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Antifungal activity of phenolic monoterpenes and structure-related compounds against plant pathogenic fungi

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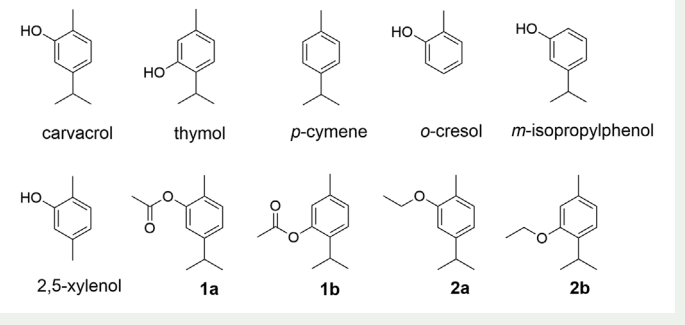
ABSTRACT

The aim of this work is to explore the possibility of using the phenolic monoterpenes (PMs) as leading compounds with antifungal activity against plant disease. The *in vitro* antifungal activities of carvacrol and thymol against seven kinds of plant pathogenic fungi were evaluated on mycelium growth rate method, and the results showed that carvacrol and thymol exhibited broad spectrum antifungal activity. Structure requirement for the antifungal activity of PMs was also investigated. The preliminary conclusion was that phenolic hydroxyl and monoterpene were basic structures for the antifungal activity of PMs, and the position of phenolic hydroxyl showed less effect. Ester derivatives of carvacrol and thymol were more effective than carvacrol and thymol against plant pathogenic fungi. We suggested that carvacrol, thymol and their ester derivatives could potentially be used as new fungicide leading compounds.



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Carvacrol; Thymol; Phenolic monoterpenes; Antifungal activity; Structure requirement

1. Introduction

The two phenolic monoterpenes (PMs), Carvacrol (2-methyl-5-(1-methylethyl)-phenol) and thymol (5-methyl-2-(1-methylethyl)-phenol), are widely found in aromatic plants such as genus *Origanum* (Stefanakis et al. 2013), *Thymus* (Porte and Godoy 2008), *Lippia*

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(Cervantesmartínez 2014) and *Satureja* (Yousefzadi et al. 2012; Wesolowska et al. 2015). PMs and the PMs-rich essential oils are generally considered to have good biological activities, such as antibacterial, antioxidant, antifungal, insecticidal and acaricidal activity (Friedman 2014; Suntres et al. 2015). The antimicrobial activity can potentially be applied to keep food fresh (Rattanachaikunsopon and Phumkhachorn 2010;), store fruits (Pérez-Alfonso et al. 2012) and inhibit fungal infection (Taha and Azeiz 2010). The PMs are generally produced as a chemical defence mechanism against phytopathogenic microorganisms and used as potential antifungal active ingredients against plant pathogen fungi (Vázquez et al. 2001; Soković et al. 2002; Numpaque et al. 2011).

It is reported that, the action mechanism of the antifungal effect of PMs may cause by impairing biosynthesis of ergosterin and disrupting membrane integrity of fungi (Ahmad et al. 2011), which are essential process to the vital activity of most pathogenic fungi. Therefore, it attracted our interests to study the inhibitory effect of PMs against plant pathogenic fungi in agriculture, and the antifungal activities of carvacrol and thymol were evaluated by mycelium growth rate method against seven species of fungi which cause widely damage to crops. In previous studies, the phenolic hydroxyl group of PMs is considered to be essential for their antibacterial activity (Ultee et al. 2002; Ben et al. 2006; Veldhuizen et al. 2006). Because of the different physiological regulatory mechanisms, the agent may show different effect and action mechanism against different microorganisms. Aim to confirm the structure requirement for the antifungal activity of PMs, we investigated the influence of removing or changing the substituting group of PMs on their antifungal activity.

In the present work, the hyphae growth inhibition ratios of PMs and their structure-related compounds (Figure 1) had been evaluated by mycelium growth rate method. Essential structure for the antifungal activity of PMs was preliminarily investigated by comparing the hyphae growth inhibition ratios of target compounds against each target pathogen, respectively.

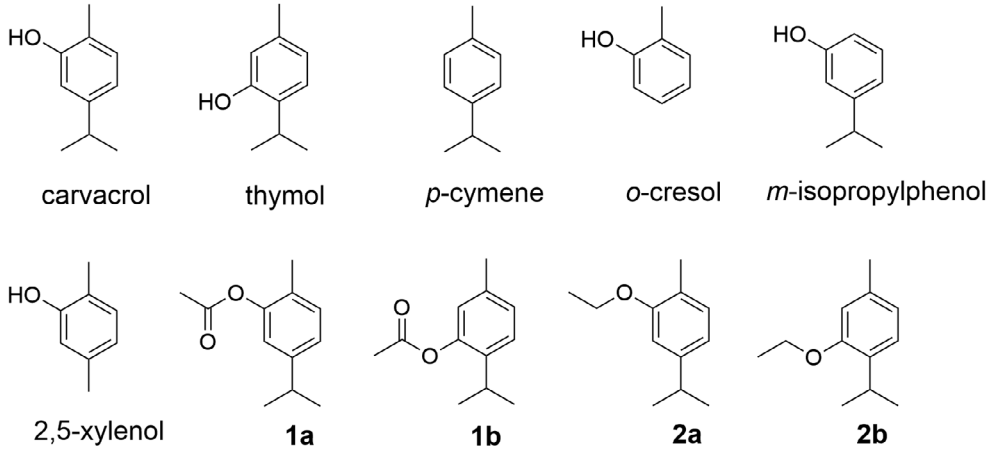

Figure 1. Chemical structures of PMs and structure-related compounds used in this study.

2. Results and discussion

2.1. Chemistry

The reaction route for synthesising ether and ester derivatives of PMs (compound **1a**, **1b**, **2a** and **2b**) is outlined in Figure 2. The preparations of **1a**-**1b** and **2a**-**2b** were performed according to the reported methods, respectively (Narkhede et al. 2008; Mathela et al. 2010). All the synthesised compounds were identified on the basis of ^{1}H-NMR,^{13}C-NMR and HR-ESI-MS analyses.

2.2. Antifungal activity

The hyphae growth inhibition ratios of carvacrol and thymol against seven kinds of fungi are listed in Table 1. The two PMs, carvacrol and thymol, played a similar role in antifungal activity against target pathogens. The inhibition ratios of the two PMs against target pathogens at high concentration were significantly higher than that at low concentration. And just as predicted, the two PMs showed broad antifungal spectrum, they had various degrees of inhibition effect against both Oomycetes (*Phytophthora capsici* and *Phytophthora nicotianae*) and Hyphomycetes (*Alternaria solani*, *Botrytis cinerea*, *Fusarium oxysporum*, *Pyricularia grisea*, and *Rhizoctonia solani*). And, the two PMs showed rather good antifungal activity against *B. cinerea* and *R. solani*, the inhibition ratio of carvacrol was higher than 90% against *B. cinerea* at 50 μg mL^{-1}, which was equal to the commercial fungicide chlorothaloni.

The inhibition ratios of PMs structure-related compounds (*p*-cymene, *o*-cresol, *m*-isopropylphenol, 2,5-xylenol) and four synthesised compounds against five kinds of Hyphomycetes are listed in Table 2. The compounds without methyl or isopropyl groups (*o*-cresol, *m*-isopropylphenol and 2,5-xylenol) showed lower antifungal activity than carvacrol and thymol. *P*-cymene, a monoterpene without hydroxyl group, was also lower effective than carvacrol and thymol. The antifungal activity of PMs had been changed by introducing substituent groups to their phenolic hydroxyl groups. As generally observed, ester derivatives (compound **1a** and **1b**) showed higher inhibition effect against target pathogens, and ether derivatives (compound **2a** and **2b**) showed negative effects against target pathogens. The hyphae growth inhibition ratios of compound **1a** and **1b** against *B. cinerea* and *R. solani* were higher than 90% at 50 μg mL^{-1}, which were equal to or better than the commercial fungicide chlorothaloni.

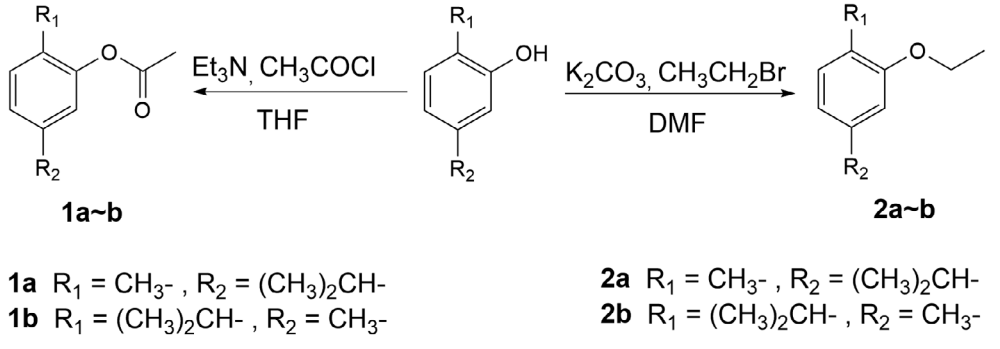

Figure 2. Synthesis of carvacrol and thymol ether and ester derivatives.

Table 1. Antifungal activity of carvacrol and thymol against seven kinds of fungi.

Compounds	Concentration(μg·mL^{-1})	Hyphae growth inhibition ratios (%) (n = 3)						
		A. solani	*B. cinerea*	*F. oxysporum*	*P. grisea*	*P. capsici*	*P. nicotianae*	*R. solani*
Carvacrol	10	9.59 ± 2.46	51.92 ± 1.70	3.08 ± 1.06	2.94 ± 0.66	−2.02 ± 1.72	−1.04 ± 0.35	39.20 ± 4.94
	50	60.42 ± 2.75	87.93 ± 2.23	42.72 ± 0.57	26.68 ± 2.38	37.30 ± 2.03	32.60 ± 1.15	78.76 ± 1.98
Thymol	10	8.44 ± 1.89	43.46 ± 6.20	1.29 ± 0.94	0.81 ± 0.73	8.04 ± 0.38	4.38 ± 3.08	26.60 ± 2.25
	50	50.73 ± 1.53	**90.50 ± 1.31**	41.50 ± 2.66	34.90 ± 3.39	42.95 ± 2.72	41.03 ± 1.63	88.21 ± 1.65
Chlorothalonil*	10	57.89 ± 0.87	60.77 ± 1.77	45.13 ± 2.49	50.53 ± 1.16	31.07 ± 5.47	34.20 ± 4.55	**95.14 ± 0.80**
	50	72.44 ± 2.97	76.65 ± 1.67	50.63 ± 4.26	78.90 ± 0.86	62.78 ± 4.26	57.55 ± 2.74	**97.22 ± 1.35**

Note: The compound which showed higher inhibition ratio were highlighted in bold.
*Chlorothalonil were used as the positive controls.

Table 2. Antifungal activity of PMs structure-related compounds against five kinds of Hyphomycetes.

Compounds	Concentration (μg·mL^{-1})	Hyphae growth inhibition ratios (%) (n = 3)				
		A. solani	*B. cinerea*	*F. oxysporum*	*P. grisea*	*R. solani*
p-cymene	10	9.68 ± 0.94	8.12 ± 7.69	12.08 ± 1.73	9.24 ± 5.79	0.46 ± 3.99
	50	10.22 ± 3.85	11.29 ± 9.62	19.93 ± 9.97	12.74 ± 3.40	4.78 ± 5.48
o-cresol	10	0.25 ± 1.76	10.99 ± 1.23	12.89 ± 0.67	0.53 ± 2.75	15.36 ± 5.47
	50	0.51 ± 2.24	17.26 ± 3.08	27.17 ± 13.04	12.18 ± 0.08	73.99 ± 1.03
m-isopropyl-phenol	10	−0.38 ± 0.38	18.12 ± 3.32	0.71 ± 4.47	3.25 ± 3.62	19.16 ± 8.24
	50	28.70 ± 1.51	88.48 ± 0.75	36.28 ± 2.42	5.01 ± 0.45	76.24 ± 1.33
2,5-xylenol	10	0.63 ± 2.72	39.51 ± 8.15	2.86 ± 0.01	5.54 ± 1.99	52.41 ± 4.90
	50	1.15 ± 0.38	39.86 ± 4.53	6.44 ± 2.14	26.81 ± 4.37	73.13 ± 2.25
1a	10	15.20 ± 4.81	42.63 ± 10.94	10.37 ± 1.01	24.94 ± 0.99	44.10 ± 12.23
	50	67.88 ± 8.13	**100.00 ± 0.00**	41.60 ± 4.25	58.10 ± 3.57	**96.30 ± 3.57**
1b	10	12.14 ± 4.47	46.15 ± 1.33	1.51 ± 1.94	5.45 ± 3.59	37.77 ± 12.00
	50	62.22 ± 9.87	**99.63 ± 0.64**	35.54 ± 6.25	48.83 ± 3.26	**96.65 ± 3.07**
2a	10	3.14 ± 4.07	3.00 ± 0.35	0.64 ± 2.27	0.37 ± 1.87	3.98 ± 2.08
	50	3.78 ± 2.72	22.17 ± 6.06	3.53 ± 0.73	2.87 ± 2.49	20.30 ± 2.60
2b	10	4.38 ± 2.84	2.17 ± 1.25	7.77 ± 4.57	0.95 ± 1.71	2.38 ± 4.76
	50	3.79 ± 1.23	2.35 ± 1.54	12.43 ± 5.42	1.83 ± 0.61	15.33 ± 4.06
Chlorothalonil*	10	57.89 ± 0.87	60.77 ± 1.77	45.13 ± 2.49	50.53 ± 1.16	**95.14 ± 0.80**
	50	72.44 ± 2.97	76.65 ± 1.67	50.63 ± 4.26	78.90 ± 0.86	**97.22 ± 1.35**

Note: The compound which showed higher inhibition ratio were highlighted in bold.
*Chlorothalonil were used as the positive controls.

The *in vitro* antifungal activities of PMs and their ester derivatives were equal to or better than the commercial fungicide chlorothalonil against *B. cinerea* and *R. solani*. We suggested that carvacrol, thymol and their ester derivatives could potentially be used as new fungicide leading compounds. The earliest idea of choosing both Oomycetes and Hyphomycetes as target fungi was to make a more reliable conclusion for the antifungal spectrum of PMs. Instead, the two PMs showed antifungal effect against two kinds of Oomycetes. As is well known, ergosterol biosynthesis is absent in physiological progress of Oomycetes. The results are conflicted with the conclusion in the study of PMs against several resistant *Candida* strains (Ahmad et al. 2011). Past studies have also explained the mechanisms of PMs inhibit fungi growth by studying their effect on TOR (Target of Rapamycin) pathway (Rao et al. 2010). Additional antifungal assay and physiological experiment will be considered to investigate the specific mechanism of PMs on fungi growth in our further study.

In this study, we determined phenolic hydroxyl and alkyl group of PMs were essential for their antifungal activity and the position of phenolic hydroxyl on benzene ring showed less effect. Previous studies suggested that hydroxyl and alkyl group of PMs provide appropriate hydrophobicity, and the hydroxyl group is essential for their hydrogen bonding and proton-release ability which are important for their ability to cause the cell death (Ultee et al. 2002; Ben et al. 2006), and it is reported that thymol ester derivatives show higher activity in comparison to thymol, whereas the carvacrol ester derivatives are much less active than carvacrol (Mathela et al. 2010). However, the antifungal assay results were different in our study, ester derivatives of PMs without a free hydroxyl group were even more effective than their parent molecule. While we could come to just a preliminary conclusion according to limited compounds, more derivatives of PMs should be synthesised to make a completely structure–activity relationships study.

3. Experimental section

3.1. Antifungal assays

The *in vitro* antifungal activities of target compounds were determined by the mycelium growth rate method according to the methods described in paper (Kim et al. 2003). The panel of microorganisms included five kinds of Hyphomycetes (*A. solani*, *B. cinerea*, *F. oxysporum*, *P. grisea*, and *R. solani*) and two kinds of Oomycetes (*P. capsici* and *P. nicotianae*). These strains were separately maintained on Potato Dextrose Agar (PDA) or Carrot Decoction Agar (CA) slants at −10 °C. All of the strains used for antifungal activity assays were incubated on *Petri* dishes containing the PDA medium in advance. Each test compounds were dissolved in acetone, and the result solution was added to PDA flat-medium (1/100, $V_{acetone}/V_{PDA}$) at 40~60 °C, and then cooled to room temperature to prepare the poisoned medium. At the same time, medium supplemented with equivalent quantities of acetone were set up as control, and chlorothalonil was used as a positive control group. Afterwards, a mycelium agar disc (5 mm diameter) of the tested strains were inoculated to poisoned and non-poisoned medium, and all *Petri* dishes were incubated at constant temperature of 25~26 °C in dark, each compound was repeated three times.

$$\text{Percentage mycelial inhibition}n = [(C - T)/C] \times 100$$

Where C is the mean colony diameter for the control sets and T is the mean colony diameter for the treatment sets. The data from each part were analysed using percentage, mean, standard deviation ($n = 3$) statistical methods based on the IBM SPSS statistics 22.0 software.

3.2. Chemistry

The reagents (carvacrol, thymol, *p*-cymene, *o*-cresol, *m*-isopropylphenol, 2,5-xylenol) and solvents for reaction were purchased from Aladdin (China) or Sinopharm Chemical Reagent Co., Ltd (China). Solvents for extraction and chromatography were of technical grade and distilled prior to use. Reactions were monitored by thin layer chromatography (TLC) using silica gel coated glass slides (silica gel 60 GF 254, Qingdao Haiyang Chemical, China). Detections were conducted under UV (254 nm). ^{1}H- and ^{13}C-NMR spectra were recorded on a Bruker Avance III 500 NMR spectrometer. The chemical shifts (δ) were reported in ppm with reference to internal TMS, and coupling constants (J) were given in Hz. ESI-MS spectra were recorded on a Thermo Fisher Scientific TSQ Endura MS.

3.2.1. Synthesis of 5-isopropyl-2-methylphenyl acetate (1a)

Acetyl chloride (86.35 mg, 1.1 mmol, 1.1 equiv) in 1.1 mL THF was added to a solution of carvacrol (150 mg, 1 mmol, 1.0 equiv) and triethylamine (111 mg, 1.1 mmol, 1.1 equiv) in anhydrous THF (1 mL) over 1 min at 0 °C. The reaction mixture was stirred at 0 °C for 15 min and then at room temperature for 3 h. After the reaction was completed, 10 mL of water was added to the reaction mixture, and extracted with EtOAC (2 × 25 mL). The organic layer was washed with water, and dried over anhydrous sodium sulphate. The solvent was evaporated *in vacuo*, and the crude product was purified by silica gel colunm chromatography (Petroleum ether : EtOAC = 10:1) to give compound **1a** in 86% yield.

3.2.2. Synthesis of 2-ethoxy-4-isopropyl-1-methylbenzene (2a)

To a solution of carvacrol (150 mg, 1 mmol, 1.0 equiv) in DMF (2 mL) potassium carbonate (207 mg, 1.5 mmol, 1.5 equiv) and bromoethane (130 mg, 1.2 mmol, 1.2 equiv) were added. The reaction mixture was stirred at room temperature for 10 h. The progress of reaction was monitored by TLC. After completion, 10 mL of water was added to the reaction mixture, and extracted with EtOAC (3 × 25 mL). The organic layer was washed with water, brine and dried over anhydrous sodium sulphate. The solvent was evaporated *in vacuo*, and the crude product was purified by silica gel colunm chromatography (Petroleum ether : EtOAC = 10:1) to give compound **2a** in 90% yield.

Ester and ether derivatives of thymol (Compound **1b** and **2b)** were prepared as described for compound **1a** and **2a**, with yields of 93% and 88%, respectively.

4. Conclusions

The antifungal activities of PMs and their structure-related compounds against several plant pathogenic fungi were tested by mycelium growth rate method. We suggested that carvacrol, thymol and their ester derivatives could potentially be used as new fungicide leading compounds. We determined hydroxyl and alkyl group of PMs were essential for their antifungal activity. It was important for synthetic research work of PMs derivatives to get higher antifungal activity compounds.

Supplementary material

Spectral data relating to this article can be found in the online versions as Appendix.

Disclosure statement

No potential conflict of interest was reported by the authors.

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