3. Discussion

By introduction of a short, medium, or long prenylated chain at one of the hydroxyl functionalities of *trans*-cinnamic acids **1**–**3**, a series of prenylated cinnamic derivatives **4**–**13** was prepared to test the hypothesis that differences in the chemical and physical proprieties would influence the antifungal activity of the compounds against clinical *Fusarium* spp. The presence of a conjugated double bond confers particular conformational and electronic characteristic to these compounds strongly influenced by the phenol-OH group in para position. In order to observe the effect of the hydroxylated aromatic ring on the fungicide activity, a set of cinnamic esters **10**–**12** was prepared by functionalisation of the corresponding carboxylic acid with citronellol, while a set of allyl cinnamic ethers **4**–**6** was prepared with the aim to evaluate the influence of the phenolic-OH group in para position to the alpha, beta-unsaturated carboxylic chain. No significant differences were observed in the synthesis and yields of each set of compounds with different aromatic rings, whereas lower yields in esters in comparison with ethers were achieved, evidencing the higher reactivity of the phenolic-OH group.

The Food and Drug Administration (FDA) considers *trans*-cinnamic acids **1**–**3** as "generally recognized as safe" (GRAS), enabling their use in the field of food additives [40]. Compounds **1**–**3** are commercially available at an affordable price and can been obtained by direct extraction from plants biomass where the compounds are the main components [41] or by chemical and biotechnological processes [42]. Besides cinnamic acids **1**–**3**, prenylated cinnamic ester **13** and ethers **7** and **9** are plant components, whose extracts were studied for their remarkable biological properties [43]. In particular, compounds **9** and **13** are present in propolis, a source of valuable compounds with antioxidant and antimicrobial activity [44]. Compound **9** is not toxic to human cells and presents antitumoral and anti-inflammatory activities, in addition to acting as an inhibitor of biofilm formation by oral pathogenic bacteria [45]. While 4′-geranyloxy ferulic acid **9** is generally extracted from citrus fruit, quinoa seeds, and several vegetable oils, compound **13** was only detected in propolis extract. Propolis, produced by honeybees, is a very complex mixture composed of 50% resin, 30% wax, 10% essential oil, and 5% of polyphenols as flavonoids, terpenes, fatty acids, stilbenes, β-steroids, cinnamic acids, and their prenylated derivatives [46,47]. Change in chemical composition of propolis is frequently observed [44]. A few studies have been conducted on the antifungal and antibiofilm activity of propolis against onychomycosis caused by *Fusarium* spp. [48,49], but no studies aimed to identify the active component of the propolis extract against these fungi.

In a previous article reporting the activity of natural phenols against clinical *Fusarium* spp., we observed that the percentage of growth inhibition measured in liquid medium (Vogel's) and solid (PDA) was comparable [50]. To achieve full solubilisation of compounds **1**–**13** at 0.5 mM, the preliminary screening was carried out with a sustainable solid medium based on gellan/water, where each compound was solubilized in a 0.1% water/gellan solution. In the preliminary in vitro screening of compounds **1**–**13** against *Fusarium* spp., cinnamic acids **1**–**3** were generally ineffective, whereas significant growth inhibition was achieved by prenylated derivatives **4**–**13**, evidencing ester **13** as the most active. Among the *Fusarium* spp. investigated, *F. solani* was the most resistant to compounds **1**–**13**, whereas *F. verticillioides* was the most sensitive, in accordance with data present in the literature for these species. In fact, *Fusarium* spp. are increasingly reported as resistant to many antifungal compounds in vitro; among them, *F. solani* is considered as the most resistant taxon, albeit some reports pointed out that the resistance may be species- and even isolate-dependent [51].

The antifungal activity of compounds **1**–**13** may be explained by the key role played by some moieties of their structure. The prenylated chain present in compounds **4**–**13** has the ability to penetrate and to accumulate within the fungal cell membrane, resulting, according to the size, in the disruption of its integrity as generally acknowledged for prenylated phenols [36–38]. The position and size of the prenylated chain in the studied compounds appear crucial for their antifungal activity. Although we did not perform a

proper bioavailability assay, we observed a detrimental effect on the fungal membrane when treated with compound **13** (Table 4).

Esters were more active than ethers as inhibitors of all tested *Fusarium* spp. Ester **13** was definitely more active than the corresponding ether **7** containing the same 3,3′-dimethyl allyl moiety, even though an identical lipophilicity was measured for both compounds (LogP 3.04). In the esters series, the alcoholic unit represented by a 3,3′-dimethyl allyl chain (compound **13**) was more active than the citronellyl one (compound **10**), a substituent that significantly increases the lipophilicity of the molecule (LogP 4.9).

We suppose that different prenylated chains may change the bioavailability of the compound influencing the hydrolytic degradation of the prenylated esters within the fungal cell. In fact, hydrolytic degradation, mediated by fungal enzymes, of the esters in the parent cinnamic acid and the corresponding alcohol cannot be ruled out. In esters **11** and **10**, a too long prenylated chain could be partially metabolized by the fungus at the first stages of contact, whereas in ester **13**, the hydrolysis would take a longer time, allowing it to reach sensitive compartments of the fungal cell where the prenyl alcohol may exert its antifungal activity. A similar effect has been reported by farnesol on *F. keratoplasticum*, which is associated with biofilm formation in hospital water systems and internal pipelines: this prenylated alcohol has a remarkable anti-biofilm activity; causes the destruction of hyphae and of the extracellular matrix; and prevents the adhesion of conidia, filamentation, and the formation of biofilm [52].

Compounds **4**–**13** contain an α,β-unsaturated Michael acceptor pharmacophore effective in interacting with nucleophiles present in the fungal cell; nevertheless, this feature is not exhaustive for the antifungal activity. The presence of a free phenol-OH in para position would play a key role in the radical scavenging and stabilisation of the radical by electronic delocalisation along the structure. In general, we observed that compounds with a catechol and guaiacyl ring favouring an intramolecular hydrogen bond and hampering the availability of the H donor to scavenge radicals were less active as antimycotic (compounds **5**, **8**, and **12**).

The antifungal activity of compound **13** was compared with that of TRB and AmB, two of the most effective conventional fungicides for clinical use [53]. TRB and AmB were applied at clinical dosage ranging between 2–256 μM and 1–135 μM, respectively, whereas compounds **13** was amended at concentrations comprised between 7.8 and 500 μM. Both TRB and AmB interact at the level of fungal cell membrane, the first one by inhibiting squalene epoxidase, a key step along the ergosterol biosynthesis pathway, and the second one by a complex interaction with phospholipid bilayers [54]. Our results demonstrate that compound **13** presents MIC and LD_{50} values against *F. verticillioides* 115 and *F. oxysporum* 89 that are consistent with those reported for AmB. Similarly, while AmB was indeed the most effective compound in terms of MIC and LD_{50} against *F. keratoplasticum* 93 and *F. solani* 96, the antifungal efficacy of compound **13** against these members of the FSSC was comparable to that of terbinafine. Besides its remarkable biological activity, ester **13** presents some attractive advantages; that is, it is as natural compound with a simple structure, a straightforward synthesis, low production cost with easy recovery of the starting materials. Considering the increasing frequency of multi-drug resistance patterns in opportunistic *Fusarium* spp. [55], the development of compound **13** as an effective antifungal drug represents a valuable alternative to the conventional therapeutic agents in onychomycosis treatment.

The results of this study provide useful insights to the optimal design of the structure of cinnamic esters with improved antifungal properties. Although cinnamic acids and their derivatives have been studied on some plant pathogenic fungi [56], to the best of our knowledge, no investigation was conducted on prenylated cinnamic esters and ethers on clinical *Fusarium* spp., thereby offering an intriguing opportunity in drug repositioning strategy.

4. Materials and Methods

4.1. Chemical Synthesis

4.1.1. General

Unless otherwise noted, starting materials and reagents were obtained from commercial suppliers and were used without further purification. Melting points were determined on a Büchi 530 apparatus and are uncorrected. All ^{1}H-NMR and ^{13}C-NMR spectra were recorded in $CDCl_3$ (if not otherwise indicated) solution at 399.94 MHz and 75.42 MHz, respectively, with a Varian VXR 5000 spectrometer (Varian, Palo Alto, CA USA). Chemical shifts are given in ppm (δ); multiplicities are indicated by s (singlet), d (doublet), t (triplet), q (quartet), m (multiplet), or dd (doublet of doublets). Elemental analyses were performed using an elemental analyser model 240 C (Perkin-Elmer, Walthan, MA USA). Acetone was freshly distilled from $CaCl_2$. Flash chromatography was carried out with silica gel 60 (230–400 mesh; VWR; Radnor, AF, USA) eluting with appropriate solution in the stated v:v proportions. Analytical thin-layer chromatography (TLC) was performed with 0.25 mm thick silica gel plates (Sigma Aldrich, Munich, Germany). All reactions were monitored by TLC performed on 0.2 mm thick silica gel plates (60 F254 Merck). Microwave reactions were carried out on a MW instrument (CEM-Discover SP MW, Matthews, NC, USA). Melting points were determined on a 530 apparatus (Büchi, Flawil, Switzerland) and are uncorrected. The purity of all new compounds was judged to be >98% by ^{1}H-NMR spectral determination. Lipase from *Candida antarctica* (Novozym 435 CAL-B) is immobilized on a macroporous polyacrylic resin beads (recombinant, expressed in *Aspergillus niger*, activity ≥ 5000 PLU/g (propyl laurate units/g) and purchased from Merck (Milan, Italy). Compound **8** was prepared according to the literature [57].

Lipophilicity of the compounds was estimated using the logarithm of the partition coefficient for *n*-octanol/water (log P), which was calculated using 403 ChemBioDraw Ultra 13.0.

4.1.2. General Procedure for the Synthesis of Compounds **10**–**13**

Ethyl chloroformate (2 eq for **10**, **12**, and **13** or 3 eq for **11**) and triethylamine (2 eq for **10**, **12**, and **13** or 3 eq for **11**) were added to a suspension of appropriate cinnamic acid (1 eq) in dichloromethane (10 mL) and stirred for 1 h at −30 °C until all of the starting material disappeared, as determined by TLC. Appropriate alcohol (1 eq) and 4-dimethylaminopyridine (0.2 eq) were then added, and the mixture was stirred at room temperature for 6 h. The reaction mixture was acidified with hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL), and the organic phases were combined and dried over anhydrous sodium sulphate. The product was then concentrated under reduced pressure and filtered on a pad of silica gel using dichloromethane as eluent to give a yellow oil. The oil was diluted in dichloromethane (15 mL) and piperidine (30 eq) was added at 0 °C. The reaction mixture was stirred at room temperature for 3 h, acidified with hydrochloridric acid (10% solution), and extracted with dichloromethane (3 × 50 mL), and the organic phases were combined and dried over anhydrous sodium sulphate. The crude product was concentrated under reduced pressure and purified by flash chromatography using a 1:1 mixture of petroleum ether/ethyl acetate as eluent to give the pure ester.

(E)-3,7-Dimethyloct-6-en-1-yl 3-(4-hydroxyphenyl)acrylate ***10***: oil; 44%; $[\alpha]_D^{20}$ 0.5 (c = 0.9, $CHCl_3$); ^{1}H-NMR δ 0.94 (d, *J* = 6.4 Hz, 3H), 1.22 (m, 1H), 1.38 (m, 1H), 1.51 (m, 1H), 1.60 (m, 1H), 1.61 (s, 3H), 1.67 (s, 3H), 1.76 (m, 1H), 1.99 (m, 2H), 4.26 (m, 2H), 5.11 (m, 1H), 6.28 (d, *J* = 16.0 Hz, 1H), 6.81 (m, Ar, 2H), 7.42 (m, Ar, 2H), 7.63 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 17.66, 19.43, 25.40, 25.73, 29.54, 35.48, 36.99, 63.40, 114.97, 115.99, 124.57, 126.63, 130.07, 131.37, 145.12, 158.51, 168.44; Anal. Calcd. for $C_{19}H_{26}O_3$: C, 75.46; H, 8.67; Found: C, 75.44; H, 8.60.

(E)-3,7-Dimethyloct-6-en-1-yl 3-(3,4-dihydrophenyl)acrylate ***11***: brown solid; 47%; mp 100–101 °C; $[\alpha]_D^{20}$ 2.9 (c = 0.4, $CHCl_3$); ^{1}H-NMR δ 0.93 (d, *J* = 6.4 Hz, 3H), 1.21 (m, 1H), 1.36 (m, 1H), 1.52 (m, 1H), 1.59 (m, 1H), 1.60 (s, 3H), 1.67 (s, 3H), 1.74 (m, 1H), 1.99 (m, 2H),

4.30 (m, 2H), 5.09 (m, 1H), 6.28 (d, *J* = 15.6 Hz, 1H), 6.88 (d, *J* = 8.4 Hz, Ar, 1H), 6.99 (dd, *J* = 2.1, 8.4 Hz, Ar, 1H), 7.12 (d, *J* = 2.1 Hz, Ar, 1H), 7.59 (d, *J* = 15.6 Hz, 1H); ^{13}C-NMR δ 17.66, 19.42, 25.37, 25.72, 29.51, 35.42, 36.97, 63.61, 114.44, 115.14, 115.46, 122.46, 124.52, 127.17, 131.42, 144.03, 145.56, 146.78, 168.69; Anal. Calcd. for $C_{19}H_{26}O_4$: C, 71.67; H, 8.23; Found: C, 71.60; H, 8.26.

(E)-3,7-Dimethyloct-6-en-1-yl 3-(4-hydroxy-3-methoxyphenyl)acrylate ***12***: oil; 37%; $[\alpha]_D^{20}$ 0.6 (c = 0.4, $CHCl_3$); ^{1}H-NMR δ 0.93 (d, *J* = 6.8 Hz, 3H), 1.22 (m, 1H), 1.37 (m, 1H), 1.52 (m, 1H), 1.60 (m, 1H), 1.61 (s, 3H), 1.67 (s, 3H), 1.73 (m, 1H), 2.01 (m, 2H), 3.99 (s, 3H), 4.23 (m, 2H), 5.09 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 6.89 (d, *J* = 8.4 Hz, Ar, 1H), 7.02 (d, *J* = 2.0 Hz, Ar, 1H), 7.06 (dd, *J* = 2.0, 8.4 Hz, Ar, 1H), 7.59 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 17.64, 19.44, 25.39, 25.71, 29.54, 35.55, 37.01, 55.90, 62.94, 109.32, 114.74, 115.61, 123.03, 124.59, 127.01, 131.31, 144.67, 146.78, 147.30, 168.41; Anal. Calcd. for $C_{20}H_{28}O_4$: C, 72.26; H, 8.49; Found: C, 72.34; H, 8.40.

(E)-3-Methylbut-2-en-1-yl 3-(4-hydroxyphenyl) acrylate ***13***: oil; 45%; ^{1}H-NMR δ 1.73 (s, 3H), 1.77 (s, 3H), 4.71 (d, *J* = 7.2 Hz, 2H), 5.41 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 6.87 (m, Ar, 2H), 7.38 (m, Ar, 2H), 7.64 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 18.08, 25.82, 61.73, 114.91, 115.99, 118.42, 126.69, 130.07, 139.50, 145.19, 158.42, 168.36; Anal. Calcd. for $C_{14}H_{16}O_3$: C, 72.39; H, 6.94; Found: C, 72.45; H, 6.96.

4.1.3. General Procedure for the Synthesis of Compounds **14**–**17**

In a 30 mL glass pressure microwave tube, equipped with a magnetic stirrer bar, a few drops of concentrated sulphuric acid were added to a solution of hydroxycinnamic acid (*p*-coumaric acid or caffeic acid or ferulic acid) (1 eq) in methanol (for **14**–**16**) or ethanol (for **17**) (10 mL). The mixture was subjected to microwave irradiation (power: 150 W; temperature: 80 °C for **14**–**16** and 98 °C for **17**) for 15 min, basified with aqueous sodium bicarbonate (5% solution), and extracted with dichloromethane (3 × 5 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated to dryness to give the pure ester.

(E)-Methyl 3-(4-hydroxyphenyl)acrylate ***14***: white solid; 82%; mp 125–127 °C ([58] 132–134 °C); ^{1}H-NMR δ 3.79 (s, 3H), 5.37 (bs, 1H), 6.28 (d, *J* = 16.0 Hz, 1H), 6.85 (m, Ar, 2H), 7.42 (m, Ar, 2H), 7.63 (d, *J* = 16.0, 1H); ^{13}C-NMR δ 51.75, 114.52, 115.91, 125.98, 129.89, 144.79, 158.12, 167.65. Anal. Calcd. for $C_{10}H_{10}O_3$ C, 67.41; H, 5.66; Found: C, 67.53; H, 5.56.

(E)-Methyl 3-(3,4-dihydroxyphenyl)acrylate ***15***: brown solid; 88%; mp 155–156 °C ([59] 160 °C); ^{1}H-NMR δ 3.80 (s, 3H), 6.25 (d, *J* = 16.0 Hz, 1H), 6.87 (d, *J* = 8.4 Hz, Ar, 1H), 7.01 (dd, *J* = 2.0, 8.4 Hz, Ar, 1H), 7.07 (d, *J* = 2.0 Hz, Ar, 1H), 7.58 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 51.78, 114.33, 115.27, 115.49, 122.46, 127.52, 143.74, 145.03, 146.28, 168.18. Anal. Calcd. for $C_{10}H_{10}O_4$; C, 61.85; H, 5.19; Found: C, 62.05; H, 5.78.

(E)-Methyl 3-(4-hydroxy-3-methoxyphenyl)acrylate ***16***: brown solid; 92%; mp 62–64 °C ([60] 65 °C); ^{1}H-NMR δ 3.75 (s, 3H), 3.83 (s, 3H), 6.14 (bs, 1H), 6.25 (d, *J* = 16.0 Hz, 1H), 6.86 (d, *J* = 8.0 Hz, Ar, 1H), 6.96 (d, *J* = 2.0 Hz, Ar, 1H), 6.96 (dd, *J* = 2.0, 8.0 Hz, Ar, 1H), 7.58 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 51.64, 55.84, 109.52, 114.72, 114.81, 122.98, 126.78, 145.12, 146.92, 148.12, 167.92. Anal. Calcd. for $C_{11}H_{12}O_4$ C, 63.45; H, 5.81; Found: C, 63.51; H, 5.76.

(E)-Ethyl 3-(4-hydroxyphenyl)acrylate ***17****:* brown solid; 87%; mp 70–72 °C ([61] 73–74 °C); ^{1}H-NMR δ 1.32 (t, *J* = 7.1 Hz, 3H), 4.26 (q, *J* = 7.1 Hz, 2H), 6.39 (d, *J* = 16.0 Hz, 1H), 7.35 (m, Ar, 2H), 7.47 (m, Ar, 2H), 7.58 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 14.43, 60.74, 119.14, 124.58, 129.54, 132.22, 133.52, 143.29, 166.78. Anal. Calcd. for $C_{11}H_{12}O_3$: C, 68.74; H, 6.29; Found: C, 68.81; H, 6.34.

4.1.4. Procedure for the Synthesis of Compound **13** with Lipase

To a solution of **17** (1 eq) in cyclohexane (2.5 mL), 3,3-dimethylallyl alcohol (2 eq) was added. The reaction mixture was stirred at 60 °C for 15 min at a speed of 300 rpm. The reaction was initiated by adding a known fixed quantity of lipase (100 mg). The progress of the reaction was monitored by TLC using a 1:1 mixture of petroleum ether/ethyl acetate as eluent. After three days, the starting material was still present and another aliquot of

lipase (100 mg) was added, and the mixture was left stirring at 60 °C for two additional days. The reaction mixture was filtered over Buchner funnel, solvent concentrated under reduced pressure, and purified by flash chromatography using a 1:1 mixture of petroleum ether/ethyl acetate as eluent to obtain compound **13** (0.19 g, 80% yield).

4.1.5. General Procedure for the Synthesis of Compounds **4**, **6, 7**, and **9**

Compound **14** or **15** or **16** (1 eq) was dissolved in dry acetone (15 mL) and then anhydrous potassium carbonate (1 eq) and appropriated alkenyl bromide (1 eq) were added. The resulting mixture was stirred at 50 °C for 12 h, then sodium hydroxide 2 N (15 mL) was added and the reaction mixture was stirred at 90 °C for an additional 3 h. The cooled solution was acidified to pH 2 with hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated to dryness to give the pure ether.

(E)-3-(4-(Allyloxy)phenyl)acrylic acid ***4***: white solid; 85%; mp 161–162 °C ([62] 160 °C); ^{1}H-NMR δ 4.56 (d, *J* = 5.2 Hz, 2H), 5.29–5.44 (series of m, 2H), 6.03 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H), 6.91 (m, Ar, 2H), 7.49 (m, Ar, 2H), 7.73 (d, *J* = 16 Hz, 1H); ^{13}C-NMR δ 68.85, 114.72, 115.13, 118.08, 126.91, 130.07, 132.66, 146.67, 160.73, 172.55. Anal. Calcd. for $C_{12}H_{12}O_3$ C, 70.57; H, 5.92; Found C, 70.78; H, 5.87.

(E)-3-(4-(Allyloxy)-3-methoxyphenyl)acrylic acid ***6***: white solid; 53%; mp 152–154 °C ([63] 151–153 °C); ^{1}H-NMR δ 3.91 (s, 3H), 4.66 (m, 2H), 5.30–5.44 (series of m, 2H), 6.08 (m, 1H), 6.29 (d, *J* = 16.0 Hz, 1H); 6.86 (d, *J* = 8.4 Hz, Ar, 1H), 7.08 (dd, *J* = 2.0, 8.4 Hz, Ar, 1H), 7.09 (d, *J* = 2.0 Hz, Ar, 1H), 7.71 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 55.92, 69.70, 110.08, 112.71, 114.90, 118.51, 122.92, 127.14, 132.61, 147.01, 149.52, 150.46, 172.72. Anal. Calcd. for $C_{13}H_{14}O_4$ C, 66.66; H, 6.02; Found: C, 66.87; H, 6.12.

(E)-3-(4-((3-Methylbut-2-en-1-yl)oxy)phenyl)acrylic acid ***7***: white solid; 69%; mp 146–147 °C ([16] 148–150 °C); ^{1}H-NMR δ 1.73 (s, 3H), 1.79 (s, 3H), 4.52 (d, *J* = 6.4 Hz, 2H), 5.47 (s, 1H), 6.28 (d, *J* = 16 Hz, 1H), 6.89 (d, *J* = 8.8 Hz, Ar, 2H), 7.47 (d, *J* = 8.4 Hz, Ar, 2H), 7.73 (d, *J* = 16 Hz, 1H); ^{13}C-NMR δ 25.81, 29.19, 64.91, 114.62, 115.04, 119.11, 126.67, 130.04, 138.73, 146.60, 161.04, 171.36. Anal. Calcd. for $C_{14}H_{16}O_3$ C, 72.39; H, 6.94; Found C, 72.59; H, 6.03.

(E)-3-(4-(((E)-3,7-Dimethylocta-2,6-dien-1-yl)oxy)-3-methoxyphenyl)acrylic acid ***9****:* white solid; 75%; mp 59–60 °C ([64] 60–61 °C); ^{1}H-NMR δ 1.63 (s, 3H), 1.70 (s, 3H), 1.77 (s, 3H), 2.01–2.24 (series of m, 4H), 3.95 (s, 3H), 4.62 (m, 2H), 5.11 (m, 1H), 5.44 (m, 1H), 6.33 (d, *J* = 16.0 Hz, 1H), 6.86 (d, *J* = 7.6 Hz, Ar, 1H), 7.01–7.15 (series of m, Ar, 2H), 7.37 (d, *J* = 16.0 Hz, 1H); ^{13}C-NMR δ 16.70, 17.70, 25.70, 26.20, 39.53, 55.91, 65.82, 109.91, 112.52, 114.71, 119.11, 123.01, 123.71, 126.81, 131.84, 141.22, 147.01, 149.52, 150.83, 172.11. Anal. Calcd. for $C_{20}H_{26}O_4$ C, 72.70; H, 7.93; Found: C, 72.80; H, 7.92.

4.1.6. Synthesis of Compound **5**

(E)-3-(4-(Allyloxy)-3-hydroxyphenyl)acrylic acid ***5****:* Compound **15** (0.5 g, 2.57 mmol) was dissolved in dry acetone (15 mL) and then anhydrous potassium carbonate (0.35 g, 2.57 mmol) and allyl bromide (0.31 g, 2.57 mmol) were added. The resulting mixture was stirred at 50 °C for 12 h. The cooled solution was acidified to pH 2 with hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated. The crude product was purified by flash chromatography using a mixture of 3:1 petroleum ether/acetone as eluent to give compound **18** as a white solid (0.47 g, 78%).

(E)-Methyl 3-(4-(allyloxy)-3-hydroxyphenyl)acrylate ***18***: mp 95–96 °C ([65] 94–95 °C); ^{1}H-NMR δ 3.78 (s, 3H), 4.65 (d, *J* = 5.6 Hz, 2H), 5.33–5.43 (series of m, 2H), 5.69 (s, 1H), 6.08 m, 1H), 6.30 (d, *J* = 15.6 Hz, 1H), 6.83 (d, *J* = 8.4 Hz, Ar, 1H), 6.99 (dd, *J* = 2.0 8.4 Hz, Ar, 1H), 7.14 (d, *J* = 2.0 Hz, Ar, 1H), 7.61 (d, *J* = 15.6 Hz, 1H); ^{13}C-NMR δ 51.59, 69.80, 11.84, 113.21, 115.94, 118.80, 121.64, 123.13, 128.20, 132.26, 144.60, 146.02, 147.39, 167.68. Anal. Calcd. for $C_{14}H_{16}O_4$ C, 67.73; H, 6.50; Found: C, 67.52; H, 6.42. To compound **18** (0.47 g, 2.00 mmol) in a 3:1 solution of MeOH:H_2O, sodium hydroxide 2 N (15 mL) was added and the mixture was stirred at 90 °C for 3 h. The cooled solution was acidified to pH 2 with

hydrochloridric acid (10% solution) and extracted with dichloromethane (3 × 50 mL). The collected organic phases were dried over anhydrous sodium sulphate and evaporated to dryness to give the pure **5** as white solid (0.33 g, 75%); mp 184–185 °C; ^{1}H-NMR δ (acetone d_6) 4.66 (m, 2H), 5.26 (m, 1H), 5.46 (m, 1H), 6.07 (m, 1H), 6.32 (d, *J* = 16 Hz, 1H), 7.01 (d, *J* = 8.4 Hz, Ar, 1H), 7.09 (dd, *J* = 2.4, 8.4 Hz, Ar, 1H), 7.19 (d, *J* = 2.4, Hz, Ar, 1H), 7.56 (d, *J* = 16 Hz, 1H); ^{13}C-NMR δ 64.41, 112.87, 113.95, 115.94, 117.05, 121.08, 128.03, 133.44, 144.61, 147.08, 148.52, 167.09. Anal. Calcd. for $C_{12}H_{12}O_4$ C, 65.45; H, 5.49; Found: C, 65.52; H, 5.32.

4.2. Fungal Strains and Culture

Two monosporic isolates collected from human samples from an Italian hospital (Table 2) were selected as representative of each *Fusarium oxysporum*, *F. solani*, and *F. fujikuroi* species complexes. Conidial suspensions of each strain were pre-cultured in a carboxymethyl cellulose medium (CMC; [66]) for 5 days on a rotary shaker at 24 °C and 180 rpm. Cultures were filtered through four layers of sterile cheesecloth, and spores were collected by centrifugation, adjusted to 1 × 10^6 colony-forming units (CFU)/mL in sterile water, and used as inoculum.

4.3. Evaluation of the Antifungal Activity of Compounds **1**–**13** *in FMM Solid Medium*

A total of 13 phenolic compounds (Table 1) were tested for their antifungal activity against the six *Fusarium* spp. isolates (Table 2) in Fusarium minimal medium (FMM) [67]. Each phenolic compound was resuspended in H_2O/gellan 0.1 % solution and sonicated at room temperature for 1 h at 80 Hz (Elmasonic P 180 H, Elma Schmidbauer GmbH, Germany). Solid FMM with nitrate sodium $NaNO_3$ as nitrogen source was distributed into Ø90 mm Petri dishes (15 mL/Petri dish) and amended with each compound at a final concentration of 0.5 mM at a temperature of 45 °C. Ten microliters of the conidial suspension of each strain were spotted onto the center of the Petri dish amended FMM. Antifungal activity of each compound was measured after 5 d of growth at 25 °C in the dark and expressed as the colony diameter (percentage relative to control). Three replicates were prepared for each isolate/inhibitor combination and the experiment was repeated once.

*4.4. Evaluation of the Antifungal Activity of p-Coumaric Acid 3,3′-Dimethyl Allyl Ester (***13***) in FMM Liquid Medium*

The conventional antifungal drugs used in the study were AmB and TRB. AmB was purchased by Sigma Aldrich (A2942, Germany) as a standard solution. TRB was extracted from Terbinafine Hexal 250 mg tablets by fine crushing and dissolution in a solution 1:2 (v:v) dichloromethane and water. The emulsion was stirred at room temperature until two phases clearly appeared. The organic phase was extracted and dried on Na_2SO_4 and the TRB was recovered in neat form after evaporation of the solvent under vacuum. NMR spectra of the solid extract confirmed the presence of TRB with a purity ≅98%. TRB was dissolved in 60% ethanol/H_2O (*v*/*v*), while AmB was diluted with water to reach the desired concentration and was frozen in aliquots at −20 °C. AmB and TRB concentrations were selected according to clinical dosage and standard experimental procedures with some modifications [68].

The minimal inhibitory concentration (MIC) and the lethal dose 50 (LD_{50}) of each strain were assayed by a standardized micro-dilution method in the 96-well plate. Two-fold serial dilutions of each antifungal agent in liquid FMM in a total volume of 200 µL were tested. Further, 10 µL of fungal spore suspension (4 × 10^6 CFU/mL) was added. A blank control with water was used for each treatment. The optical density mOD of each microplate well was measured at 2 h intervals during 72 h of incubation with a microplate spectrophotometer SpectrostarNano (Euroclone, Germany) at a 595 nm wavelength. The inhibitory activity of each compound was expressed as MIC, representing the lowest concentration of active ingredient (µM) that is sufficient to inhibit the absorbance signal, whereas the LD_{50} of each compound was calculated as the concentration of active ingredient

(μM) able to reduce by 50% the mOD_{595} signal. The experiments were repeated at least two times in quadruplicate.

4.5. Optical Microscopy Examination

A drop (15 μL) of the total volume present on the wells corresponding to the MIC of each isolate/compound combination was pipetted after 72 h of incubation onto a glass slide. A clean glass cover slip was placed on the sample prepared with emulsion oil. Each slide was examined at 100× for the presence of mycelium, branched hyphae, fialides, microconidia, and germinating spores, using an optical microscope (LEICA ICC50) at a scale of 20 μm.

4.6. Data Acquisition and Analysis

In the first screening, an analysis of variance (one-way ANOVA) followed by multiple comparisons by Tukey HSD test at the significance level $p < 0.05$ using Minitab for Windows, release 17 was performed.

In the second screening, data were recorded and analysed with Mars Data Analysis Software, BMG Labtech, and exported to Microsoft Excel for generation of the graphs. Graphs for the determination of the MIC and LD_{50} (Figures S4–S9) were generated for an incubation time between 0 h and 48 h because, after this interval time, the drug free-test (control) curve started to reach the stationary phase for almost all strains investigated. Optical microscopy images were captured and treated with LAS V4.13 Leica application software.

5. Conclusions

The design of cinnamic derivatives **4**–**13** was focused on both electronic and steric modification of the parent compounds **1**–**3** by esterification and etherification reaction with bioactive prenylated chains, with the aim to enhance the antifungal activity of the final compound. Compounds **1**–**3** are commercially available at a reasonable price and offer a successful example of repositioning of natural compounds. In this study, we provided data that may contribute to increasing the knowledge about the promotion of the importance of antifungal susceptibility testing. *p*-Coumaric acid 3,3′-dimethyl allyl ester **13**, a component of propolis, showed good antifungal activities against *Fusarium* spp., causing onychomycosis, and identifies prenylated hydroxy cinnamic acids as interesting pharmacophore for developing new drugs effective against this pathology. The activity of this compound will be investigated over a larger number of isolates belonging to different species complexes and haplotypes. Furthermore, the mechanism of action of compound **13** needs to be fully characterized and possibly tested in combination with other bioactive molecules that may be enabled to reach their target within the fungal cell.

Noteworthy, this study cannot be adopted as a clinical guideline, and the MIC values obtained must be tested in an appropriately designed clinical study.

Supplementary Materials: The following are available online. Figures S1–S3: Antifungal activity of compounds **1**–**13** against FOSC, FFSC, and FFSC, respectively. Figures S4–S9: MIC and LD_{50} ranges expressed as absorbance (milliOD) at 595 nm at 48 h of ester **13** (A), TRB (B), and AmB (C) against FOSC, FSSC, and FFSC strains, respectively.

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Sample Availability: Samples of the compounds **1**–**13** are available from the authors.

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