Article

Propolis Hydroalcoholic Extracts: Biochemical Characterization and Antifungal Efficacy

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Abstract: The present study investigated the antifungal potential of hydroalcoholic extracts of propolis against the causal agent of grey mould, *Botrytis cinerea*, by in vitro and in vivo assays. Five different propolis from different Italian regions were subjected to hydroalcoholic extraction using different ethanol concentrations and extraction methods. The preliminary bio-assay showed significant inhibitory effects on *B. cinerea* mycelial growth of propolis extracts obtained using 90% ethanol and subjected to sonication. The calculation of EC50 values, based on the demonstrated efficacy of non-volatile and volatile metabolites of propolis extracts, was useful to understand the main fraction involved in the antifungal activity of the samples and to perform the in vivo assay on grape and blueberry fruits. Three of the propolis extracts showed a high amount of genistein. Conversely, the other two propolis showed a fair amount of apigenin, caffeic acid, chrysin, ferulic acid, kaempferol, luteolin, p-coumaric acid and quercetin. From the volatile analysis of propolis, the main compounds detected were α-cadinol, α-eudesmol, calamenene, cadinol, benzyl acetate, benzyl benzoate and benzyl alcohol. The results suggest that propolis extracts have potential as an effective postharvest antifungal treatment, with varying degrees of efficacy depending on the extraction method and the type of propolis metabolites.

Keywords: propolis; biodiversity; grey mould; volatile compounds; polyphenols

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1. Introduction

Usually, management of fungal pathogens primarily relies on the use of synthetic fungicides [1]. However, excessive fungicide use poses serious food safety and environmental risks due to persistent chemical residues and may also lead to the development of pathogen resistance phenomena [2]. In response to these concerns, the European Plant Health Regulation (EU 2016/2031) [3] and the European Green Deal have been introduced to accelerate the transition to a more sustainable food system. These initiatives advocate a 50% reduction in the use of pesticides and a 25% increase in the area devoted to organic farming by 2030. As a result, there is growing interest in exploring alternative management strategies, such as the use of natural compounds, to address the challenges posed by synthetic fungicides.

Propolis is produced by bees (*Apis mellifera*), and it is renowned for its mechanical and antimicrobial properties [4]. For these reasons, it emerges as a promising solution to the above-reported challenges [5]. Comprising over 300 different compounds, including phenols, tannins, polysaccharides, terpenes and fatty acids, propolis offers a rich source of bioactive compounds having potential antifungal properties [6]. It is important to note that the composition of propolis varies considerably depending on the geographical area and the type of substrate from which the raw materials are derived. This variability poses a challenge in achieving a definitive biochemical characterization [7]. In addition, propolis can be considered as an adjuvant substance for use in agriculture because it enhances plant defences [8]. Several studies have investigated the efficacy of propolis extracts in preserving the quality of fruits and vegetables [9], applied topically or incorporated into edible coatings or food formulations [10]. Propolis was shown to prevent lipid oxidation and improve the shelf life of citrus fruits [11]. Thus, propolis holds promise as a natural and sustainable alternative for the management of postharvest fruit grey mould [11].

Botrytis cinerea, the causal agent of grey mould symptoms, has the ability to infect over 1400 different hosts [12] and causes significant agricultural and economic losses worldwide. The impact of this pathogen is particularly pronounced in the postharvest phase, where infections often lie dormant until favourable environmental conditions trigger widespread deterioration [13]. This dormancy poses a significant challenge to crop production and postharvest management, as apparently healthy produce may only show signs of infection after harvest, resulting in significant losses [14]. *Botrytis cinerea* of berries presents a multifaceted challenge, because it affects crops during both pre- and postharvest phases [15]. In the field, the pathogen can infect different plant parts, such as flowers and young fruits. However, it is during postharvest handling, transport and storage that the true extent of grey mould disease becomes apparent [16]. Grapes, being highly perishable, are susceptible to damage and the presence of *B. cinerea* can rapidly lead to visible grey mould formation on the fruit surface. This not only affects the quality and marketability of the grapes but also accelerates rotting, resulting in significant economic losses for growers and traders [17]. Similarly, blueberry (*Vaccinium* spp.) crops face the same pathogen infection in the field and during postharvest storage, where *B. cinerea* causes significant production losses [18–20].

Based on these broad premises, the aim of the present study was to evaluate the efficacy of different propolis, collected in different environments, against *B. cinerea* through in vitro and in vivo experiments on grapes and blueberries. In addition, one of the main objectives was to characterize the phenolic and volatile profile of each propolis tested in order to study in depth the biodiversity related to the efficacy against the target pathogen.

2. Materials and Methods

2.1. Propolis and Fruit

Five different propolis were used in the experiments. Propolis samples were collected in the Friuli Venezia Giulia, Umbria, Tuscany and Veneto regions (Table 1). Ten grams of each propolis was ground using liquid nitrogen and stored at −20 °C until use.

Table 1. Propolis samples: geographical location and experimental code.

Sample	Code	Geographical Coordinates	Region
Propolis 1	PFvg1	46°7′28.623″ N/13°10′41.169″ E	Friuli Venezia Giulia
Propolis 2	PFvg2	46°15′31.257″ N/13°20′45.296″ E	Friuli Venezia Giulia
Propolis 3	PU3	42°59′25.794″ N/12°12′42.415″ E	Umbria
Propolis 4	PV4	45°42′45.43″ N/11°21′24.16″ E	Veneto
Propolis 5	PT5	43°35′12″ N/11°37′43″ E	Tuscany

Grape *cv* 'Thompson Seedless' and blueberry *cv* 'Bluecrop' were bought in the local market at the right maturity stage (18 and 13° brix, respectively) (Atago Refractometer PAL-3, Tokio, Japan) and were manually selected for those without any visible diseases or defects and homogeneous in size.

2.2. Pathogen

Botrytis cinerea strain Bc1 belonged to the mycological collection of the University of Udine. The pathogen, isolated from diseased grapes, was cultivated on oatmeal agar (60 g oatmeal, 10 g sodium nitrate, 30 g sucrose and 12 g agar per 1 L of Sterile Distilled Water, SDW) (Oxoid, Basingstoke, UK) and incubated at 25 °C for 8 days. Conidia were collected and suspended in SDW containing 0.05% (v/v) Tween 80. The suspension was adjusted to a final concentration of 1×10^3 and 1×10^5 conidia mL^{-1} using a haemocytometer, depending on the assay.

2.3. Propolis Extracts

In sterile vials (50 mL), 1 g of each powdered propolis and 5 mL of ethanol solution (30%, 60%, and 90%) (v/v) were mixed, as reported by El-Sakhawy [21], with some modifications. Each vial was covered with aluminium foil. To obtain the propolis extracts, two different methods were used. One set of samples was put on a rotary shaker (150 rpm) for one week according to the procedure followed by Zin et al. [22] with some modifications, while the other set was subjected to sonication for 20 min and agitated for 24 h at room temperature. After 24 h and 7 d, the hydroalcoholic propolis extracts were carefully collected using a syringe, sterilized with a 0.22 µm filter (Millipore, Merck, Burlington, MA, USA), transferred into sterile tubes (2 mL) and stored at −20 °C until use.

2.4. Bio-Assay: Effectiveness of Hydroalcoholic Propolis Against B. cinerea

Fungal pathogen suspension was infused on PDA medium (50 mL per square plate, 10 cm × 10 cm) reaching a final concentration of 1×10^5 conidia mL^{-1}. For each hydroalcoholic extract, 80 microlitres was placed in a hole (6 equidistant holes per treatment) punched in the medium with a 5 mm sterile cork-borer. Control plates were inoculated with the same volumes of ethanol (30%, 60%, 90%). After incubation at 20 °C for 72 h, a clear zone around the holes, the index of fungal growth inhibition, was detected. The area of these halos was measured using Fiji package for Image J 1.54f software, after image acquisition (800 dpi) using a scanner (Epson perfection 2400 photo, Epson, Los Alamitos, CA, USA). The efficacy of the extracts was considered for the subsequent experiments and the chemical analysis. One plate was used for each condition and the controls. The experiment was conducted twice.

2.5. Volatile and Non-Volatile Fraction of Propolis Extracts: Efficacy on B. cinerea Conidial Germination

Propolis extracts obtained after 24 h of extraction in ethanol 90% were chosen for their efficiency. Hence, 0 ppm (control), 3.12 ppm, 6.5 ppm, 12.5 ppm, 25 ppm, 50 ppm, 100 ppm, 150 ppm and 200 ppm of each extract were used as baseline. To assess the efficacy of the extracts' volatile metabolites, PDA (potato dextrose agar, 39 g L^{-1} of SDW) (Oxoid, Basingstoke, UK) plates were spread with 100 µL of *B. cinerea* conidial suspension (1×10^3 conidia mL^{-1}). Sterile filter papers (Whatmann No. 1, 90 mm diameter) were carefully positioned inside of the plate covers and 100 µL of each propolis extract concentration was placed using a microsyringe, as reported by Di Francesco et al. [23]. Subsequently, the dishes were hermetically sealed and incubated at 20 °C for 48 h in the dark.

To evaluate the effectiveness of the propolis' non-volatile metabolites on *B. cinerea*, PDA plates were inoculated with 100 µL of the pathogen conidial suspension (1×10^3

conidia mL^{-1}) [23], and 1 h after the application, 100 µL of each propolis concentration was placed. The plates were incubated at 20 °C for 48 h. For both experiments, the sample unit was represented by three plates. Ethanol solution (90%) was placed on the filters and on the plates as control. The experiments were conducted twice.

2.6. Propolis EC50 Values

To define the EC50 value of each propolis extract depending on the modes of action, nine different concentrations were used to create a baseline. The EC50 values were calculated on the bases of *B. cinerea* conidial germination inhibition (%) for both volatile and non-volatile metabolites of the propolis extracts. The following formula was applied:

$$(\%)\ \text{inhibition} = \frac{(\text{Control} - \text{Treatment})}{(\text{Control})} \times 100$$

The control and treatment are, respectively, the number of *B. cinerea* CFUs (colony-forming units) untreated and treated with the propolis extracts.

The EC50 value of each propolis was calculated using the probit analysis applied to the percentage of conidial germination inhibition [24].

2.7. Biochemical Characterization of Propolis Metabolites

2.7.1. Volatile Compounds

The propolis samples' volatile compounds were extracted by HS-SPME with a divinylbenzene/carboxen/polydimethyl siloxane (DVB/CAR/PDMS) fibre (df 50/30 µm; 2 cm length) (Supelco, Bellefonte, PA, USA), chosen on the basis of previous studies [25]. The SPME fibre was conditioned before use as recommended by the manufacturer. Sampling was carried out on 1.00 ± 0.03 g of propolis extract, precisely weighed in a 20 mL headspace vial and kept at 40 °C for 60 min under constant agitation. An Internal Standard IS 2-methyloctynoate was used to monitor sampling repeatability (i.e., response fluctuation ≤ 20% Relative Standard Deviation). An IS working solution was prepared at 0.100 g/L in dibutyl phthalate and stored at −18 °C in sealed vials. To preload the IS onto the SPME fibre, 5 µL of the working solution was placed in a 20 mL glass sealed vial and exposed to the SPME device for 5 min at 40 °C. After extraction, the SPME device was automatically transferred to the split/splitless injection port of the GC × GC system, kept at 270 °C, and thermal desorption lasted for 5 min. Samples were analysed in four replicates randomly distributed. Comprehensive two-dimensional GC analyses were carried out using an Agilent 7890B GC chromatograph (Agilent Technologies, Wilmington, DE, USA) coupled with a Markes BenchTOF Select™ mass spectrometer featuring Tandem Ionization™ (Markes International, Llantrisant, UK). The GC transfer line was set at 270 °C. The TOFMS was tuned for single ionization at 70 eV, and the scan range was set between 35 and 350 *m*/*z* with a spectrum acquisition frequency of 100 Hz. The thermal modulator was a loop-type, two-stage KT 2004 (Zoex Corporation, Houston, TX, USA) cooled with liquid nitrogen and controlled by Optimode, v2.0 (SRA Instruments, Cernusco sul Naviglio, Milan, Italy). The modulation period (PM) was set at 4 s, while the hot-jet pulse duration was set at 350 ms. The cold-jet stream at the mass flow controller (MFC) was programmed to linearly reduce the total flow (i.e., 20 L/min) from 35% to 5% along the analytical run.

The column set consisted of a 1D HeavyWax™ column (100% polyethylene glycol—PEG; 30 m × 0.25 mm dc × 0.25 µm df) coupled with a 2D DB17 column (50% phenyl-methylpolysiloxane; 1.0 m × 0.10 mm dc × 0.10 µm df), both supplied by Agilent Technologies (Wilmington, DE, USA). A fused-silica capillary loop (1.0 m × 0.1 mm dc) was used in the modulator slit. SilTite™ µ-unions (Trajan Scientific and Medical, Melbourne, Australia) were used to connect the columns with the capillaries. The GC split/splitless

injector port was set at 270 °C and operated in split mode with a 1:5 split ratio. A specially design liner for SPME thermal desorption was used to improve the transfer of the analytes to the 1D column and to limit band broadening in space. Helium was used as the carrier gas at a nominal flow of 1.0 mL/min. The oven temperature programme was set as follows: from 50 °C (1 min) to 260 °C (10 min) at 3.5 °C min^{-1}. The n-alkanes solution for I^Ts determination was analysed under the following conditions: split/splitless injector in split mode, split ratio: 1:50, injector temperature: 270 °C, and injection volume: 1 µL. Analyte identification was performed by combining retention data (carried out using experimental I^T with ± 10 unit tolerance vs. tabulated ones) and comparing electron ionization (EI)-MS spectral signature with reference spectra in commercial and in-house databases by using the NIST identity search algorithm with direct match factor (DMF) and reverse match factor (RMF) scores threshold ≥ 900.

2.7.2. Non-Volatile Compounds

Phenolic compounds have been quantified using UPLC (Waters Acquity UPLC system; Milford, MA, USA) coupled to a QqQ-MS/MS (Waters Xevo TQMS; Milford, MA, USA) according to the method reported in Vrhovsek et al. [26]. In reference [26], detailed information about detection limits, linear dynamic ranges and a complete validation study for each compound can also be found. To ensure appropriate quantification of the compounds, extracts were injected at different dilutions (1, 1:10; 1:100) to both remain within the linear dynamic ranges and avoid detector saturation.

2.8. In Vivo Experiments

The in vivo experiments were conducted to assess the volatile and non-volatile efficacy of propolis extracts against *B. cinerea* on grapes and blueberries. Berries were washed in 1% sodium hypochlorite (NaOCl) solution for 1 min and rinsed three times for 1 min in water before air drying [27]. Infected or damaged berries were excluded. The fruits were then placed on sterile grids inside sterile boxes (30 cm × 20 cm × 10 cm, L × W × H). For each propolis extract, the relative EC50 values were considered for both experiments.

To verify the efficacy of the volatile compounds, 5 µL of conidial suspension of the pathogen (1×10^5 conidia mL^{-1}) [27,28] was applied to an artificial wound (2 × 2 × 2 mm) previously inflicted on the berry by a flame-sterilized needle. The berries on a sterile grid were placed in a box containing six sterile paper filters (90 mm, Ø), each inoculated with 100 µL of each propolis solution concentrated at the relative EC50 value. The same amount of SDW was used for the controls. The boxes were sealed with Parafilm and incubated at 20 °C for biofumigation and 80% R.H. Disease incidence was assessed 5 d after pathogen inoculation.

For the non-volatile metabolite assay, the berries, arranged on a sterile grid, were wounded (2 × 2 × 2 mm) with a flame-sterilized needle and inoculated with 5 µL of each propolis extract at the wound site. After 1 h, 5 µL of conidial suspension (1×10^5 conidia mL^{-1}) [27,28] was inoculated into the same wound. The same procedure was followed for the control, using SDW instead of propolis. The storage parameters were the same previously mentioned. Three replicates of 8 berries were used for each treatment and the control. The experiments were conducted two times.

2.9. Statistical Analysis

The data were analysed using one-way analysis of variance. Mean separation was conducted using Tukey's test (α = 0.05). The data were reported as mean values ± standard error (SE). For the bio-assay, the fungal inhibition halos were elaborated using Fiji distribution of ImageJ version 1.54 [29]. The EC50 of each propolis was calculated using

the probit analysis applied to the percentage of conidial germination inhibition [24]. All the analyses were performed using Minitab version 17 software.

The association between non-volatile metabolites and EC50 efficacy was studied by Orthogonal Projections to Latent Structures (OPLS) regression, calculating the coefficients to identify the most influential variables driving the response [30]. The OPLS model was developed using the software Simca 17 (Sartorius Stedim Data Analytics AD).

3. Results

3.1. Bio-Assay: Effectiveness of Hydroalcoholic Propolis Against *B. cinerea*

The assay showed the inhibitory activity against *B. cinerea* mycelial growth of the different propolis extracts, obtained by two methods of extraction, as reported in Section 2.3. Figure 1 shows a pronounced antifungal activity of propolis extracted using ethanol solution at 90% and in particular that obtained by sonication and 1 d of incubation (Supplemental Figure S1). The greatest area of mycelial inhibition was recorded for the extract PU3 (331.2 mm^2), followed by PFvg1 (246.2 mm^2) and PFvg2 (227.2 mm^2), all, respectively, obtained via the sonication method. The lowest inhibition halos (mm^2), within the same extraction mode, were displayed by PV4 (182.9 mm^2) and PT5 (168.1 mm^2). Even in the case of the 7 d extraction, 90% ethanol solution was found to be more effective. No fungal inhibition activity was recorded for the solvent solutions (Supplemental Figure S1).

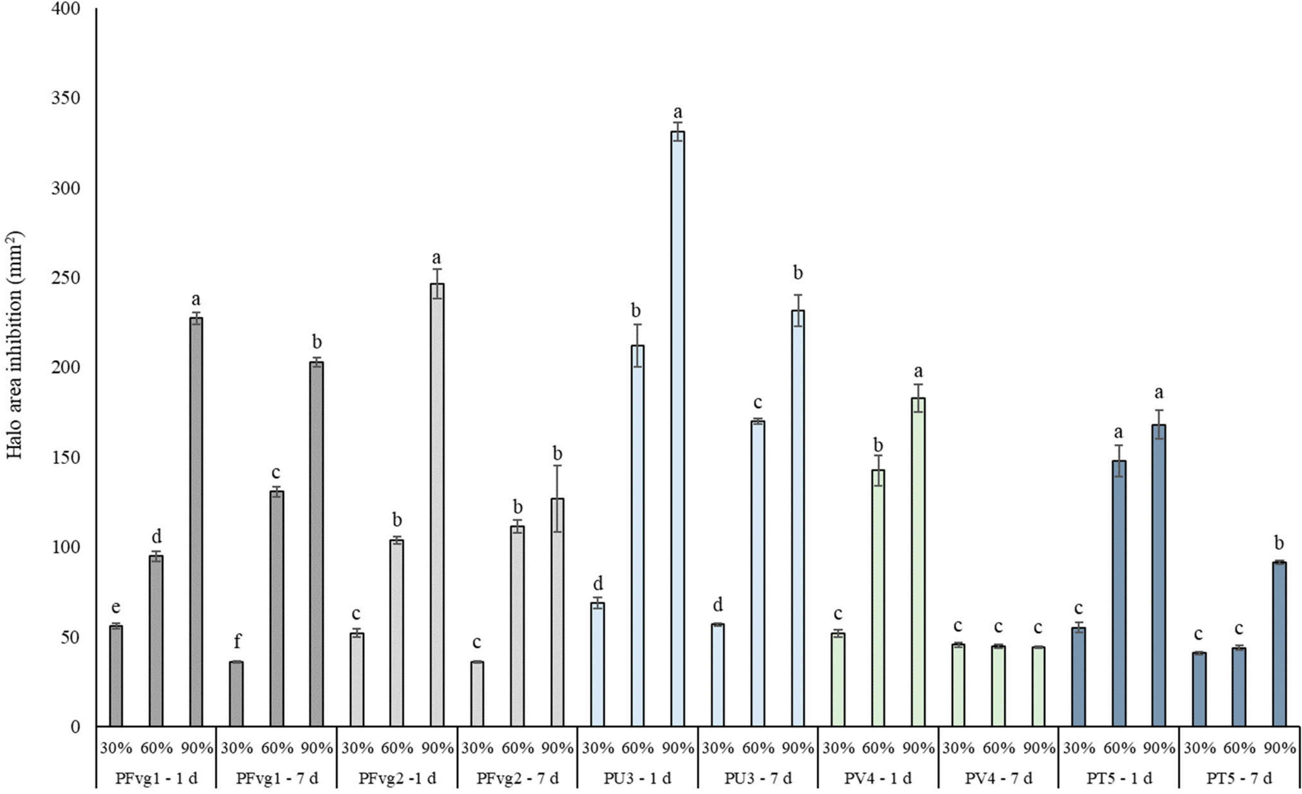


Figure 1. Area (mm^2) of halo inhibition of *Botrytis cinerea* growth by different propolis (PFvg1, PFvg2, PU3, PV4, PT5) extracts obtained, respectively, with 30%, 60% and 90% ethanol solution and with 7 and 1 days of extraction. The last method was preceded by 20 min of sonication. Data are the mean of six replicates ± standard error. Different letters indicate significant differences within the propolis according to Tukey's test (α = 0.05).

3.2. Volatile and Non-Volatile Fraction of Propolis Extracts: Efficacy on B. cinerea Conidial Germination

The experiments were conducted using propolis extracts obtained using ethanol 90% for 1 d of incubation following 20 min of sonication, which was the most effective method as reported in the preliminary bio-assay results. Nine concentrations, ranging between 0 (control) and 200 ppm, of each extract were subsequently tested for their efficacy through the production of volatile and non-volatile metabolites against the conidial germination of *B. cinerea*.

In Figure 2, *B. cinerea* colony-forming units (CFUs), affected by the volatile fraction of the propolis extracts at different concentrations, are reported. The highest inhibition on CFU growth was recorded at a concentration of 200 ppm for all the propolis extracts: 85.61% (PFvg1), 92.46% (PFvg2), 78.1% (PU3), 57.53% (PV4) and 69.86% (PT5). The volatile compounds of propolis extracts PV4 and PT5 were less effective than those of the other extracts. However, impressive results were displayed by PFvg1 and PFvg2 starting from the lowest tested concentration. In this regard, both propolis extracts inhibited CFUs, respectively, by 32.87% and 61.64% at a concentration of 3.12 ppm, compared to the control. A more scalar inhibition trend was observed for the other propolis.

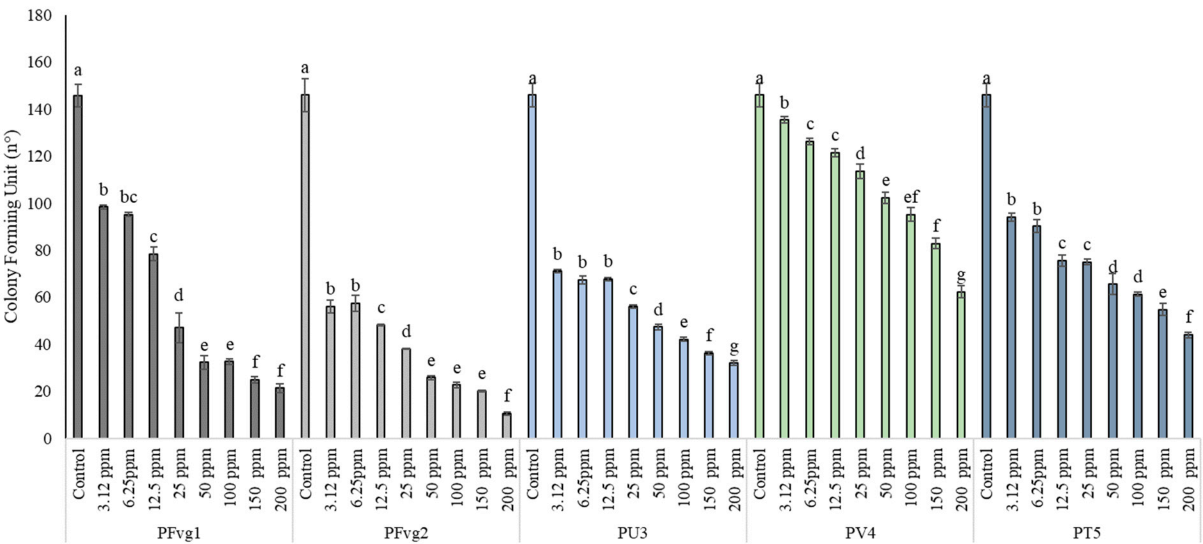


Figure 2. Effect of volatile metabolites produced by PFvg1, PFvg2, PU3, PV4 and PT5 propolis extracts obtained using 90% ethanol solution and 1 day of extraction, preceded by 20 min of sonication. Nine concentrations, ranging between 0 ppm (control) and 200 ppm, were tested on *Botrytis cinerea* colony-forming unit (CFU) growth. The data are the mean of six replicates ± standard error. Different letters indicate significant differences within the propolis according to Tukey's test (α = 0.05).

Regarding the non-volatile metabolites assay, four out of five propolis extracts were able at different concentrations to completely inhibit fungal CFUs. Propolis extract PFvg1 completely inhibited *B. cinerea* CFU growth starting from 50 ppm. A total inhibition was recorded starting from a concentration of 100 ppm for PU3 and PV4. With PT5 propolis, 95% of inhibition was reached at 200 ppm. Both propolis PFvg1 and PU3, at 25 ppm, were able to inhibit *B. cinerea* CFUs by almost 90% (Figure 3).

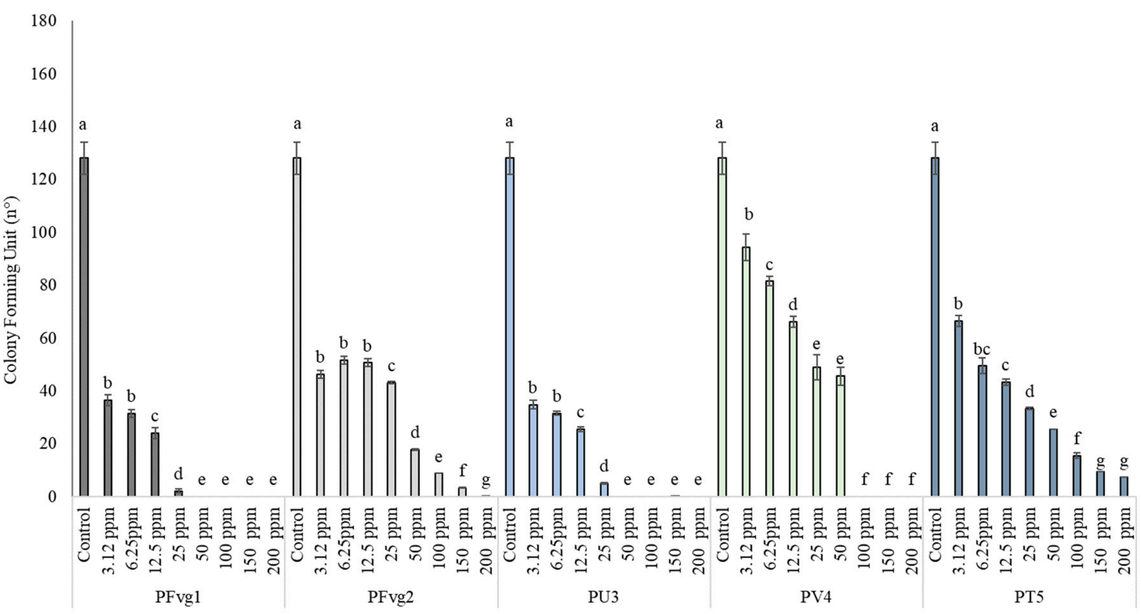


Figure 3. Effect of non-volatile metabolites produced by PFvg1, PFvg2, PU3, PV4 and PT5 propolis extracts obtained using 90% ethanol solution and 1 day of extraction, preceded by 20 min of sonication. Nine concentrations, ranging between 0 ppm (control) and 200 ppm, were tested on *Botrytis cinerea* colony-forming unit (CFU) growth. The data are the mean of six replicates ± standard error. Different letters indicate significant differences within the propolis according to Tukey's test (α = 0.05).

By the obtained results, the effective dose (EC50) of each propolis extract concerning the mode of action (volatile and non-volatile) was calculated using the probit values (Table 2).

Table 2. EC50 values (mg mL^{-1}) of the five different propolis extracts per mode of action (volatile and non-volatile).

		EC50 (mg mL^{-1})
Propolis	**Volatile**	**Non-Volatile**
PFvg1	3.45	2.39
PFvg2	1.36	4.58
PU3	63.88	2.24
PV4	211.25	10.03
PT5	24.94	4.66

From the results, the non-volatile metabolites were seen to be the most effective, showing the lowest values, ranging from 2.24 to 10.03 mg mL^{-1}, respectively, for PU3 and PV4. Regarding the volatile metabolites, propolis extracts PFvg1 and PFvg2 were the most active, as demonstrated by the lowest EC50 values, 3.45 and 1.36 mg mL^{-1}, respectively.

3.3. Propolis Biochemical Composition: Volatile Metabolites and Polyphenols

Propolis hydroalcoholic extract VOCs were analysed via SPME/GC–MS analysis. A total of 77 compounds were detected (Supplemental Table S1). Fifty-six, identified as the most significant compounds, together with retention times ($^{1}t_R$ and $^{2}t_R$) are reported in Table 3; analyte chromatographic responses expressed as 2D peak normalized area (Normalized Area) are reported in Supplemental Table S2. In all the five propolis, the presence of sesquiterpene alcohols such as α-cadinol, α-eudesmol, calamenene and cadinol was detected, followed by aromatic compounds (benzyl benzoate and benzyl alcohol).

The propolis PU3, PV4 and PT5 showed more similarities in the volatilome compared to PFvg1 and PFvg2. Also, their volatilome results were composed of a higher number of compounds: PU3 (52), PV4 (48) and PT5 (47).

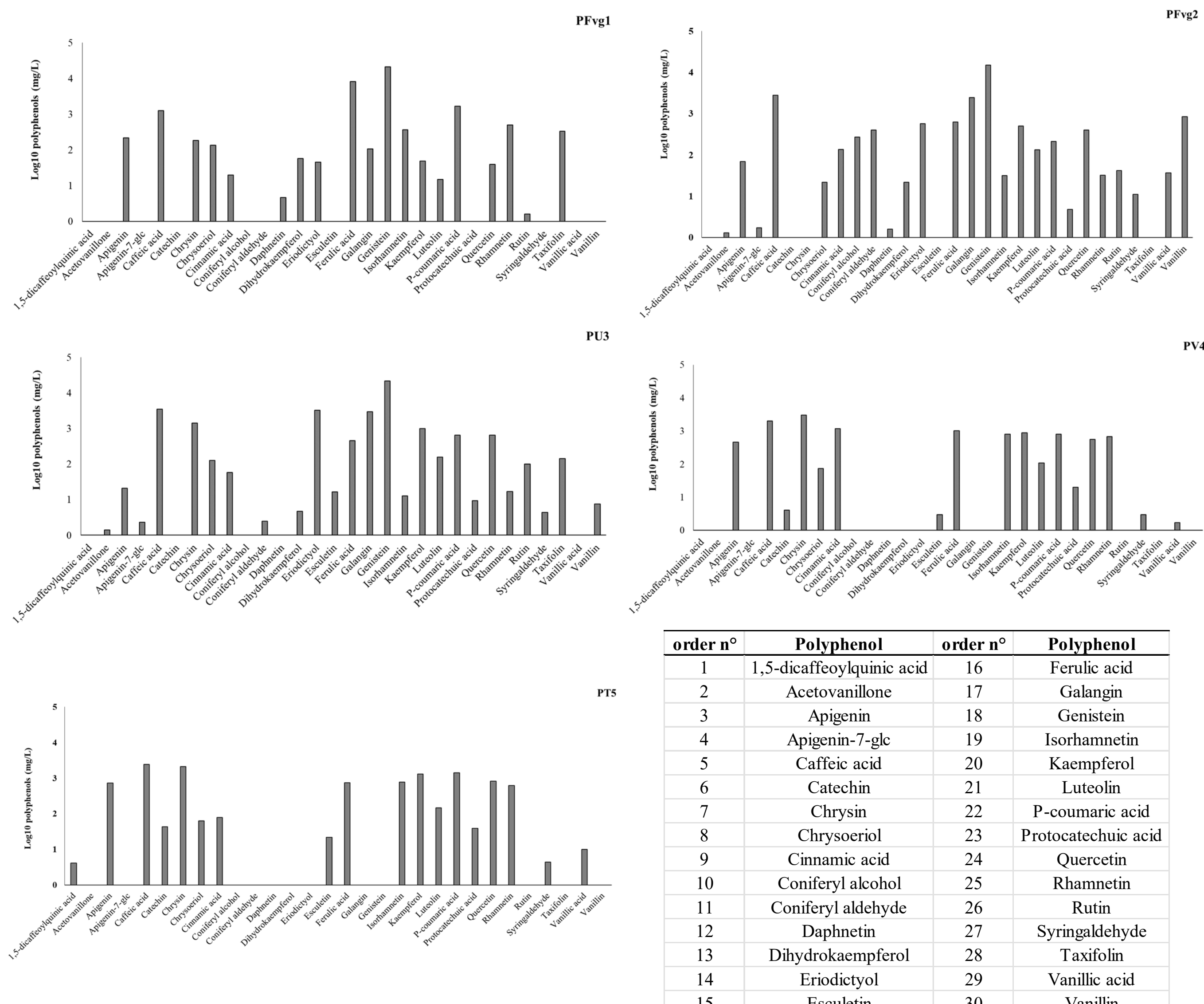
Table 3. Volatile organic compounds of propolis (PFvg1, PFvg2, PU3, PV4, PT5) analysed via HS-SPME and GC–MS gas phase. In bold are the compounds detected (√) in all samples.

			Absolute Area (AA)				
Compound	**$^{1}t_{R}$ (min)**	**$^{2}t_{R}$ (s)**	**PFvg1**	**PFvg2**	**PU3**	**PV4**	**PT5**
1,4-Dihydronaphthalene	30.51	0.75	√	√	√	√	√
1-Methyl-5-nitro-4-phenylsulfonylimidazole	26.67	0.77	√	√	√	√	√
2-Butanone, 4-phenyl-	37.92	0.70	-	-	√	√	√
2-Buten-1-ol, 3-methyl-, acetate	17.24	0.80	-	-	√	√	√
2-Buten-1-ol, 3-methyl-, acetate	17.01	0.84	-	-	√	√	-
2-Butenal, 3-methyl-	15.63	0.62	-	-	√	√	√
2-Methoxy-4-vinylphenol	47.26	0.41	-	√	-	-	√
3-Buten-1-ol, 3-methyl-	17.73	0.38	√	√	√	√	√
3-Buten-2-one, 4-phenyl-	45.13	0.59	-	-	√	√	√
3-Methyl-3-buten-1-ol, acetate	15.14	0.83	-	-	√	√	√
Acetic acid	24.83	0.15	√	√	√	√	√
Acetic acid, 2-phenylethyl ester	36.67	0.70	√	-	√	√	√
Acetic acid, phenylmethyl ester	33.95	0.63	-	√	√	√	√
Benzaldehyde	27.13	0.55	√	√	√	√	√
Benzaldehyde, 3-hydroxy-4-methoxy-	56.13	0.34	√	√	-	-	√
Benzene, 1,3-dimethyl-	14.49	0.91	-	-	√	√	√
Benzene, 4-ethenyl-1,2-dimethoxy-	42.73	0.62	-	-	√	√	√
Benzeneethanol	39.38	0.40	√	√	√	√	√
Benzoic acid	54.17	0.17	√	√	√	√	√
Benzoic acid, ethyl ester	32.00	0.74	√	√	√	√	√
Benzyl alcohol	38.35	0.33	√	√	√	√	√
Benzyl benzoate	57.67	0.64	√	√	√	√	√
Butanoic acid, 2-methyl-	32.07	0.24	-	-	√	√	-
Cadinol	47.25	0.99	√	√	√	√	√
Calamenene	37.13	1.50	√	√	√	√	√
Cinnamic acid, p-methoxy-, methyl ester	56.41	0.59	-	-	√	-	-
Cubenol	43.80	1.25	√	-	-	√	√
Decanoic acid	49.25	0.36	-	√	√	√	√
Decanoic acid, ethyl ester	30.84	1.75	-	-	√	√	√
Epicubenol	43.66	1.20	-	-	√	√	√
Ethanone, 1-(2-methylphenyl)-	32.17	0.66	√	√	√	√	√
Ethyl 3-phenylpropionate	38.73	0.80	√	√	√	√	√
Ethyl cinnamate, trans	45.60	0.68	√	√	√	√	√
Farnesol	42.93	1.02	-	-	√	-	√
Gleenol	42.93	1.16	-	-	√	-	√
Indole	53.33	0.31	√	√	√	√	√
Nonanal	22.25	1.34	√	√	√	√	√
Nonanoic acid	46.48	0.34	√	√	√	√	√
Phenol, 4-ethyl-	46.67	0.32	-	-	√	-	√
Styrene	17.29	0.71	√	√	√	√	√
Tiglic acid	37.66	0.23	√	√	√	√	√
tau-Cadinol	46.53	1.03	√	√	√	√	√
trans-Cinnamaldehyde	43.07	0.55	-	√	√	√	-
α-Calacorene	39.66	1.29	-	√	√	√	-
α-Cadinene	35.86	1.66	-	√	√	√	-
α-Cadinol	48.13	0.99	√	√	√	√	√

Table 3. *Cont.*

			Absolute Area (AA)				
Compound	**^1t_R (min)**	**^2t_R (s)**	**PFvg1**	**PFvg2**	**PU3**	**PV4**	**PT5**
α-Copaene	25.65	2.69	-	-	√	-	√
α-Eudesmol	47.92	1.01	√	√	√	√	√
α-Humulene	32.02	1.95	-	√	√	√	-
β-Calacorene	39.19	1.30	-	-	√	-	√
β-Cyclocitral	30.44	1.14	-	-	√	√	-
γ-Amorphene	32.57	1.85	-	√	√	√	√
γ-Cadinene	34.83	1.72	-	√	√	√	√
γ-Gurjunene	31.18	2.10	-	-	√	√	√
δ-Cadinene	34.73	1.74	-	√	√	√	√
δ-Cadinol	46.99	1.00	√	-	√	√	√

In Figure 4, the polyphenol composition of each propolis extracts is reported. In total, 30 polyphenols were identified, differently distributed depending on the sample.



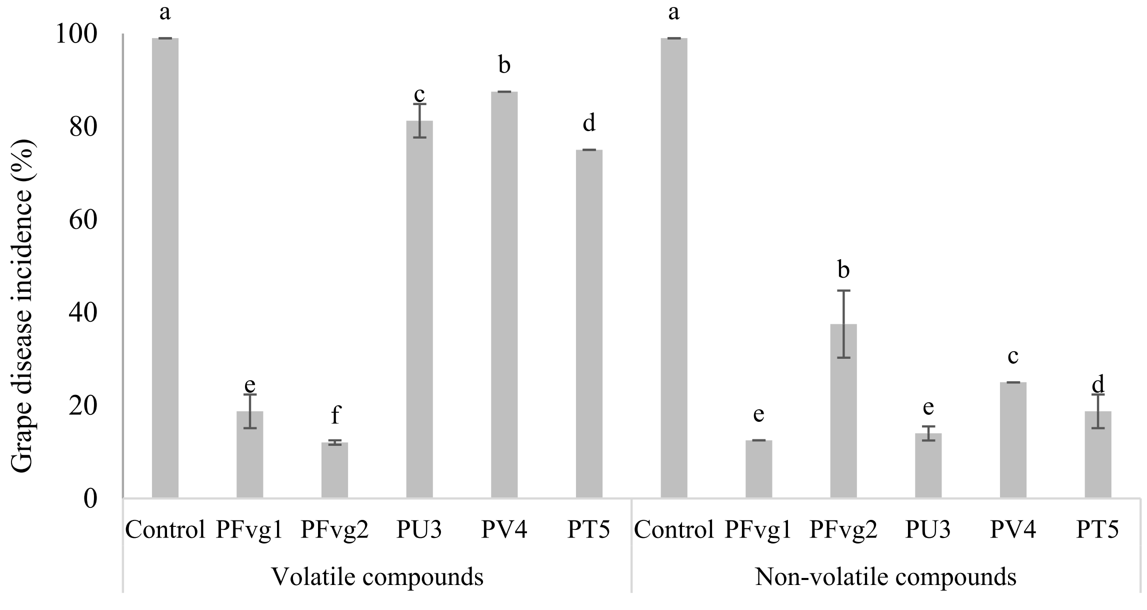
order n°	Polyphenol	order n°	Polyphenol
1	1,5-dicaffeoylquinic acid	16	Ferulic acid
2	Acetovanillone	17	Galangin
3	Apigenin	18	Genistein
4	Apigenin-7-glc	19	Isorhamnetin
5	Caffeic acid	20	Kaempferol
6	Catechin	21	Luteolin
7	Chrysin	22	P-coumaric acid
8	Chrysoeriol	23	Protocatechuic acid
9	Cinnamic acid	24	Quercetin
10	Coniferyl alcohol	25	Rhamnetin
11	Coniferyl aldehyde	26	Rutin
12	Daphnetin	27	Syringaldehyde
13	Dihydrokaempferol	28	Taxifolin
14	Eriodictyol	29	Vanillic acid
15	Esculetin	30	Vanillin

Figure 4. Propolis polyphenol composition defined using UPLC coupled to a QqQ-MS/MS (Log_{10} polyphenol mg L^{-1}).

In more detail, propolis PFvg1, PFvg2 and PU3 showed a high amount of genistein, respectively, of 21.2, 15.1 and 22.0 mg mL^{-1}. Conversely, genestein was absent in the PV4 and PT5 extracts. In the case of the PV4 and PT5 extracts, chrysin and caffeic acid were the most present compounds: 3.0 mg mL^{-1} and 2.1 mg mL^{-1}, and 2.0 mg mL^{-1} and 2.4 mg mL^{-1}, respectively. However, other polyphenols known to be a substantial part of the polyphenolic composition of propolis were detected, such as apigenin, kaempferol, luteolin, *p*-coumaric acid, quercetin and vanillin. Propolis PFvg2 and PU3 displayed a wide array of 25 detected polyphenols, followed by PFvg1, PV4 and PT5, respectively, with 19, 17 and 18 compounds.

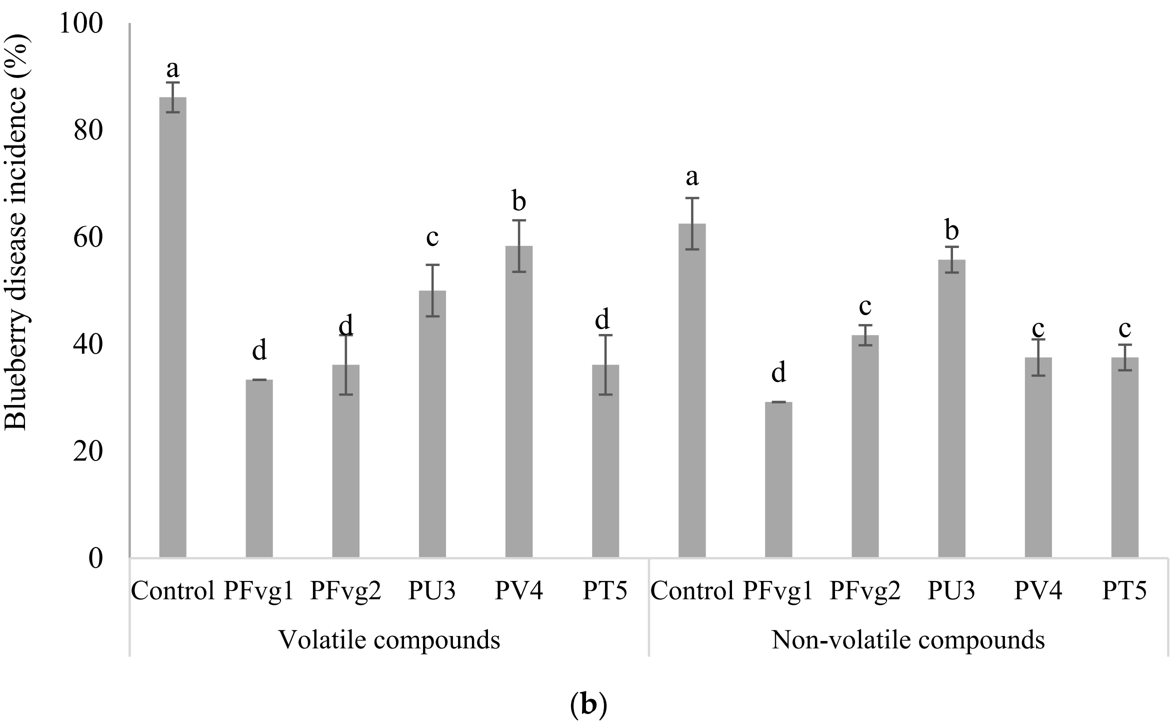
3.4. In Vivo Experiments

To evaluate the in vivo effect of the volatile and non-volatile metabolites of the propolis extracts, both, at the EC50 concentrations, were tested against grey mould on grapes and blueberries, respectively. Regarding table grapes, generally non-volatile metabolites showed the best results if compared to volatile (Supplemental Figure S2). Figure 5a shows the percentage of disease incidence. By propolis extract volatile metabolites, an inhibition of 81.2% and 88%, if compared to the control, was registered for PFvg1 and PFvg2. The other propolis extract volatile metabolites displayed an inhibition by 18.7% (PU3), 12.5% (PV4) and 25% (PT5) compared to the control. Regarding blueberries (Figure 5b), grey mould disease incidence was inhibited by 60.4% on average for PFvg1, PFvg2 and PT5 propolis extract volatile metabolites. Also, for the non-volatile, PFvg1 confirmed the highest inhibition value (47.8%) compared to the others, which showed a significant inhibition of 38.7%, on average.



(**a**)

Figure 5. *Cont.*



(**b**)

Figure 5. Effect of volatile and non-volatile metabolites produced by PFvg1, PFvg2, PU3, PV4 and PT5 propolis extracts obtained using 90% ethanol solution and 1 day of extraction, preceded by 20 min of sonication, on *Botrytis cinerea* disease incidence (%) on table grapes (**a**) and blueberries (**b**). Different letters indicate significant differences within the propolis according to Tukey's test (α = 0.05).

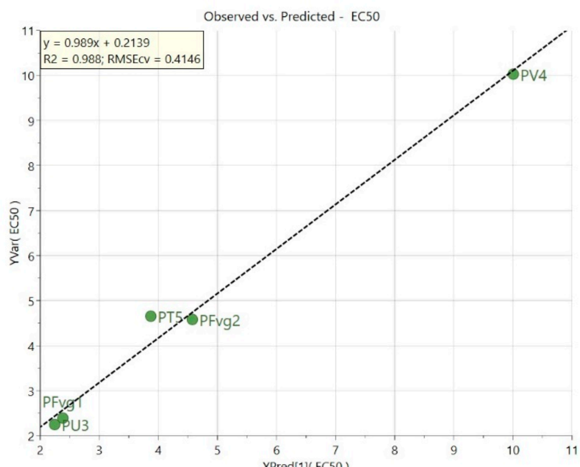
3.5. Metabolites Potentially Effective Against B. cinerea

Figure 6 illustrates the analysis conducted using OPLS to correlate the non-volatile metabolites to the EC50 values recorded for the different tested extracts. In the Left Plot (Figure 6), the scatter plot shows the observed versus predicted values of the EC50 for each sample. The R^2 value of 0.9878 indicates an excellent fit, meaning the model predicts the EC50 values with high accuracy. The low RMSECV (Root Mean Squared Error of Cross Validation) of 0.414564 further supports the model's accuracy in predicting EC50 values. Even though the predictive variance explained during leave-one-out cross-validation is close to 1, the limited number of samples does not allow for a generalization of the model due to the strong risk of overfitting; therefore, the results shown should be considered preliminary. The Right Plot of Figure 6 shows the coefficients of different chemical components found in the propolis extracts that influenced the EC50 values. The compounds with negative coefficients are of particular interest, as they appear to lower the EC50, indicating they may enhance the bioactivity of the propolis extracts.

Chrysin, a natural dietary polyphenol and flavonoid found in flowers, honeycombs and mushroom species that has multiple biological and pharmacological activities [31], has the highest positive coefficient, indicating it significantly decreases bioactivity (higher EC50). Its high coefficient suggests a negative influence on the potency of the extract, although previous research highlights chrysin's antioxidant and anti-inflammatory properties [31]. Naringenin also has a large positive coefficient, although slightly lower than that for chrysin, implying it contributes substantially to reducing bioactivity of the propolis extract. Known for its antioxidant and anti-cancer properties [32] and antifungal activity [33], naringenin appears to contrast the extract's potency.

With a significant negative coefficient, luteolin is compound that boosts bioactivity by reducing the EC50. This supports its known biological effects, including antifungal activity supressing mycelial growth and pathogenicity of *B. cinerea* and *P. expansum* [34]. Apigenin

also shows a substantial negative coefficient, suggesting an improved bioactivity of the extract, confirming its antifungal activity [35]. Chrysoeriol, while contributing less than the above, still shows a negative coefficient, indicating a role in enhancing bioactivity. Kim et al. [36] reported chrysoeriol's antifungal activity as well. Several compounds, including vanillin, rhamnetin, quercetin, protocatechuic acid and p-coumaric acid, have near-zero or positive coefficients with relatively low magnitude. This suggests that these compounds either do not significantly influence the EC50 value or may slightly reduce the potency of the extract.



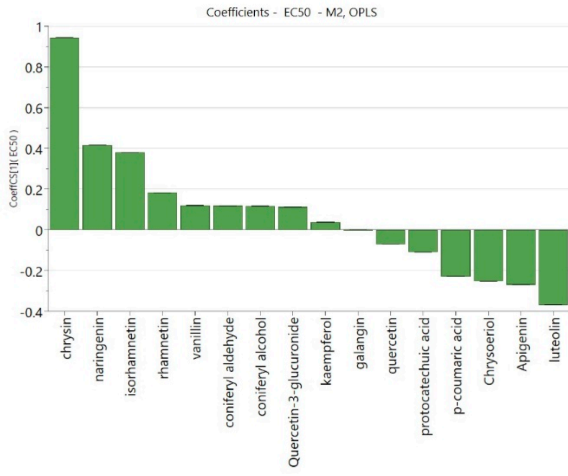


Figure 6. OPLS (Orthogonal Projections to Latent Structures) model analysing propolis extracts to predict their bioactivity, represented by the EC50 values. (**Left Plot**): relationship between observed and predicted EC50 values for each propolis sample. (**Right Plot**): coefficients of the model.

4. Discussion

The present research confirms that propolis is chemically rich, and this fact, as well as its antimicrobial effectiveness, varies according to plant, environment, season and bee type [37]. The composition of propolis is crucial for understanding its biological role [8]. Until now, according to the available data, propolis' antimicrobial and antifungal efficacy has been attributed principally to polyphenols and flavonoids [38]. The propolis extracts used in the present study displayed a wide range of polyphenols. Our results confirmed the data reported by Da Silva Cunha et al. [39] that showed how the efficiency of extraction by maceration was less effective than that achieved by ultrasounds. Prolonged extraction by sonication leads to degradation of phenolics along with flavonoids, as reported by Gullian and Terrats [40]. Ethanol is an organic solvent with intermediate polarity, so exposure to higher temperature can contribute to increased substance solubilization [41]. However, ethanol has a higher efficiency than water in the extraction of phenols and polar compounds. Also, it is the substance most used for the extraction of bioactive compounds from propolis [42].

In particular, Pfvg1, PFvg2 and PU3 reported a high amount of genistein, regardless of their geographical origin. Andrade et al. [43] reported that genistein and chrysin are commonly found in propolis and are among the predominant bioactive constituents. In the present study, propolis extracts PV4 and PT5 did not exhibit the presence of galangin and genestein and on average displayed a fair amount of content of apigenin, caffeic acid, chrysyn, ferulic acid, kaempferol, luteolin, *p*-coumaric acid and quercetin. Mirzoeva et al. [44] attributed the possible mechanism of inhibition of protein synthesis and cell membrane functions to quercetin.

Propolis hydroalcoholic extracts PFvg1, PFvg2 and PU3, during in vitro assays, showed a notable inhibitory effect on *B. cinerea*, compared to the other extracts. PFvg1 propolis extract displayed a high level of ferulic acid. This is known to exert an antimicrobial action that cause cell membrane disfunction and changes in cellular morphology [44]. However, together with the other polyphenols, it provides synergistic activity against microbial organisms. In the evaluation of the efficacy of propolis extracts in inhibiting conidial germination of *B. cinerea*, also the propolis volatile metabolites were considered. Conversely to non-VOCs, volatile metabolites showed a gradual inhibition against the pathogen, with the best results observed at higher concentrations for all the extracts. The antifungal effect of propolis increased with increasing doses [45]. Concerning this, Abo-Elyousr et al. [45] have reported that higher propolis doses were effective against *Sclerotinia sclerotiorum* and *Penicillium digitatum* of citrus fruit.

Usually, volatile compound concentration was low in the propolis. However, the volatilome is closely connected to propolis biological activity as well as it being a key element in its characterization [4]. From the VOC propolis analysis, the main detected compounds in the five propolis belong to the sesquiterpene alcohols class (α-cadinol, α-eudesmol, calamenene, cadinol) and to aromatic compounds (benzyl acetate, benzyl benzoate). Our results are confirmed by a study by Bankova et al. [8] that reported the abundance of sesquiterpenes in most of the studied European propolis. In addition, Jerkovic and Mastelić [46] reported that β-eudesmol and benzyl benzoate were found to be the main constituents of the essential oil derived from *Populus nigra*, including nonanoic acid and tiglic acid, mainly found in *Geraniaceae* and *Euphorbiaceae* plants. Compounds such as benzyl alcohol, farnesol, α- and δ-cadinene and α-eudesmol were reported as active compounds against fungal and bacterial microorganisms [47,48]. Despite the wide diversity of volatile compounds detected in each propolis, the non-volatile EC50 values were found to be lower than the volatile, suggesting their higher efficacy against the fungal pathogen.

In in vivo experiments, the PFvg1 extract showed the highest significant reduction compared to the control and to the other propolis. In particular, on grapes, both the PFvg1 volatile and non-volatile metabolites displayed the best efficacy against the fungal pathogen. Many studies support the efficacy of propolis treatments in reducing the incidence rates of fungal pathogens. Kahramanoğlu et al. [49] demonstrated that propolis extracts, in particular through their antioxidant activity and high content of anthocyanin, significantly contribute to the reduction of grey mould on pomegranate fruits. The antimicrobial efficacy of propolis was also reported for mango [50], papaya [51] and citrus fruit [52].

The literature suggests that the positive influence of propolis on the postharvest storability of food is due to its hydrophobic composition and high phenolic concentration, which also provides the ability to create a biodegradable barrier on the fruit surface [52]. This effect reduces the transpiration and respiration of the fruit and, at the same time, improves the shelf life of the products [52]. In fact, the presence of antioxidant and antimicrobial compounds in propolis makes it an excellent product having a dual function. In the present study, we have also proposed the potential use of propolis hydroalcoholic extracts as a biofumigation treatment. The studies dedicated to the bioactivity of propolis volatiles are relatively scarce, most of them dealing with antimicrobial properties [8]. However, it is necessary to verify the potential and importance of propolis volatiles as propolis effective components [8].

The results suggest that hydroalcoholic propolis extracts have potential as effective inhibitors of *B. cinerea*, with distinct degrees of efficacy in the function of extraction methodology and on propolis composition.

5. Conclusions

In conclusion, our research sheds light on the potential of propolis extracts as effective inhibitors of *B. cinerea*, addressing a critical need for sustainable postharvest disease management in grape and blueberry production. Propolis, rich in active compounds and recognized for its antimicrobial properties, emerges as a promising alternative to synthetic fungicides. Our findings demonstrated that the inhibitory effects of the extracts on *B. cinerea* strictly depend on the extraction method, ethanol concentration and geographical origin of the propolis. Notably, the propolis extracts exhibited significant inhibitory activities, particularly when extracted using sonication and after 24 h of extraction. Furthermore, the efficacy of these extracts in inhibiting fungal growth was highlighted, with both non-volatile and volatile metabolites showing promising results. Assessing the EC50 values will also be useful for future uses of the extracts, perhaps simulating medium- and large-scale applications on fruit. Through the OPLS model, we identified luteolin and apigenin as major contributors to the efficacy of the propolis extracts, while chrysin and naringenin play a negative role in enhancing bioactivity. These findings highlight the potential of specific flavonoids in propolis extracts that could be targeted for their health benefits. These results open up new perspectives for future research to test the efficacy of the single detected polyphenols against the main fruit fungal pathogens.

Overall, the present study contributes to the growing body of literature on natural compounds for plant disease management and highlights the potential of propolis as a sustainable solution for postharvest diseases. However, propolis composition and its enzymatic activity still need to be studied in detail. Also, the organoleptic characteristics of treated fruits should be studied in detail. Further information will help to understand the possible introduction of propolis extracts in postharvest formulations or technologies aimed at a sustainable, environmentally and human-health-friendly strategy for fruit fungal disease control.

Supplementary Materials: The following supporting information can be downloaded at https://www.mdpi.com/article/10.3390/horticulturae11020122/s1. Figure S1: Antifungal activity against *Botrytis cinerea* of propolis PFvg1 and PFvg2, extracted with ethanol solution at 90% by sonication and 1 d of incubation and the relative control (ethanol solution at 90%).; Figure S2: Effect of propolis PV4 non-volatile and volatile metabolites against *Botrytis cinerea* on grape; Table S1: Volatile organic compounds (VOCs) produced by analyzed propolis extracts; Table S2: Volatile organic compounds of propolis (PFvg1, PFvg2, PU3, PV4, PT5) together with ^{1}D and ^{2}D retention times (^{1}tR; ^{2}tR) and normalized 2D peak areas.

Author Contributions: Conceptualization, A.D.F.; methodology, A.D.F., E.A., C.C., A.A., A.C. and S.M.; formal analysis A.D.F. and E.A.; investigation, A.D.F., A.S., R.C., E.A., C.C., A.A., A.C. and S.M.; resources, A.D.F., E.A. and C.C.; data curation, A.D.F., R.C., E.A. and C.C.; writing—original draft preparation, A.S., A.D.F. and E.A.; writing—review and editing, A.D.F., E.A., C.C., A.C. and R.C.; project administration, A.D.F. All authors have read and agreed to the published version of the manuscript.

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Conflicts of Interest: The authors declare that they have no conflicts of interest.

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