

Article

Antifungal effect of magnolol and honokiol from Magnolia officinalis on Alternaria alternata causing tobacco brown spot

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Abstract: The phenol compounds of magnolol and honokiol were separated from Magnolia officinalis, identified by LC-MS, ¹H- and ¹³C- NMR. The magnolol and honokiol were found to be effective against Alternaria alternata, Rhizoctonia solani KChn, Fusarium moniliforme Sheld, Penicillium expansum (Link) Thom, Valsa mali Miyabe et Yamada and Fusarium oxysporum Schlecht with more than 60.80% of growth inhibition rates. Their antifungal activity and potential antifungal mechanisms were also investigated. The results showed that magnolol and honokiol were effective against Alternaria alternata with minimum inhibitory concentration (MIC) and minimum fungicidal concentration (MFC) of 3 mg/mL and 5 mg/mL for magnolol, respectively, and 1 mg/mL and 3 mg/mL for honokiol, respectively. These two phenol compounds caused distortion of the mycelia and an obvious increase in cell membrane permeability of A. alternata through the determination of conductivity. These results suggest that magnolol and honokiol were potential antifungal agents for treatment of fungal disease infections in the application point.

Keywords: Magnolia officinalis; antifungal activity; Alternaria alternata; minimum inhibitory concentration (MIC); minimum fungicidal concentration (MFC)

1. Introduction

Magnolia officinalis, is an important herb in traditional Chinese and Japanese medicine, in which it has been used as treatment reagent of gastrointestinal disorders, thrombotic stroke, allergic disease, typhoid fever, anxiety and nervous disturbance, etc [1]. M. officinalis contains a diverse group of biologically active compounds, such as magnolol, honokiol, 4-O-methylhonokiol, obovatol and other neolignan compounds, which systemically showed anti-cancer, anti-inflammatory, anti-oxidant activity [2-6].

Tobacco is an important economic plant in the world for cigarette production. Alternaria alternata, the causal agent of tobacco brown spot, is one of the most damaging diseases of tobacco and causes heavy losses around the world [7-9]. In China, the annual incidence area of A. alternata is about 100,000 hm², leading to economic losses [10]. After infection by A. alternata, the small yellowish-brown round spots appear on the tobacco leaves. Initially, the spots are blade and change into larger circle latterly [11], which could significantly affect the quantity and quality of tobacco

[12–14]. To control this disease, the chemical reagents, for example dimetachlone have been used widely in the field [13]. However, chemical agents have the potential to increase the fungicide resistance in plant pathogens and cause the ecological pollution in the environment, which may be a serious health risks to animals and human being. Indeed, many countries have restricted the application of chemical fungicides to control plant diseases [15]. Therefore, the plant-derived fungicides are promising alternatives, since they are found to be largely non-phytotoxic, systematic, easily biodegradable and environmentally safe.

In recent years, many studies have been conducted on the antifungal activity of plant-derived fungicides against *A. alternata*. A kind of naphthoquinone and plumbagin (29) isolated from leaves of *Nepenthes ventricosa* inhibited the growth of *A. alternata* [16]. Jing et al has found that eugenol, essential oil from the buds of *Syringa oblata* Lindl, exhibited complete inhibition of mycelial growth against *A. alternata*, suggesting its potential application as a fungicide [14].

The extracts of *M. officinalis* showed potent antifungal activity against many plant fungi pathogen. However, its antifungal activity against *A. alternata* have not been reported. Here, we identify the compounds of magnolol and honokiol from *Magnolia officinalis*. Their antifungal effect on *Alternaria alternata*, *Rhizoctonia solani* KChn, *Fusarium moniliforme* Sheld, *Penicillium expansum* (Link) Thom, *Valsa mali* Miyabe et Yamada and *Fusarium oxysporum* Schlecht in vitro were also examined. We found that these two active compounds are able to efficiently inhibit on mycelium growth and cell membrane permeability of *A. alternata*.

2. Results

2.1. Identification of Magnolol and Honokiol from *M. officinalis*

Two compounds magnolol and honokiol were purified from *M. officinalis* using the preparative high performance liquid chromatography (HPLC). The HPLC curve revealed that the components eluted in peaks A (honokiol) and B (magnolol) (Figure 1). Based on the HPLC analysis and the elution curve, the purities of the components were 98%. The structures of honokiol and magnolol were identified by $^1\text{H NMR}$, $^{13}\text{C NMR}$ and HR-MS spectra as follows (Figure 2).

Magnolol (5,5'-diallyl-2,2'-dihydroxybiphenyl): colorless solid; $^1\text{H NMR}$ (500 MHz, CD_3OD): δ (ppm) 3.35 (d, $J = 6.7$ Hz, 4H), 4.86 (s, 2H), 5.02–5.10 (m, 4H), 5.95–6.03 (m, 2H), 6.88 (d, $J = 8.2$ Hz, 2H), 7.04–7.06 (m, 4H); $^{13}\text{C NMR}$ (125 MHz, CD_3OD): δ (ppm) 40.3, 115.5, 117.4, 127.6, 129.7, 132.6, 133.1, 139.3, 153.2; HR-MS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{O}_2$ ($[\text{M}+\text{H}]^+$) 267.1378, found 267.1382.

Honokiol (5,5'-diallyl-2,4'-dihydroxybiphenyl): colorless solid; $^1\text{H NMR}$ (500 MHz, CD_3OD): δ (ppm) 3.30 (d, $J = 6.7$ Hz, 2H), 3.41 (d, $J = 6.5$ Hz, 2H), 4.86 (s, 2H), 5.00–5.11 (m, 4H), 5.93–6.09 (m, 2H), 6.79–7.26 (m, 6H); $^{13}\text{C NMR}$ (125 MHz, CD_3OD): δ (ppm) 35.3, 40.4, 115.3, 115.5, 116.8, 127.3, 128.7, 129.1, 129.9, 131.4, 131.5, 131.9, 132.5, 138.4, 139.5, 153.2, 155.5; HR-MS (ESI): m/z calcd for $\text{C}_{18}\text{H}_{19}\text{O}_2$ ($[\text{M}+\text{H}]^+$) 267.1378, found 267.1373.

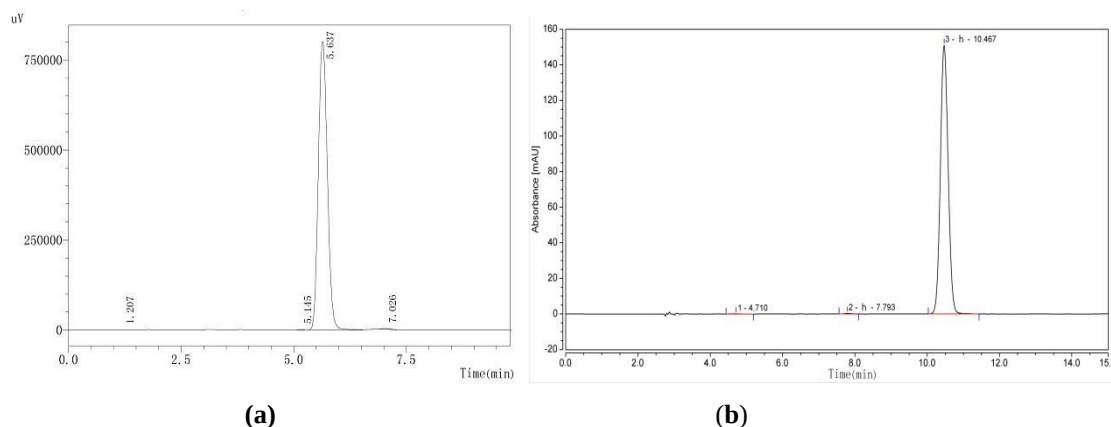


Figure 1. HPLC chromatogram in magnolol and honokiol, (a) magnolol; (b) honokiol.

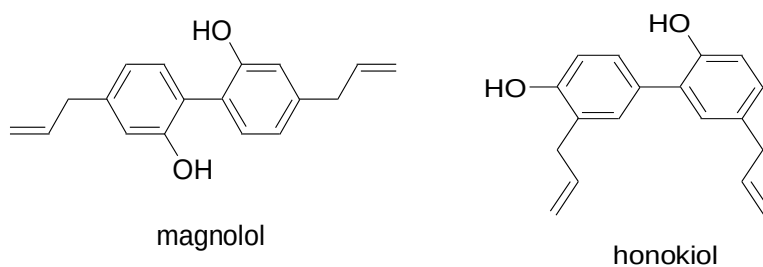


Figure 2. Structure of the compound identified as magnolol and honokiol.

2.2. The Inhibitory Efficiency of Magnolol and Honokiol against 6 Phytopathogens

To verify whether magnolol and honokiol are able to inhibit fungi pathogens, the inhibitory efficiency of magnolol and honokiol against 6 fungal pathogens was tested and shown in Table 1. We found that the magnolol (3 mg/mL) was effective against *Alternaria alternata*, *Rhizoctonia solani* KChn, *Fusarium moniliforme* Sheld, *Penicillium expansum* (Link) Thom, *Valsa mali* Miyabe et Yamada and *Fusarium oxysporum* Schlecht with the inhibition rates of 100%, 60.80%, 90.09%, 74.87%, 100% and 73.26% respectively. The honokiol (1 mg/mL) treatment was showed efficient inhibition effect on pathogens above with the inhibition rate of 100%, 74.65%, 100%, 88.69%, 100% and 91.68% correspondingly.

Table 1. The inhibitory efficiency of magnolol and honokiol against 6 other phytopathogens.

Pathogen	Mycelial growth inhibition rate (%)	
	magnolol	honokiol
<i>Alternaria alternata</i>	100.00a	100.00a
<i>Rhizoctonia solani</i> KChn	60.80 ± 3.62d	74.65 ± 0.83d
<i>Fusarium moniliforme</i> Sheld	90.09 ± 1.04b	100.00a
<i>Penicillium expansum</i> (Link) Thom	74.87 ± 1.64c	88.69 ± 2.34c
<i>Valsa mali</i> Miyabe et Yamada	100.00a	100.00a
<i>Fusarium oxysporum</i> Schlecht	73.26 ± 1.28c	91.68 ± 1.22b

Values are presented as mean ± SD. Different letters in the same column indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test.

2.3. Minimum Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC) of Magnolol and Honokiol for *A. alternata*

Magnolol and honokiol inhibited the mycelial growth of *A. alternata* in a dose-dependent manner (Table 2). The mycelial growth was considerably reduced when the concentrations of magnolol and honokiol increased, indicating that magnolol and honokiol have an inhibitory effect on *A. alternata*. When magnolol concentration was 0.1 mg/mL, the mycelial radial growth inhibition rate was 77.31%. At 3 mg/mL (MIC), magnolol completely inhibited the growth of *A. alternata*, whereas at 5 mg/mL (MFC), it induced fungicidal activity. The MIC and MFC values of magnolol against *A. alternata* were 3 mg/mL and 5 mg/mL, respectively. The MIC and MFC values of honokiol were 1 mg/mL and 3 mg/mL, respectively. The MIC value of honokiol (1 mg/mL) was significantly lower than that of magnolol (3 mg/mL). In addition, we found that there was a significant positive correlation between concentration of magnolol and honokiol and inhibitory effects on mycelial growth of *A. alternata*.

Table 2. Inhibition of magnolol and honokiol at different concentration on Mycelial growth.

Concentration (mg/mL)	Mycelial growth inhibition (%)	
	magnolol	honokiol
0.00	0.00g	0.00f
0.001	6.67±3.18f	8.69±0.81e

0.005	16.13±3.98e	20.45±2.29d
0.01	22.84±2.88d	26.13±1.63c
0.10	77.31±2.09c	91.48±1.56b
1.00	90.22±3.02b	100.00a
3.00	100.00a	100.00a
5.00	100.00a	100.00a
7.00	100.00a	100.00a

Values are presented as mean ± SD.

Different letters in the same column indicate significant difference at $P < 0.05$ level by Duncan's new multiple range test.

2.4. The Inhibition Effects of Magnolol and Honokiol on Morphology and Mycelial of *A. alternata*

After 3 days of inoculation, we observed that the colony on the plates containing magnolol at 3 mg/mL and honokiol at 1 mg/mL (Figure 3b and 3c) were significantly decreased comparing with the control (PDA plate) (Figure 3a). Correspondingly, the effects of magnolol and honokiol on the morphology of *A. alternata* were examined through scanning electron microphotography (SEM), as shown in Figure 3d, 3e and 3f. Comparison with the control, we observed that the magnolol and honokiol cause morphological changes in *A. alternata* by tightly entangled hyphae, abnormal shape, distortion.

The mycelia of *A. alternata* were showing distorted, aggregated and partly squashed when growing on the plant containing the magnolol at the MIC of 3 mg/mL (Figure. 3e), whereas it is not the case of honokiol (Figure. 3f). Shrunken and distorted mycelia were observed when *A. alternata* is growing on the plant with honokiol at the MIC of 1.00 mg/mL (Figure. 4f).

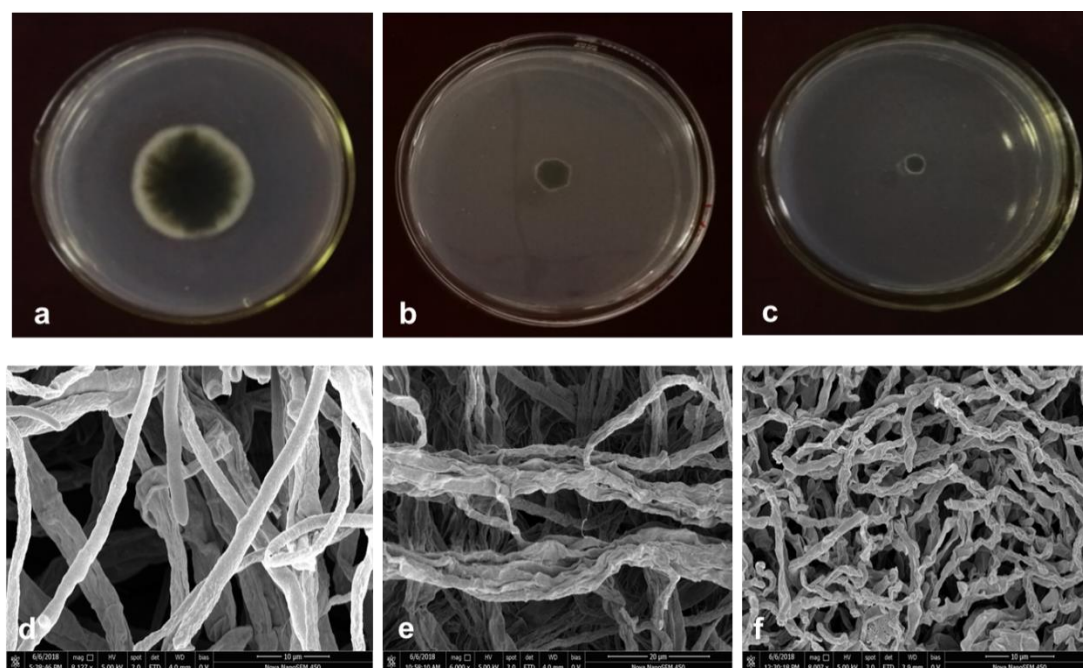


Figure 3. Observation of hypha morphology of *A. alternata* by electron microphotography. The colony of *A. alternata* on PDA plates are shown in a,b and c, a. the control (PDA plate); b. PDA plate containing the magnolol at 3 mg/mL; c. PDA plate containing the honokiol at 1 mg/mL. Hypha morphology of *A. alternata* on the PDA plate are shown in d, e, f; d. the control (PDA plate); e. PDA plate containing the magnolol at 3 mg/mL; f. PDA plate containing the honokiol at 1 mg/mL.

2.5. Effects of Magnolol and Honokiol on Cell Membrane Permeability of *A. alternata*

To address whether both magnolol and honokiol could inhibit the hypha grow by affecting the cell membrane permeability of *A. alternata*, we tested the effects of magnolol (3mg/mL) and honokiol

(1mg/mL) on cell membrane permeability of *A. alternata* for a period of 0-5 h (Figure. 4). The results showed that the cell membrane permeability were significant ($P < 0.05$) increased when *A. alternata* growing on the PDA plate containing magnolol (3mg/mL) and honokiol (1mg/mL) at different time points. The conductivity value in the *A. alternata* suspensions treated with magnolol (3 mg/mL) for 1 h was 196.44 $\mu\text{m/cm}$, which is much higher than that of the control (97.31 $\mu\text{m/cm}$). The conductivity value under the honokiol treatment at (1mg/mL) was 235.32 $\mu\text{m/cm}$.

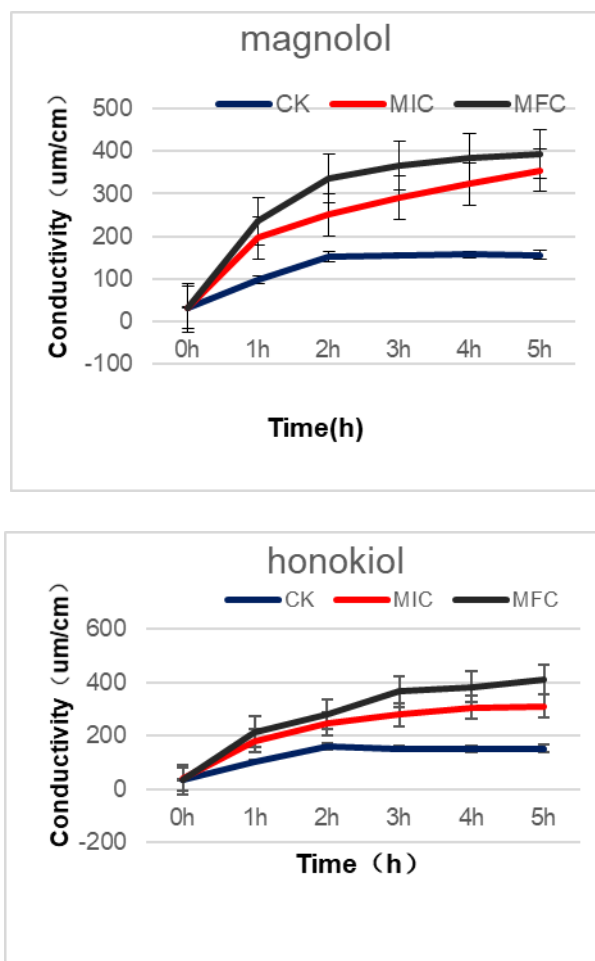


Figure 4. Effects of magnolol and honokiol on the extracellular conductivity of *A. alternata* (—): control; (—): MIC; (—): MFC).

3. Discussion

Given the reason that the disadvantage of synthetic mechanical fungicides, environmentally friendly and biodegradable botanical fungicide need to be developed in order to produce the green and safe food products for animals and human beings [15]. Previously, magnolol and honokiol are major secondary metabolites produced in *Magnolia* spp. and the composition ratios are 1.25% and 1.81% respectively [17-18]. Several reports showed that magnolol and honokiol are bearing the activities of antibacterial, anti-inflammatory, antifungal, anti-cancer, apoptosis induction and osteoclastogenesis inhibition [3,6,12,19-22]. For example, they exhibit potent antifungal activities against human fungal pathogens, such as *Trichophyton mentagrophytes*, *Candida albicans* and *Cryptococcus neoformans* [23]. In plant, it has been shown that magnolol and honokiol could efficiently inhibit *Pyricularia oryzae*, *Phytophthora infestans*, *Puccinia recondita* and *Colletotrichum capsici* [24]. Even if various biological activities of the neolignans isolated from *Magnolia* spp. have been reported, their antifungal activity against *A. alternata* in vitro has not been reported yet. In this study, we isolated magnolol and honokiol from stem bark of *Magnolia officinalis* and their antifungal activity against *A. alternata* were investigated.

Our data are suggesting that magnolol (3 mg/mL) and honokiol (1 mg/mL) could efficiently inhibit the mycelia growth of *A. alternata*, *Rhizoctonia solani* KChn, *Fusarium moniliforme* Sheld, *Penicillium expansum* (Link) Thom, *Valsa mali* Miyabe et Yamada and *Fusarium oxysporum* Schlecht in vitro with inhibition rates of more than 60.80%. This is in agreement with the antifungal activity of magnolol and honokiol on *Pyricularia oryzae*, *Phytophthora infestans*, *Puccinia recondita* and *Colletotrichum capsici* [24]. Thus, we conclude that magnolol and honokiol are potential antifungal agents against the plant fungal diseases.

The MIC and MFC values of magnolol against *A. alternata* were 3 mg/mL and 5 mg/mL, respectively, and the MIC and MFC values of honokiol were 1 mg/mL and 3 mg/mL, respectively, which are not similar to the previously reported data [22–24]. Sun et al. evaluated the antifungal activities the synergy of magnolol and honokiol and found they had the same MICs of 16–32 µg/mL against the clinically isolated strains [22]. Bang et al. found the MICs of two neolignan compounds were 25 µg/mL against *C. albicans* KCTC 1940 and *Epidermophyton floccosum* KCTC 1246 [23]. The MIC of magnolol and honokiol against *Pyricularia oryzae*, *Phytophthora infestans*, *Puccinia recondita* and *Colletotrichum capsici* are higher than that reported concentration of 300 mg/mL [24]. These differences might be due to their different sensitivities to magnolol and honokiol.

The possible antifungal mechanisms of magnolol and honokiol remain unclear. Here, through the hyphae morphology observation by SEM, we found that the *hypae* form of *A. alternata* were alerted under the pressure of magnolol and honokiol as shown in Figure 3e and 3f. These results are consistent with the previous study [25–27]. We assume that the mycelium changes may generally occur because of an increase in the permeability of cells, which resulted in the leakage of small molecular substances, ions, lesions and discrepancies in cell metabolism [28]. The marked leakage of cytoplasmic material is commonly used as an indication of gross and irreversible damage to the cytoplasmic and plasma membranes [28–31].

To verify the hypothesis, the cell membrane permeability after treatment of *hypha* with magnolol and honokiol were tested by conduct meter at different time points (0, 1, 2, 3, 4, 5, and 6 h). The results showed that the cell membrane permeability significantly increased when the samples were treated with magnolol and honokiol at different concentrations. For example, The conductivity value in the *A. alternata* suspensions with magnolol at MIC (3 mg/mL) and honokiol (1mg/mL) for 1 h was 196.44 um/cm and 180.34 um/cm, comparing with the value at 32.08 um/cm and 37.18 um/cm for 0 h; In general, conductivity value increased with the time increasing. These findings indicate that the antifungal activity of magnolol and honokiol may associated with the irreversible damage to the cytoplasmic membranes of *A. alternata*, which resulted from the ions leakage of the cells and an imbalance in osmotic pressure of the intra- and extra-cellular membrane may occurred as well.

4. Materials and Methods

4.1. Chemicals and Media

Acetonitrile (MeCN) and pyridine were purchased from Sigma Chemical Co (USA). Glucose and agar were from Shanghai Bioindustrial Engineering Co. Ltd (Shanghai, China) to prepare the PDA (potato dextrose agar) medium. Methanol, petroleum ether, quicklime, hydrochloric acid were supplied from Sinopharm group chemical reagent co. LTD (Beijing, China). Chloroform was from Sulebao technology co. LTD (Beijing, China). All the chemicals were of analytical grade.

Magnolia officinalis bark were purchased from the Jihetang Pharmacy (Yangling, China).

4.2. Fungal Strains

Alternaria alternata, *Rhizoctonia solani* KChn, *Fusarium moniliforme* Sheld and *Penicillium expansum* (Link) Thom were obtained from the Shaanxi Institute of Microorganism, Shaanxi Province, China. *Valsa mali* Miyabe et Yamada was provided by the State Key Laboratory of Crop Stress Biology for Arid Areas, Northwest A&F University, Yangling, Shanxi, China. *Fusarium oxysporum* Schlecht was provided by the Laboratory of Phytopathology, Inner Mongolia Agricultural University, Huhhot Inner Mongolia, China. All fungus were grown on PDA medium at 28 °C.

4.3. Isolation and purification of active compounds and structure analysis

The dried (100 g) stem bark of *M. officinalis* were ground into power and extracted three times with 90% Methanol. The resulted crude extract (1500 mL) was dried to paste and incubate with two times quicklime for 24 h. Washed three times using five times diluted hydrochloric acid and concentrated. After the concentrate step, the extracts were dissolved in chloroform and isolated with three times of alkaline water. The crystal was filtered from the solution with adjusting PH value to 7. After dried at a low temperature, the solution that was filtered from the crystal with petroleum for 1h was naturally cooled to room temperature, and the final product was analyzed by a liquid chromatography-mass spectrometry (LC-MS) (UltiMate3000 BioRS Systems, Thermo Fisher, Massachusetts, USA). The structures was identified by a Bruker Avance III spectrometer (Bruker, Billerica, MA, USA) (500 MHz for the frequency of ^1H and 125 MHz for ^{13}C in Norell tubes of 5 mm id).

4.4. The Inhibitory Efficiency of Magnolol and Honokiol Against 6 Fungal Pathogens

Antifungal activities of magnolol and honokiol were tested against *Alternaria alternate*, *Rhizoctonia solani* KChn, *Fusarium moniliforme* Sheld, *Penicillium expansum* (Link), *Valsa mali* Miyabe et Yamada and *Fusarium oxysporum* Schlecht. The pathogens were inoculated on the central of PDA plates containing magnolol (3mg/mL) and honokiol (1mg/mL) respectively and incubated at $25 \pm 2^\circ\text{C}$ for 3 days, the colony diameter measured. Each treatment was performed in triplicates. To calculate the percentage of mycelia growth inhibition (MGI), the following formula was used:

$$\text{Mycelium inhibition(\%)} = [(D_c - D_t) / D_c] \times 100$$

Where D_c (cm) and D_t (cm) is the mean colony diameter for the control and treatment sets, respectively.

4.5. The Determination of Minimum Inhibitory Concentration (MIC) and Minimal Fungicidal Concentration (MFC)

Effects of magnolol and honokiol on the mycelial growth of *A. alternate* were measured in vitro according to the agar dilution method [32]. Magnolol and honokiol (5 mL) were added to sterilized Petri dishes (90 mm diameter) to the concentrations of 0.001, 0.005, 0.01, 0.10, 1.00, 3.00, 5.00, 7.00 mg/mL, and mixed with PDA medium (15 mL). As the control, PDA dishes were added with the same volume of distilled water instead of magnolol and honokiol. A 6 mm diameter disc of *A. alternate* mycelia was inoculated into the center of plate. After incubation for 3 days at $25 \pm 2^\circ\text{C}$. The colony diameter was measured as described in 5.4. Each treatment was repeated for three times.

The lowest concentration that completely inhibited the growth of the fungus was considered the ~~minimum inhibitory concentration (MIC)~~. The ~~minimum fungicidal concentration (MFC)~~ was regarded as the lowest concentration that prevented growth of the pathogen after a following 72 h incubation at $28 \pm 2^\circ\text{C}$ in a fresh PDA plate, indicating more than 99.5% killing of the original inoculums [33].

4.6. Effects of Magnolol and Honokiol on Mycelium Growth of *A. alternate*

Seven day-old of *A. alternate* on PDA containing magnolol (3mg/mL) and honokiol (1mg/mL) were used for SEM observations. Cut about $7 \times 5 \times 3$ mm segments from the plates and washed three times using normal saline. After keeping the mycelium in 2.5% glutaraldehyde for 2 hours at 4°C , samples were washed with 0.1 mol/L phosphate buffer (pH 7.0) for four times. The samples were dehydrated in an ethanol series (30%, 50%, 70%, and 90%) for 20 min for each wash, and in absolute ethyl alcohol for 30 min. Mycelium were observed under a scanning electron microscope following dried in liquid CO_2 . All mycelium samples were views in a Nova NanoSEM 450 (FEI, America) operating at 5.00 kV of magnification.

4.7. Effects of Magnolol and Honokiol on Cell Membrane Permeability of *A. alternate*

The cell membrane permeability of *A. alternata* were detected referenced by Lee et al [34]. *A. alternata* suspension was incubated in a PDB medium and shaken at 27°C at 160 rpm for 48 h. Then magnolol (3mg/mL) and honokiol (1mg/mL) were added in to suspensions, 5 mL mixture solution was centrifuged at 10,000 rpm for 15 min. The supernatant conductivity was tested by conductometer in 0, 1, 2, 3, 4, 5, and 6 h, respectively. Each sample was repeated for three times.

4.8. Statistical Analysis

All data were presented as the mean $\pm$ SD by measuring four independent replicates. The data were analyzed using Data Processing System 15.10 (China). To test the significance of the differences between four means was applied using the Duncan's new complex range method at the 5% level.

5. Conclusions

In this study, magnolol and honokiol were purified from the *M. officinalis*, and identified by ^1H - and ^{13}C -NMR and HR-MS. Magnolol and honokiol showed an antifungal activity against *A. alternata*. The antifungal activity can be associated with the hypha distortion that resulted from the disruption of the cell membrane integrity. Therefore, magnolol and honokiol could be potentially used as antifungal agents against plant fungal pathogens.

Supplementary Materials: The HPLC chromatograms of magnolol and honokiol, and spectra of LC-MS, ^1H -, ^{13}C - and DEPT-NMR spectra of magnolol and honokiol are available in Supplementary Materials.

Author Contributions: For Yahan Chen, Meihuan Lu and Dongsheng Guo isolated and purified of active compounds and analyzed the structure; Dan Miao performed MIC and MFC test; Yingyan Zhai tested effects of magnolol and honokiol on mycelium; Jianying Yue and Chenhong Yuan analyzed effects of magnolol and honokiol on cell membrane permeability and the inhibitory against 6 fungal pathogens; Derong An and Mingmin Zhao designed the experiments and supervised the study. Mingmin Zhao and Yahan Chen wrote the manuscript.

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Sample Availability: Samples of the compounds are available from the authors.



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