with data previously reported [39]; ESIMS (+) *m*/*z*: 181 $[M + H]^+$, consistent with the molecular formula $C_9H_8O_4$.

Rosmarinic acid: ^{1}H NMR spectrum (Figure S6) in agreement with data previously reported [40]; ESIMS (+) *m*/*z*: 361 $[M + H]^+$, consistent with the molecular formula $C_{18}H_{16}O_8$. $[\alpha]^{20}_D$ + 61° (c 0.2, MeOH); $[\alpha]^{20}_D$ + 68° (c 0.2, MeOH) lit. [41].

2.5. Evaluation of G. hederacea Extracts and Secondary Metabolites against P. viticola

Residues of the organic extracts (obtained in *n*-hexane, CH_2Cl_2 and EtOAc) and the secondary metabolites (namely carvacrol, cirsimaritin, apigenin, methyl caffeate, caffeic acid and rosmarinic acid) were resuspended in dimethyl sulfoxide (DMSO, 0.5% *v*/*v*) in order to obtain a final concentration of 500 µg/mL. These solutions were evaluated for the protection of grapevine leaf discs against *P. viticola* following the procedure reported above. A DMSO solution (0.5% *v*/*v*), water and copper hydroxide (Coprantol Hi Bio, Syngenta; 2 g/L) were used as controls. Five replicates (Petri dishes) containing five grapevine leaf discs each were used for each treatment, and the experiment was repeated.

2.6. Statistical Analysis

The data of efficacy tests on grapevine leaf discs were analyzed using Statistica 9 software (StatSoft, Tulsa, OK, USA). After validation of the normal distribution (K-S test, $p > 0.05$) and variance homogeneity (Levene's test, $p > 0.05$) of the data, an analysis of

variance (ANOVA) was carried out using Tukey's HSD test ($\alpha = 0.05$) to detect significant differences among treatments.

3. Results and Discussion

3.1. Activity of G. hederacea Aqueous Extracts against P. viticola on Grapevine Leaf Discs

The anti-oomycete activity of aqueous extracts from the aerial parts of *G. hederacea* against *P. viticola* was evaluated in a leaf disc bioassay (Figures 1 and S7). The application of *G. hederacea* aqueous extracts determined a remarkable reduction of grapevine downy mildew severity (11.4%) compared to that in grapevine leaf discs treated with distilled water (78.0%). Although significantly different, the ability of *G. hederacea* aqueous extracts to reduce grapevine downy mildew severity was similar to the protection achieved by the application of the copper-based fungicide (2.3%). To the best of our knowledge, this is the first evidence of anti-oomycete activity by *G. hederacea* aqueous extracts. These results showed that *G. hederacea* produces compounds active against *P. viticola*. Thus, the isolation and identification of those compounds is essential to get insights into their characterization.

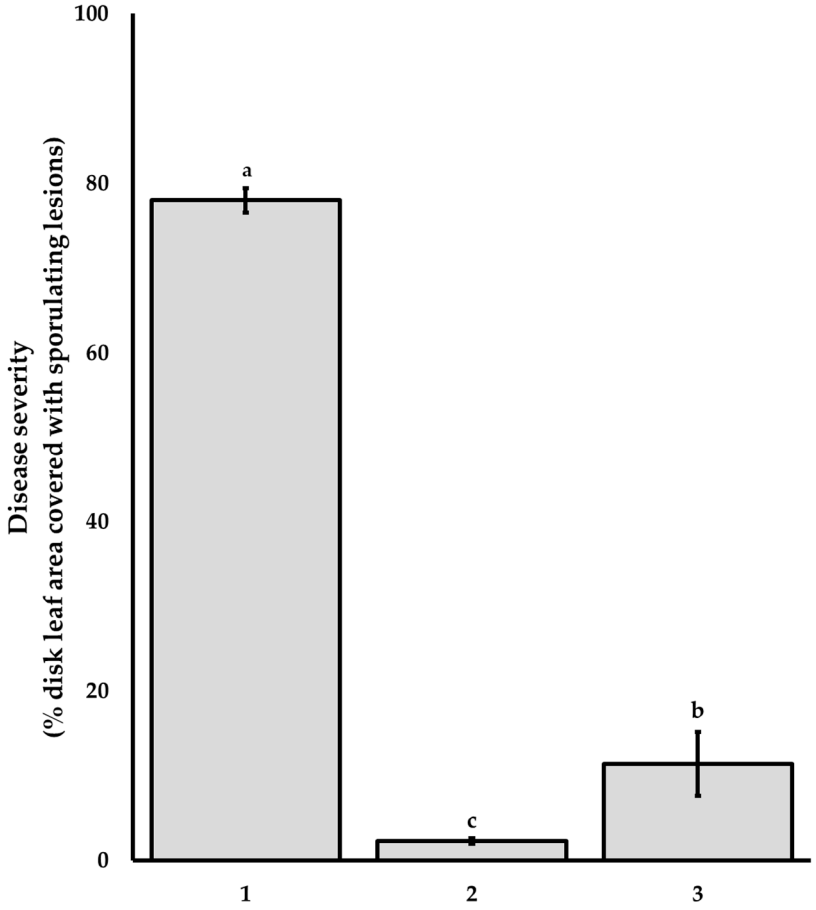

Figure 1. Efficacy of *Glechoma hederacea* aqueous extracts in controlling *Plasmopara viticola* on grapevine leaf discs. The treatments evaluated were: (1) grapevine leaf discs treated with distilled water, (2) grapevine leaf discs treated with copper hydroxide (2 g/L) and (3) grapevine leaf discs treated with *G. hederacea* aqueous extracts. The columns represent the mean ± standard error of five replicates (Petri dishes) containing five grapevine leaf discs each. Columns with different letters are significantly different according to Tukey's HSD test ($\alpha = 0.05$).

3.2. Bioguided Isolation and Supressive Activity of Pure Compounds

Dried aerial parts of *G. hederacea* were macerated in a H_2O/MeOH (1/1, *v*/*v*) solution and extracted with solvents of increasing polarity (namely *n*-hexane, CH_2Cl_2 and EtOAc) as described in Section 2.4. The weight of the collected extracts increased with the polarity

of the solvent (98.6 mg for *n*-hexane, 105.5 mg for CH_2Cl_2 and 170.3 mg for EtOAc). The anti-oomycete activity of these extracts against *P. viticola* was subsequently evaluated (Figure 2).

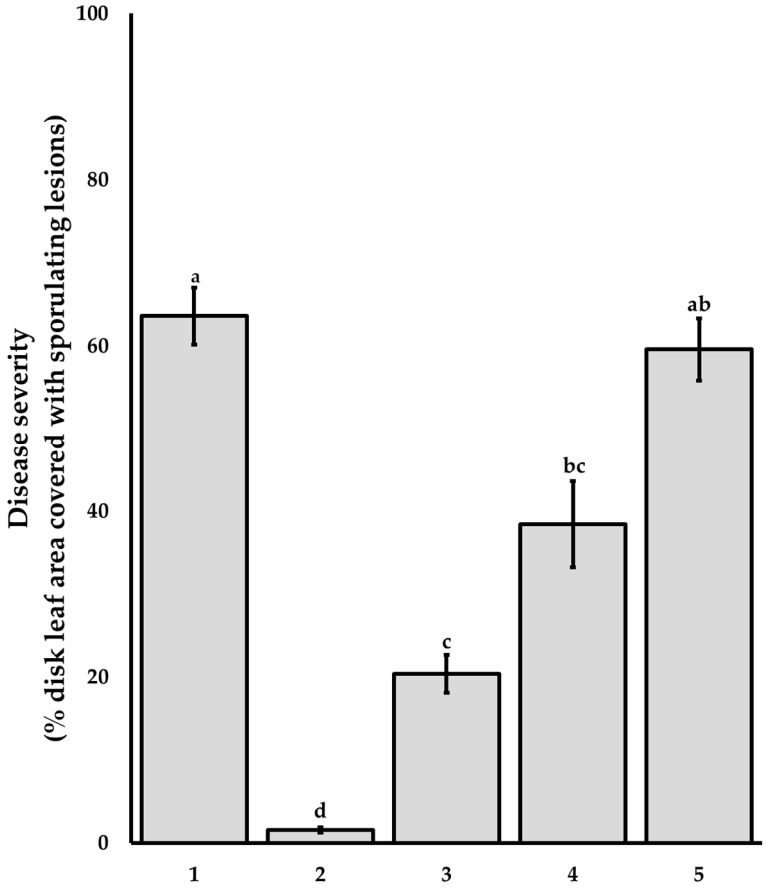

Figure 2. Efficacy of organic extracts of *Glechoma hederacea* in controlling *Plasmopara viticola* on grapevine leaf discs. The treatments evaluated were: (1) grapevine leaf discs treated with a DMSO solution (0.5% *v*/*v*), (2) grapevine leaf discs treated with copper hydroxide (2 g/L), (3) grapevine leaf discs treated with a *G. hederacea* organic extract in CH_2Cl_2, (4) grapevine leaf discs treated with a *G. hederacea* organic extract in *n*-hexane and (5) grapevine leaf discs treated with a *G. hederacea* organic extract in EtOAc. Organic extracts of *G. hederacea* were evaluated at a final concentration of 500 µg/mL. The data are presented as the mean ± standard error of five replicates (Petri dishes) containing five grapevine leaf discs each. Columns with different letters are significantly different according to Tukey's HSD test ($\alpha = 0.05$).

The CH_2Cl_2 extract showed significantly lower disease severity (20.4%) compared to that of the untreated control (63.6%), followed by that of the n-hexane extract (38.4%). On the other hand, the EtOAc extract did not provide a significant reduction of disease severity (59.6%).

These results suggested the purification of the organic extracts in order to identify the low-molecular-weight metabolites produced by *G. hederacea* and evaluate their standalone anti-oomycete activity against *P. viticola*.

The *n*-hexane, CH_2Cl_2 and EtOAc extracts were purified by chromatographic means (mainly column and TLC chromatography) following the procedures described in Section 2.4. Six pure metabolites (Figure 3) were isolated and identified as three aromatic compounds (carvacrol, caffeic acid and methyl caffeate), two flavonoids (cirsimaritin and apigenin) and a polyphenolic acid (rosmarinic acid). Specifically, carvacrol was collected from the *n*-hexane extract, cirsimaritin, apigenin and methyl caffeate from the CH_2Cl_2 extract and caffeic acid and rosmarinic acid from the EtOAc extract. All the compounds contain an

aromatic system in their structure. Carvacrol is a monoterpenic phenol found in essential oils from different plant species. It has been reported as a volatile constituent of the essential oil of *G. hederacea* [42], the unique *Glechoma* species in which this metabolite has been described so far. Structurally related compounds are common metabolites described in the literature, such as the recently discovered natural carvacrol dimer [43,44].

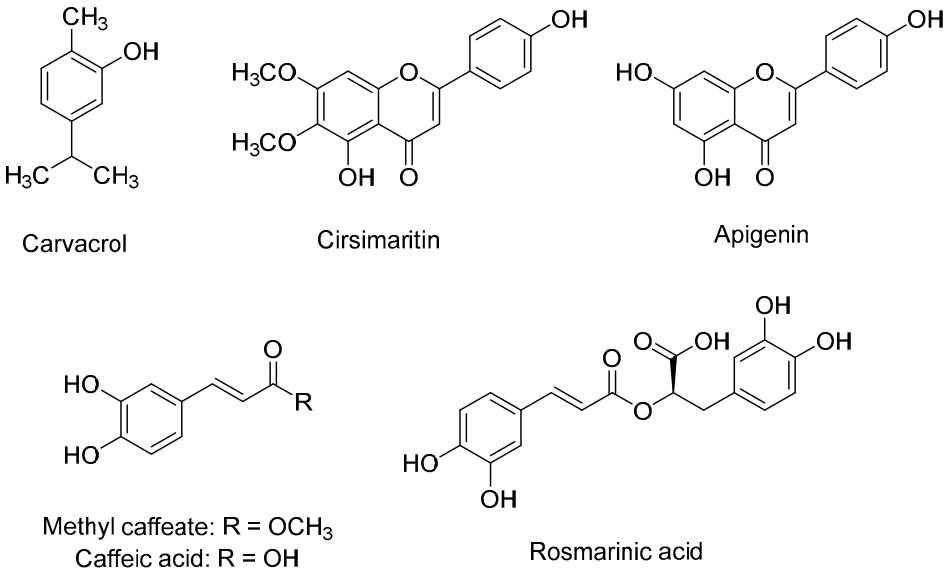

Figure 3. Structures of the metabolites isolated from the *Glechoma hederacea* extracts.

The flavonoids cirsimaritin and apigenin were also isolated, with cirsimaritin being reported for the first time from *G. hederacea* (it was previously found in *Glechoma herbaceae* [45]). Apigenin is often isolated in glycosylated forms and has already been identified in *G. hederacea* [46,47]. Herein, methyl caffeate is reported for the first time for a *Glechoma* species, while caffeic acid and rosmarinic acid, a water-soluble ester of caffeic acid, are among the most abundant phytochemicals detected in hot water extracts of *G. hederacea* [47–49].

P. viticola disease severity was evaluated after the application of the six isolated compounds at 500 µg/mL (Figure 4). The metabolites showing the highest anti-oomycete activity were caffeic acid and methyl caffeate (18.8% and 21.2% disease severity, respectively), followed by carvacrol, cirsimaritin and apigenin (48.0–41.0%), while rosmarinic acid did not display any disease reduction, with disease severity (66.0%) comparable to that of the negative control (65.6%).

Very few reports have been found regarding the biological activity of the six isolated compounds against *P. viticola*, while antifungal activity has been described for all of them [50–57]. Caffeic acid is included among the metabolites responsible for significant *P. viticola* disease reduction in a *Pinus pinaster* knot extract [17] and is reported to be related to oxidative mechanisms in response to *P. viticola* infections [58] and to constitutive resistance in the grapevine cultivar Regent [59]. On the other hand, caffeic acid has been found in considerable amounts in susceptible *V. vinifera* varieties [60]. Thus, it is probable that the contribution of caffeic acid to the resistance of grapevine varieties might be linked the phenological phase when this secondary metabolite is produced. Methyl caffeate, isolated from a methanolic extract from *Solanum torvum* Swartz., displays antimicrobial and antimycobacterial activity against several *Mycobacterium* strains [61].

Moderate disease severity reduction by carvacrol partially confirms the results displayed in a recent study on *O. vulgare* essential oils against *P. viticola*, where the anti-oomycete activity was attributed to a cell-membrane-damaging effect after interaction with membrane sterols [24]. Moreover, among the wide array of biological activities of pharmacological and agronomical interest [62,63], carvacrol has been reported for its antifungal activity against different fungal species and for its use for the development of formulations

against the oomycete *Pythium insidiosum* [64–69]. It is interesting to note that in the case of flavonoids (in this study, cirsimaritin and apigenin), a key role has been suggested for this family of compounds in the resistance of grapevine callus tissue to *P. viticola* [70].

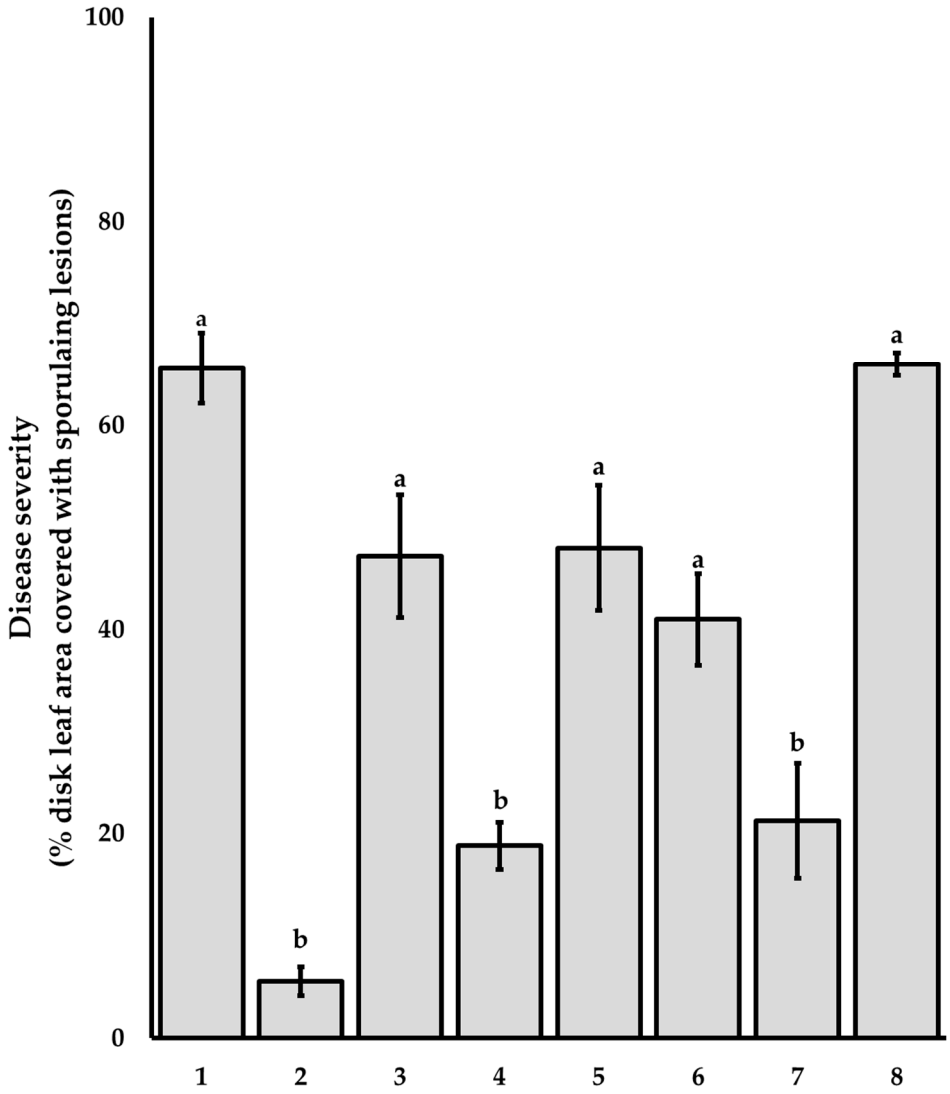

Figure 4. Efficacy of secondary metabolites extracted from *Glechoma hederacea* in controlling *Plasmopara viticola* on grapevine leaf discs. The treatments evaluated were: (1) grapevine leaf discs treated with a DMSO solution (0.5% *v*/*v*), (2) grapevine leaf discs treated with copper hydroxide (2 g/L), (3) grapevine leaf discs treated apigenin, (4) grapevine leaf discs treated with caffeic acid, (5) grapevine leaf discs treated with carvacrol, (6) grapevine leaf discs treated with cirsimaritin, (7) grapevine leaf discs treated with methyl caffeate and (8) grapevine leaf discs treated with rosmarinic acid. Secondary metabolites isolated from *G. hederacea* plant tissues and aqueous extracts were evaluated at a final concentration of 500 µg/mL. The data are presented as the mean ± standard error of five replicates (Petri dishes) containing five grapevine leaf discs each. Columns with different letters are significantly different according to Tukey's HSD test ($\alpha = 0.05$).

While the aqueous extract showed remarkable disease severity reduction (11.4%, Figure 1), among the six isolated metabolites, only caffeic acid and methyl caffeate showed comparable levels of activity (18.8% and 21.2%, respectively, Figure 4). From a structural point of view, both compounds display the same main structure, with the difference of the functionalization of the carboxylic acid group of caffeic acid.

Nevertheless, rosmarinic acid, which is an ester of two caffeic acid units, resulted as the less active metabolite (66.0%). This accentuated decrease could be explained by a minor

action of the aromatic ring in the activity, and consequently the lateral chain would play a direct action. Alternatively, the loss of activity of rosmarinic acid could be explained in terms of hindrance. The analysis of the Clog *P* values theoretically calculated for the six compounds (Table 1) could provide insights in this regard. Specifically, the lipophilicity expressed in this way shows how the value for rosmarinic acid (1.10) is between those of methyl caffeate (1.20) and caffeic acid (0.98). Thus, the different level of activity among caffeic acid, methyl caffeate and rosmarinic acid would not be related to a different level of solubility, and the potential hindrance of rosmarinic acid would be related to other features like molecular size and specificity with the target sites.

Table 1. Lipophilicity values of the six compounds isolated, calculated by the Clog *P* algorithm.

Compound	Carvacrol	Cirsimaritin	Apigenin	Methyl Caffeate	Caffeic Acid	Rosmarinic Acid
Clog *P*	3.35	2.86	2.91	1.20	0.98	1.10

It is interesting to note that two flavonoids were evaluated in this study (cirsimaritin and apigenin), obtaining similar disease severity (41.0% vs. 47.2%). The slightly improved activity for cirsimaritin might be correlated with the methoxy groups contained in its structure. Given the close Clog *P* values of these compounds (2.86 vs. 2.91), the methoxy groups would play a direct role in the reactivity of the compound with the target sites.

Another point to highlight regarding the lipophilicity of the six tested metabolites could be concluded by a global view of their Clog *P* values (Table 1). Specifically, a general decrease of activity was observed when the lipophilicity was higher, as carvacrol, cirsimaritin and apigenin showed the highest Clog *P* values (2.86–3.35), with the exception of rosmarinic acid, as previously discussed.

The higher disease reduction of the aqueous extract compared to that of the most active standalone compounds (such as caffeic acid and methyl caffeate) may be correlated to additive or synergetic action of the different compounds taken into consideration. Indeed, a previous study proved the synergistic effects of caffeic acid in relation to its antifungal activity [71]. The activity evaluation of the organic extracts (Figure 2) and the isolated compounds (Figure 4) can provide additional information. The CH_2Cl_2 extract was the most active organic extract (20.4%), and this activity must be strongly related with the action of methyl caffeate (18.8%). Cirsimaritin or apigenin, with disease severities of 41.0% and 47.2%, respectively, did not seem to negatively affect the activity of the CH_2Cl_2 extract. The *n*-hexane extract, which should contain the less polar compounds contained in the aqueous extract, also showed moderate activity (38.4%), which slightly improved that of carvacrol (48.0%), the only compound isolated from it. Thus, other potential minor compounds in the *n*-hexane extract could play a key role in the activity. Finally, the EtOAc extract showed poor activity, similarly to rosmarinic acid. Since caffeic acid showed high activity (18.8%), it could be concluded that this compound is a minor component of the EtOAC extract showing relevant anti-oomycete activity.

Research on the six isolated compounds may be useful to define these potential correlations, which would provide insights into the applicability of *G. hederacea* extracts for the control of *P. viticola* or into the development of new pesticides based on natural products.

4. Conclusions

This study explored the potential of *Glechoma hederacea* extracts and isolated compounds active against grapevine downy mildew, *Plasmopara viticola*. The aqueous extract caused significant *P. viticola* growth inhibition, close to that of the positive control. Subsequent isolation of compounds from organic extracts revealed six metabolites: three aromatic compounds (carvacrol, caffeic acid and methyl caffeate), two flavonoids (cirsimaritin and apigenin) and a polyphenolic acid (rosmarinic acid). Among these, caffeic acid and methyl caffeate exhibited the highest activity, while carvacrol, cirsimaritin and apigenin displayed

moderate activity. Notably, rosmarinic acid was inactive. The study suggests that the efficacy obtained by the aqueous extract may have resulted from synergistic or additive activity of methyl caffeate and caffeic acid with other extract components. This study highlights the potential of *G. hederacea* extracts to control *P. viticola* and the complexity of interactions among its active compounds, offering prospects for developing natural pesticides.

Supplementary Materials: The following supporting information can be downloaded at: https://www.mdpi.com/article/10.3390/agriculture14010058/s1, Figure S1: ^{1}H NMR spectrum of carvacrol in $CDCl_3$ (400 MHz), Figure S2: ^{1}H NMR spectrum of cirsimaritin in CD_3OD (400 MHz), Figure S3: ^{1}H NMR spectrum of apigenin in CD_3OD (400 MHz), Figure S4: ^{1}H NMR spectrum of methyl caffeate in CD_3OD (400 MHz), Figure S5: ^{1}H NMR spectrum of caffeic acid in CD_3OD (400 MHz), Figure S6: ^{1}H NMR spectrum of rosmarinic acid in CD_3OD (400 MHz), Figure S7: Control of *Plasmopara viticola* on grapevine leaf discs through the application of *Glechoma hederacea* aqueous extracts.

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