Communication

Antifungal Activity of Essential Oil and Main Components from *Mentha pulegium* Growing Wild on the Chilean Central Coast

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Abstract: Fungal diseases, both pre- and post-harvest, are currently difficult to control—increased antifungal resistances have further stimulated the search for natural alternatives. The objective of the present research work was to evaluate the antifungal activities of *Mentha pulegium* essential oil (EO) and its major constituents. The EO was obtained from hydro distillation of fresh leaves, and composition was determined using gas chromatography/mass spectrometry (GC/MS). The main components were identified as pulegone (29.33%), menthol (28.79%), menthone (20.48%), and isopulegol (9.75%). EO and isopulegol exhibited the highest antifungal activity, with half maximal effective concentrations (EC₅₀) inhibiting mycelial activity of *Monilinia fructicola* at 24.6 µg/mL and 20.8 µg/mL, respectively, and against *Botrytis cinerea*, at 301.45 µg/mL and 333.84 µg/mL, respectively. These findings could lay the foundation for developing antifungal agents of agricultural value.

Keywords: *Mentha pulegium*; *Botrytis cinerea*; *Monilinia fructicola*; antifungal activity; essential oil

1. Introduction

Phytopathogenic fungi are a serious threat to plant health, causing a plethora of diseases that contribute substantially to overall losses in agricultural yield [1]. In addition, fungal plant pathogens are divided into two main groups: biotrophic pathogens, which form intimate interactions with plants and can persist in and utilize living tissues (biotrophs), and necrotrophic pathogens, which kill the tissue to extract nutrients (necrotrophs) [2]. *Botrytis cinerea* and *Monilinia fructicola* are the best examples of broad-host-range, necrotrophic plant pathogens. The success of these pathogens on diverse crops is attributed to the production of an extensive array of compounds, enzymes, and toxins, which singly or in combination likely interfere with common structural and functional features shared among different

plant families [3]. For instance, *B. cinerea* can infect more than 235 different plant species prevalent over geographically diverse regions, causing grey mould [4], while *M. fructicola* can infect different members of the Rosaceae family worldwide, causing brown rot [5]. The losses generated by these fungi have led farmers to combat them mainly with chemical treatments, which cause harmful effects on human and environmental health, and may result in more resistant strains and an increase in production costs [6]. Recently, many researchers have demonstrated that essential oils (EOs), due to their effectiveness, low toxicity, and low persistence in the environment, should be used as a promising form of alternative to synthetic fungicides [7].

Mentha pulegium L. (known in Chile as poleo) is a medicinal flowering plant native to Chile, Europe, North Africa, and the Middle East [8] that grows wild in the temperate regions. Traditionally, *Mentha pulegium* has been extensively utilized in traditional herbal medicine to treat several ailments, including uses as a stomachic, expectorant, diuretic, menstrual treatment, and microbial infections [9]. Various biological properties for *Mentha pulegium* have been described, including antioxidant, antitumor, insecticidal, and antimicrobial activities [10–12]. The constituents of *Mentha pulegium* oil have been the subject of numerous studies, which have shown differences in its composition depending on the region of cultivation [13,14].

Based on these considerations, the aim of this communication was to determine by means of in vitro antifungal activity bioassays the effectiveness of the *Mentha pulegium* EO and its chemical constituents against some important post-harvest diseases, such as *Botrytis cinerea* and *Monilinia fructicola*. The motivation behind this study is the wish to have natural substances that are active for possible organic control against the deterioration of fruits.

2. Materials and Methods

2.1. Chemicals

Pulegone, menthol, menthone, and isopulegol were obtained from Sigma-Aldrich, Darmstadt, Germany. All other chemicals used were analytical grade and obtained from either Sigma-Aldrich or Merck.

2.2. Plant Material

Leaves of *Mentha pulegium* were randomly selected and collected in March 2019 from the locality Laguna Verde, Valparaíso, Chile (33°03'04" S, 71°39'34" O). The sample was stored as a voucher specimen MP2019-3.24 at the Natural Products Laboratory, University of Playa Ancha.

2.3. EO Extraction and Analysis

Essential oil was extracted from 500 g of fresh plant for 6 h by hydro distillation (3.0 L, H₂O) in a Clevenger-type apparatus. The EO was dried over anhydrous sodium sulfate and stored at −20 °C until analysis. The EO component analysis was performed by gas chromatography (GC) and gas chromatography/mass spectrometry (GC/MS) using the instrumentation described below. A Thermo Scientific GC–MS system (GC: model Trace GC Ultra and MS: model ISQ, Thermo Fisher Scientific, Waltham, Massachusetts, USA) was used for the analysis of the sample. The separation was performed on a 60 m × 0.25 mm internal diameter fused silica capillary column coated with 0.25 µm film Rtx-5MS. The operating conditions were as follows: on-column injection; injector temperature, 250 °C; detector temperature, 280 °C; carrier gas, He at 1.25 mL/min; oven temperature program: 40 °C for 5 min, increase to 260 °C at 5 °C/min, and then 260 °C for 5 min. The mass detector ionization employed an electron impact of 70 eV. Recording conditions employed a scan time of 1.5 s and a mass range of 40 to 400 amu. Compounds in the chromatograms were identified by comparison of their mass spectra with those in the NIST/EPA/NIH Mass spectral Library (2008), and by comparison of their retention index with those reported in the literature [15], for the same type of column or those of commercial standards (Sigma-Aldrich, Darmstadt, Germany).

2.4. In Vitro Antifungal Activity of the EO and Main Compounds against Phytopathogenic Fungi

2.4.1. Fungal Isolates

The isolates of *B. cinerea* and *M. fructicola* were isolated directly from grape and peach sick fruits, respectively. The isolates were maintained on potato dextrose agar (PDA) medium in the dark at 22 °C. Fungal isolates were characterized based on their morphology. It is part of the collection of micro-organisms of the Laboratory of Biological Tests of the Department of Chemistry of the University Technical Federico Santa María.

2.4.2. In vitro Antifungal Activity

The antifungal activity of the *Mentha pulegium* EO and main compounds against *B. cinerea* and *M. fructicola* were tested according to a procedure previously reported [16] using radial growth rate assay in potato dextrose agar (PDA) growth [16]. Fungal inocula of *B. cinerea* and *M. fructicola* were prepared in plates with potato dextrose agar (PDA), incubated for 3 days and 1 week, respectively, at 25 °C. The oil and main compounds were dissolved in an ethanol/water solution (5% v/v) and were added to a Petri dish containing PDA medium (5 mL). The final tested concentrations were 10, 25, 50, 150, and 250 µg/mL for each treatment. PDA medium containing 1% ethanol was considered as the negative control (C⁻), whereas BC-1000® and Mystic® 520 SC, commercial fungicides (CHEMIE®, BAYER, Santiago, Chile), were used as the positive control (C⁺) at the same concentrations and under the same bioassay conditions. A mycelium agar disk (4 mm in diameter) of the pathogen's fungi were placed in the center of the PDA plates. *B. cinerea* was incubated for 3 days at 23 °C, whereas *M. fructicola* was incubated for 1 week at the same temperature in the dark. Each treatment was replicated three times. The inhibition percentages of mycelial growth for each compound were calculated and compared with the negative control as described in a previous report [17]. The effective concentration that inhibited mycelium growth by 50% (EC₅₀) was obtained for each treatment by fitting % Inhibition and concentration to a dose–response equation. The fit analysis was performed using the Origin 8.0 software [17].

2.5. Statistical Analysis

The data of Table S1 of the supplementary material and EC₅₀ of *Mentha pulegium* EO and its main compounds were reported as the mean values ± standard deviation (SD). The inhibition percentages of mycelial growth for oil and each compound were calculated and compared with the negative control as described in a previous report [16]. An analysis of variance (ANOVA) was performed on all data with a post-hoc Tukey HSD ($p \leq 0.05$).

3. Results and Discussion

3.1. Essential Oil Composition

Hydrodistillation of *Mentha pulegium* aerial parts gave yellow EO in 1.30% yield (w/w). The chemical composition of the EO is given in Table 1.

Twelve components were identified in the essential oil: 50.11% were terpenketones, 46.89% terpenyl alcohols and derivatives, 1.81% terpenes, 0.37% linear alcohol, and 0.82% unknown compounds. The EO was mainly characterized by pulegone (29.33%), menthol (28.79%), menthone (20.48%), and isopulegol (9.75%) (see Figure 1).

The chemical composition of the *Mentha pulegium* EOs has been the subject of numerous studies, which show significant differences [13,18]. However, the chemical profile of the volatile oil of the studied plant was drastically different than previously reported. In our study, the oil of *Mentha pulegium* was found to contain equivalent amounts of pulegone and menthol. It is likely that these chemical variations are due to the diverse climatic and geographical differences between *Mentha pulegium* wild habitats in different countries as well as the divergent genetic potential of

plants for compartmentalization of different biochemical pathways leading to a wide variety of oil components [19].

Table 1. Main components of *Mentha pulegium* essential oil (EO).

No.	Chemical Group	Main Components	% Area ^a	RI ^b	RIref ^c
1	Terpene	α -Pinene	0.20	940	941
2	Terpene	β -Pinene	0.20	984	985
3	Linear alcohol	3-Octanol	0.37	993	993
4	Terpenyl alcohol	Isopulegol	9.75	1147	1147
5	Terpenketone	Menthone	20.48	1153	1154
6	Terpenyl derivative	Menthofuran	1.06	1159	1160
7	Terpenyl alcohol	Menthol	28.79	1176	1176
8	Terpenketone	Isopulegone	0.30	1178	1179
9	Terpenketone	Pulegone	29.33	1255	1257
10	Terpenyl derivative	Menthyl acetate	8.35	1283	1283
11	Terpene	α -Caryophyllene	0.28	1477	1472
12	Terpene	Caryophyllene oxide	0.07	1613	1606
13		Unknown compounds	0.82		

^a Surface area of GC peak; ^b RI: Retention indices relative to C₈–C₃₆ *n*-alkanes on the Rtx-5MS capillary column ^c RIref: Retention indices reported in literature.

Figure 1. Structures of the main compounds present in the EO of *M. pulegium*.

3.2. In Vitro Antifungal Activity of the EO and Main Compounds on Mycelium Growth of *Botrytis cinerea* and *Monilinia fructicola*

In this investigation, *Mentha pulegium* EO exhibited different degrees of antifungal activity against *B. cinerea* and *M. fructicola* (Figure 2).

Figure 2. In vitro effect of *Mentha pulegium* EO on the mycelial growth of *B. cinerea* and *M. fructicola* according to different concentrations: (a) 0 µg/mL (Negative control); (b) 25 µg/mL; (c) 250 µg/mL; (d) BC-1000® 25 µg/mL for *B. cinerea*; (e) 0 µg/mL (Negative control); (f) 25 µg/mL; (g) 250 µg/mL (h) MYSTIC® 520 SC at 25 µg/mL, used as positive control for *M. fructicola*.

The effect on mycelial growth is evaluated by comparing the growth areas with that observed for the negative control. The results are expressed as a percentage of inhibition, which is calculated as the ratio of the area of *B. cinerea* and *M. fructicola* in the presence and absence of the *Mentha pulegium* EO and main compounds, and they are summarized in Table S1 (Supplementary Material). In addition, EC₅₀ values (concentration causing 50% inhibition of mycelial growth) were obtained from fitting data to a dose–response curve. The comparison between antifungal effects of EO and natural compounds were further confirmed by comparing their effective concentrations for EC₅₀ listed in Table 2.

Table 2. EC₅₀ values of *Mentha pulegium* EO and its main compounds on the in vitro mycelial growth of *B. cinerea* and *M. fructicola*.

Treatment	<i>B. Cinerea</i>		<i>M. Fructicola</i>	
	* EC ₅₀ (µg/mL) ± ** SD	*** R	* EC ₅₀ (µg/mL) ± ** SD	*** R
EO	301.45 ± 1.49 ^a	0.9541	24.6 ± 1.33 ^b	0.8916
Isopulegol	333.84 ± 2.00 ^b	0.9945	20.8 ± 1.21 ^a	0.8638
Menthone	444.19 ± 1.57 ^c	0.9801	53.4 ± 1.36 ^d	0.9342
Menthol	332.15 ± 2.27 ^b	0.9984	33.4 ± 1.23 ^c	0.8800
Pulegone	496.48 ± 1.40 ^d	0.9649	69.6 ± 1.36 ^e	0.9228
BC-1000®	238.28 ± 2.04 ^e	0.7999	-	-
Mystic® 520 SC	-	-	2.19 ± 0.75 ^f	0.7999

These values were estimated by measuring the colony diameter after of 72 h and 168 h of incubation. * EC₅₀: concentration causing 50% mycelial growth inhibition; ** SD: Standard Deviation; *** R: Pearson's correlation coefficient. Mean values followed by the different letters under different treatments within a column are significantly different to positive control at $p \leq 0.05$ according to Tukey test.

Table 2 showed that EO and isopulegol exhibited strong activity against *M. fructicola*, with EC₅₀ values of 24.6 µg/mL and 20.8 µg/mL, respectively, and moderate activity against *B. cinerea*, with EC₅₀ values of 301.45 µg/mL and 333.84 µg/mL, respectively. However, the EC₅₀ values of EO and isopulegol are significantly different ($p \leq 0.05$); EO is more active compared to isopulegol against *B. cinerea* but is less active against *M. fructicola*. Based on the EC₅₀ values of the five treatments tested, EO and isopulegol are the most active growth inhibitors against both pathogens. Their EC₅₀ values are significantly different with BC-1000® and Mystic® 520 SC as the positive controls.

On the other hand, this is the first report of isopulegol activity against these phytopathogenic fungi. Furthermore, the results indicate that menthone and pulegone presented slight inhibitory effects on *B. cinerea*, in accordance with previous reports [20,21]. Menthol showed inhibitory effects on both, although with a slightly higher inhibition against *M. fructicola*, which agrees with previous findings [21–23]. In general, the results of the inhibitory effect of *Mentha pulegium* oil is consistent with previous reports on the antifungal activity of essential oils, which showed that the inhibitory effects of essential oils tend to increase according to their major component as follows: phenols > alcohols > aldehydes > ketones > ethers > hydrocarbons [24]. Numerous literature studies have provided support for the antimicrobial activities of several of the compounds in *Mentha pulegium* oil [25]. For instance, terpene alcohols (isopulegol and menthol) have shown greater inhibition of mycelial growth than have terpene ketones (menthone and pulegone) against both pathogens. Moreover, other authors have similarly found that terpene alcohols tend to be relatively active, regardless of structural types, as a function of their hydrogen capacity and water solubility [26].

The research team expects to expand upon the most active compounds found in this study, which are promising candidates for structural modification, as starting materials for potent antifungal hybrids against globally important agricultural pathogens.

4. Conclusions

Overall, the evaluation of *Mentha pulegium* EO against the two most important fruit diseases responsible for the most post-harvest losses revealed that the essential oil and its main components

were moderate growth inhibitors. Activities toward the two pathogens differed slightly, with higher inhibition against *M. fructicola*. Our results suggest that the antifungal activity of the oil is due to the synergistic effect of the components, including some not examined thus far. In turn, *Mentha pulegium* oil may be potentially used in natural therapies to treat infectious diseases in plants, and its inhibitory abilities help confirm the ability of some terpenes—in our case, isopulegol and menthol—to inhibit *M. fructicola* at low concentrations. In addition, we highlighted that mixtures of isopulegol and menthol could represent a promising starting point for the development of antifungal agents. Combinations of these two substances in various ratios should be studied, and it will be enlightening to evaluate the interaction effects of either or both isopulegol and menthol with commercial fungicide.

Supplementary Materials: The following are available online at <http://www.mdpi.com/2073-4395/10/2/254/s1>, Table S1. Effect of on in vitro mycelial growth of *B. cinerea* and *M. fructicola* measured as a percentage of inhibition.

Author Contributions: A.M. supervised the whole study. B.S. collected the plant. X.B. performed the isolation of the EO. C.P. and P.G. performed the spectroscopic data. K.D. conceived and designed the biologic experiments. A.M., K.D., and I.M. collaborated on the discussion and interpretation of the results. K.D. and A.M. wrote the manuscript. All authors have read and agreed to the published version of the manuscript.

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