Research Article

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Discovery of novel piperidine-containing thymol derivatives as potent antifungal agents for crop protection

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Abstract

BACKGROUND: Plant fungal diseases pose a significant threat to crop production. The extensive use of chemical pesticides has led to growing environmental safety risks and pesticide resistance of various plant pathogens. Therefore, it is an urgent task to explore novel eco-friendly fungicidal agents with high efficacy to combat fungal infection.

RESULTS: In this study, we rationally designed a series of novel thymol derivatives by incorporation of the sulfonamide moiety and evaluated their biological activities against plant pathogenic fungi. The bioassay results underscored the remarkable *in vitro* antifungal activity of compounds 5m **and** 5t **against *Phytophthora capsici* (*P. capsici*), with EC_{50} values of 8.420 and 8.414 μg/mL, respectively. Their efficacies were superior to that of widely used commercial fungicides azoxystrobin (AZO, 20.649 μg/mL) and cabendazim (CAB, 251.625 μg/mL). Furthermore, compound** 5v **exhibited excellent *in vitro* antifungal activity against *Sclerotinia sclerotiorum* (*S. sclerotiorum*), with an EC_{50} value of 12.829 μg/mL, significantly outperforming AZO (63.629 μg/mL). *In vivo* bioassays demonstrated the impactful activity of compound** 5v **against *S. sclerotiorum*, achieving over 98% curative and protective efficacies at the concentration of 200 μg/mL. Further mechanistic investigations unveiled that compound** 5v **induced mycelial shrinkage and collapse in *S. sclerotiorum*, resulting in organelle damage and the accumulation of antioxidant enzyme activity.**

CONCLUSION: The significant antifungal efficacy of the prepared thymol derivatives shall encourage further exploration of compound 5v **as a promising candidate to develop novel fungicides for crop protection.**

Supporting information may be found in the online version of this article.

Keywords: antifungal activity; *P. Capsici*; *S. Sclerotiorum*; thymol derivatives; sulfonamide moiety

1 INTRODUCTION

The control of plant fungal diseases is of continuing significance in crop protection as these plant diseases can lead to a dramatic decline in both crop quality and yield, resulting in global losses of approximately 30%.[1] For instance, the oomycete pathogen *Phytophthora capsici* (*P. capsici*) is susceptible to infect pepper, a worldwide cultivated commercial crop, during both the seedling and fruit maturation period.[2,3] To date, there is no satisfactory strategy to realize highly effective control of *P. capsici* from spreading once pepper was infected. *Sclerotinia sclerotiorum* (*S. sclerotiorum*), a necrotic homologous fungus belonging to *Ascomycetes*, poses a significant threat to both the production and quality of oilseed rape.[4,5] Various solutions, including physical, chemical, and biological strategies, have been employed to protect crops from diverse fungi.[6,7] In particular, the chemical control method has notably contributed to modern plant disease management.[8–14] However, the extensive use of chemical pesticides has also led to increasing environmental safety risks and resistance of various plant pathogens.[15–17] Therefore, the continuing exploration of novel eco-friendly fungicidal agents with high efficacy to combat fungal infection remains to be a growing demand and of profound importance for crop protection.

Structural modification and molecular optimization on natural products have emerged as an attractive approach for the development of medicines and agrochemicals owing to their high efficiency, low toxicity, and broad-spectrum biological activity.[18–28] In this context, thyme has been broadly used as a flavor in foods and traditional herbal medicines for a long time.[29] It consistent

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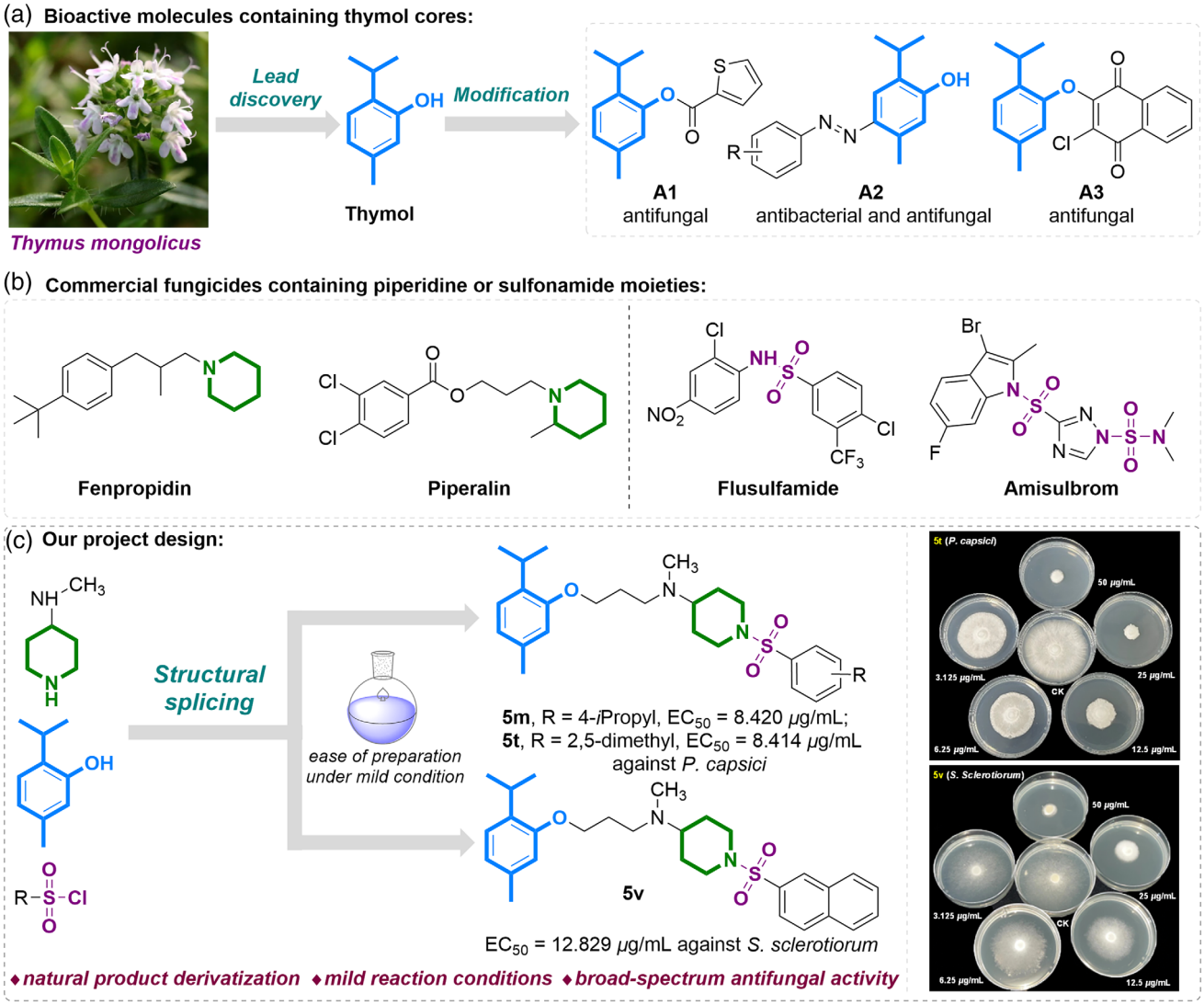


Figure 1. Representative bioactive molecules and fungicides containing thymol/piperidine/ sulfonamide cores and our project design.

with a large number of biologically intriguing terpenoids, glycosides, flavonoids as well as various phenolic acids that offer a striking opportunity for the discovery of novel lead compounds for pesticide development. Thymol (known as 2-isopropyl-5-methylphenol) is one of the major components of thyme species that are extracted from the *Lamiaceae* family plants, such as the genera of *Thymus*, *Ocimum*, *Origanuma* and others.[30] As a class of monoterpene phenol, thymol and its derivatives feature a diversity of biological activities including antimicrobial, antioxidant and anticancer properties.[31–35] For instance, studies have shown that compound **A1** exhibits both antibacterial and antifungal activities against gruesome human pathogenic bacteria and fungi in the medical field,[33] while compound **A2** shows 100% inhibitory activity against *Pyricularia oryzae* (*P. oryzae*) *in vitro* at 50 μg/mL (Fig. 1(a)).[34] Consequently, the versatile biological activities featured by thymol derivatives underscores the vast potential for development of novel antifungal pesticides.

On the other hand, highly effective fungicides featuring piperidine fragments or sulfonamide moieties continue to emerge,[36–40] as exemplified by an array of commercially available fungicides on the market such as fenpropidin, piperalin, flusulfamide and amisulbrom (Fig. 1(b)).[41] Here, we designed a series of novel antifungal chemicals by the substructural splicing of thymol, piperidine and sulfonamide cores, with thymol used as the lead structure (Fig. 1(c)). The target thymol derivatives were readily prepared through a 4-step sequence under mild reaction conditions. *In vitro* studies revealed that most of the compounds exhibit notable inhibition activity against a diverse array of plant pathogenic fungi, and the structure–activity relationship (SAR) was preliminary studied. In addition, we systematically explored the EC_{50} values and *in vivo* antifungal activities of the highly active compounds. Furthermore, studies by employment of scanning electron microscope (SEM), transmission electron microscope (TEM), and enzyme activity analyses shed primary insights on the inhibition mechanism against *S. sclerotiorum* of the hit compound.

2 MATERIALS AND METHODS

2.1 Chemicals and instruments

Commercially available materials and solvents were purchased from Energy Chemical, Aladdin, and J&K, which were used directly without additional purification. NMR spectra were acquired using

a Bruker ASCEND (AVANCE III HD 400 MHz) spectrometer. High-resolution mass spectrometer (HRMS) analysis was conducted with the Thermo Fisher Q Exactive mass spectrometer (QTOF mass analyzer). Melting points were determined on an uncorrected Beijing Tech Instrument X-4 digital display micro-melting point apparatus.

2.2 General procedure for the synthesis of compounds 5a-5y

According to the previous reports,[42,43] the target compounds **5a-5y** were synthesized from thymol through a four-step sequence. The detailed synthetic procedures were provided in the Supporting Information.

2.3 *In vitro* antifungal bioassays

The *in vitro* antifungal activity of the target compounds **5a-5y** against six plant pathogens was assessed with the mycelial growth inhibition method[44] at the concentration of 50 μg/mL. The studied plant pathogens included *Phytophthora capsici* (*P. capsici*), *Aspergillus flavus* (*A. flavus*), *Rhizoctonia solani* (*R. solani*), *Sclerotinia sclerotiorum* (*S. sclerotiorum*), *Botryosphaeria dothidea* (*B. dothidea*), *Pyricularia grisea* (*P. grisea*). The EC_{50} (50% maximal effective concentration) values were calculated based on the antifungal activities of the target compounds at concentrations of 50, 25, 12.5, 6.25 and 3.125 μg/mL, respectively. For detailed experimental procedures, please refer to the Supporting Information.

2.4 *In vivo* antifungal bioassays

According to the previous studies,[45] the curative and protective effects of compound **5v** against *S. sclerotiorum* on oilseed rape leaves were assessed at concentrations of 200 and 100 μg/mL, respectively. Azoxystrobin (AZO) was employed as the positive control. For comprehensive testing methods, please refer to the details provided in the Supporting Information.

2.5 Scanning electron microscopy (SEM)

SEM observations on the hyphae of *S. sclerotiorum* were conducted following established procedures.[46,47] The hyphae of *S. sclerotiorum* were treated with DMSO or compound **5v** (50 and 100 μg/mL) to prepare the testing samples. The scanning electron microscope (Hitachi, SU8100, Japan) was used for observation. The detailed preparing methods were provided in the Supporting Information.

2.6 Transmission electron microscopy (TEM)

TEM observations on the morphology of *S. sclerotiorum* were carried out according to the reported methods.[10,48] The morphology of *S. sclerotiorum* were treated with DMSO or compound **5v** (13 μg/mL) to prepare the testing samples. The transmission electron microscope (Hitachi, HT7800/HT7700, Japan) was used for observation. The detailed preparing methods were provided in the Supporting Information.

2.7 Enzyme activity assays

The test boxes for peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) were procured from Suzhou Comin Biotechnology Company, China. Following previously reported procedures,[49,50] ten new mycelial blocks (diameter = 5 mm) of *S. sclerotiorum* were cultured in 100 mL PDB medium at 24 ± 1 °C and 130 rpm for 3 days. Subsequently, these samples were respectively treated with compound **5v** (dissolved in DMSO) at concentrations of 50, 25, 12.5, 6.25, and 3.125 μg/mL, with three parallel treatments for each concentration. The resulting cultures were then continued under the same conditions for an additional 72 h. Afterward, the mycelium of *S. sclerotiorum* was extracted, washed three times with sterile water, and then dried with filter paper. Subsequently, 0.10 g of mycelium was weighed for the subsequent experiments. The enzyme activity of compound **5v** was measured following the kit instructions.

3 RESULTS AND DISCUSSION

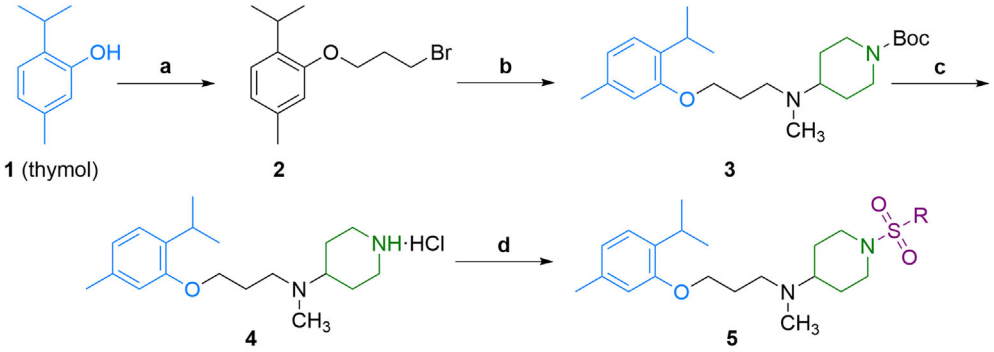
3.1 Synthesis

The target compounds **5a-5y** were synthesized from thymol through a four-step reaction sequence (Scheme 1). The synthetic pathway involved the following steps: (i) Substitution reaction of thymol (**1**) and 1,3-dibromopropane in the presence of K_2CO_3 to yield 2-(3-bromopropoxy)-1-isopropyl-4-methylbenzene (**2**); (ii) Substitution reaction between 2-(3-bromopropoxy)-1-isopropyl-4-methylbenzene (**2**) and *tert*-butyl 4-(methylamino)piperidine-1-carboxylate under basic conditions, resulting in the formation of *tert*-butyl 4-((3-(2-isopropyl-5-methylphenoxy)propyl)(methyl)amino)piperidine-1-carboxylate (**3**); (iii) Deprotection of the intermediate (**3**) under acidic condition to provide *N*-(3-(2-isopropyl-5-methylphenoxy)propyl)-*N*-methylpiperidin-4-amine hydrochloride (**4**); (iv) Final substitution reaction of (**4**) with various sulfonyl chlorides to yield the target compounds **5a-5y**. The structures of all final products were characterized by NMR and HRMS, and the corresponding characterization data was provided in the Supporting Information.

3.2 *In vitro* antifungal activity and SAR analysis

The *in vitro* antifungal activities of target compounds **5a-5y** against six plant pathogens at the concentration of 50 μg/mL were evaluated and summarized, as presented in Table 1. Bioassay results revealed that most of the prepared thymol derivatives exhibit notable antifungal activities. Particularly, compounds **5b**, **5h**, **5i** and **5v** showed notable *in vitro* antifungal activities against *P. capsici*, *A. flavus* and *S. sclerotiorum*. Moreover, compounds **5c** and **5t** displayed good *in vitro* antifungal activities against *A. flavus*, *R. solani*, and *B. dothidea*. Noteworthily, compounds **5h**, **5m** and **5t** exhibited excellent *in vitro* antifungal activities against *P. capsici* at the concentration of 50 μg/mL, with inhibition rates of 90.1, 91.5 and 92.1%, respectively, which were superior to the commercial fungicides AZO (63.7%) and CAB (19.6%). Meanwhile, compounds **5L**, **5n** and **5v** also showed good inhibition rates (84.9, 84.3 and 82.6%, respectively) against *P. capsici* at 50 μg/mL. It is worth noting that the reaction intermediates including **2**–**4** were also evaluated for their *in vitro* antifungal activities against six plant pathogens. Interestingly, with the introduction of piperidine group, the antifungal activity of these compounds was improved to varying degrees. However, the *in vitro* antifungal activities of the intermediates **2**–**4** were inferior to those of most of the target compounds.

Based on the preliminary bioassay results, the EC_{50} values of several target compounds with notable *in vitro* antifungal activities were determined (Table 2). The EC_{50} values for compounds **5m** and **5t** against *P. capsici* were 8.420 and 8.414 μg/mL, respectively, outperforming the positive controls AZO (20.649 μg/mL) and CAB (251.625 μg/mL). Additionally, the EC_{50} values of compounds **5h**, **5q**, and **5t** ranged from 8.853 to 21.954 μg/mL, demonstrating effective *in vitro* antifungal activities against *A. flavus*



Prepared compounds library

5a: R = C_6H_5;
5b: R = 2-CH_3-C_6H_4;
5c: R = 2-Cl-C_6H_4;
5d: R = 2-F-C_6H_4;
5e: R = 2-CF_3-C_6H_4;
5f: R = 3-CH_3-C_6H_4;
5g: R = 3-F-C_6H_4;
5h: R = 4-Cl-C_6H_4;
5i: R = 4-Br-C_6H_4;
5j: R = 4-NO_2-C_6H_4;
5k: R = 4-CN-C_6H_4;
5l: R = 4-CF_3-C_6H_4;
5m: R = 4-*i*Propyl-C_6H_4;
5n: R = 4-*t*Butyl-C_6H_4;
5o: R = 4-OCH_3-C_6H_4;
5p: R = 2-NO_2-4-CF_3-C_6H_3;
5q: R = 2-NO_2-4-OCH_3-C_6H_3;
5r: R = 2,4-dinitro-C_6H_3;
5s: R = 3-CF_3-4-Cl-C_6H_3;
5t: R = 2,5-dimethyl-C_6H_3;
5u: R = 2,4,6-trimethyl-C_6H_2;
5v: R = 2-naphthyl;
5w: R = 2-thienyl;
5x: R = 3-pyridyl;
5y: R = *N*,*N*-dimethylamino

Scheme 1. General synthetic routes of target compounds **5a**-**5y**. Reagents and reaction conditions: (a) 1,3-dibromopropane, K_2CO_3, acetone, reflux; (b) 1-Boc-4-methyl-laminopiperidine, K_2CO_3, CH_3CN, rt; (c) HCl, CH_3OH, rt; (d) RSO_2Cl, Et_3N, CH_2Cl_2, rt.

compared to CAB (28.283 μg/mL). Noteworthy, the EC_{50} value of compound **5v** against *S. sclerotiorum* was 12.829 μg/mL, which was significantly better than that of AZO (63.629 μg/mL). The experimental results indicated that compounds **5m** and **5t** exhibited the best *in vitro* antifungal activities against *P. capsici*, while compound **5v** demonstrated the best antifungal activity against *S. sclerotiorum*.

The SAR analysis revealed a significant impact of different substituents on the antifungal activity. Initially, compounds **5a**-**5e** were synthesized, demonstrating that electron-donating groups (CH_3, **5b**) at the 2-position of the phenyl ring, exhibited superior antifungal activity compared to electron-withdrawing groups (**5c**, **5d**, **5e**) and hydrogen atom (**5a**). Subsequently, we investigated the influence of substituents at the 3-position of the benzene ring on antifungal activity. The experimental results indicated that an electron-withdrawing group (F, **5g**) at 3-position of benzene ring exhibited higher antifungal activity than an electron-donating group (CH_3, **5f**). Despite these findings, the antifungal activity did not meet our desired levels, prompting the synthesis of target compounds with different substituents on the benzene ring (**5h**-**5o**). The antifungal activity was improved when a Cl atom was installed on the 4-position of benzene ring (**5 h**). Additionally, when the R substituent was the electron-donating isopropyl group (**5m**), its antifungal activity surpassed that of the tert-butyl group (**5n**) and OCH_3 (**5o**), although it was lower than that of compound **5h** (Cl). Further exploration focused on the antifungal activity of multiple substitution sites on the benzene ring, leading to the synthesis of compounds **5p**-**5u**. Notably, the antifungal activity peaked when electron-donating groups were present at the 2- and 5-positions of the benzene ring (2,5-dimethyl, **5t**), outperforming other substitution sites and electron-withdrawing groups. Considering the potential for enhancing antifungal activity, we explored the substitution of the benzene ring with alternative aryl or heterocyclic rings, resulting in the synthesis of compounds **5v**-**5x**. Remarkably, R substituents with 2-naphthyl (**5v**) demonstrated outstanding antifungal activity, surpassing 2-thienyl (**5w**) and 3-pyridyl (**5x**). Lastly, inspired by the sulfa structure of commercial fungicides like amisulbrom, we synthesized compound **5y** (*N*,*N*-dimethylamino). Unfortunately, this compound exhibited low antifungal activity.

In brief, when the R substituent is a benzene ring, antifungal activity was optimal with an electron-donating substituent at the 4-position, surpassing both electron-donating substituents at the 3- and 2-positions, as well as electron-withdrawing substituents at the same positions. Moreover, the benzene ring with a 2,5-dimethyl (**5t**) substituent showed the highest antifungal activity. When the R substituent is 2-naphthyl (**5v**), the antifungal activity was better than that of other aryl and heterocyclic groups. Future studies will be carried out for in-depth exploration of the antifungal activities of different naphthyl substituted derivatives.

3.3 *In vivo* antifungal activity

We selected the compound **5v** with the best *in vitro* antifungal activity to explore the control efficiency against *S. sclerotiorum* on the leaves of oilseed rape. As shown in Table 3 and Fig. 2, compound **5v** exhibited outstanding curative and protective efficacies against rape sclerotinia stem at the concentration of 200 μg/mL, with inhibition rates of 99.2 and 98.3%, respectively. These results were comparable to the performance of the commercial fungicide AZO, which exhibited 100% inhibition for both curative and protective activities. Good curative and protective activities (78.8 and 72.9%, respectively) were still observed when the concentration of the compound **5v** was decreased to 100 μg/mL, wherein the commercial fungicide AZO maintained high efficacy in both categories. Therefore, compound **5v** could be considered as a promising candidate in the development of new class of fungicides for protection and curation of *S. sclerotiorum* on oilseed rape.

3.4 Scanning electron microscopy (SEM)

To further investigate the impact of compound **5v** on the mycelial morphology of *S. sclerotiorum*, SEM observations were conducted

Table 1. *In vitro* antifungal activities of target compounds **5a**-**5y** at 50 μg/mL*

Compounds	Inhibition rate (%) ± SD (n = 3)					
	Pc	*Af*	*Rs*	*Ss*	*Bd*	*Pg*
5a	71.8 ± 1.8	51.8 ± 3.4	38.5 ± 0.5	19.5 ± 1.1	19.1 ± 1.9	36.0 ± 0.8
5b	63.3 ± 0.4	75.9 ± 0.3	61.0 ± 1.0	65.0 ± 3.2	32.9 ± 1.8	51.4 ± 0.3
5c	3.5 ± 0.9	70.7 ± 0.4	43.0 ± 0.4	78.0 ± 2.2	66.9 ± 2.5	47.7 ± 0.2
5d	77.6 ± 1.5	27.5 ± 0.4	31.2 ± 0.6	13.4 ± 2.1	31.8 ± 2.4	25.2 ± 0.5
5e	52.3 ± 3.1	75.2 ± 0.8	48.7 ± 0.4	55.0 ± 2.9	71.9 ± 0.7	65.5 ± 1.4
5f	32.0 ± 2.2	36.7 ± 2.4	12.7 ± 2.6	55.4 ± 1.8	32.4 ± 2.6	10.1 ± 0.3
5g	57.5 ± 1.5	53.6 ± 3.6	32.9 ± 1.9	22.1 ± 3.7	39.2 ± 3.4	35.4 ± 1.4
5h	90.1 ± 0.5	**99.5 ± 0.1**	18.0 ± 2.5	65.2 ± 4.9	**73.5 ± 0.3**	47.3 ± 0.4
5i	74.1 ± 1.1	63.8 ± 0.4	46.9 ± 2.6	83.6 ± 1.0	39.2 ± 1.8	49.5 ± 0.5
5j	30.7 ± 1.8	22.2 ± 0.3	6.1 ± 1.3	16.5 ± 0.7	21.0 ± 1.7	15.4 ± 0.5
5k	34.2 ± 0.9	12.8 ± 0.8	3.8 ± 2.5	60.7 ± 0.5	32.0 ± 6.3	11.1 ± 0.6
5L	84.9 ± 1.5	41.2 ± 3.3	40.0 ± 1.4	77.9 ± 0.5	30.2 ± 3.1	43.0 ± 0.3
5m	91.5 ± 0.1	61.9 ± 0.9	52.6 ± 1.5	10.7 ± 3.8	34.6 ± 1.6	**66.6 ± 0.3**
5n	84.3 ± 0.6	15.3 ± 1.7	22.1 ± 2.0	27.6 ± 2.7	28.4 ± 4.2	51.8 ± 0.4
5o	58.4 ± 0.9	74.7 ± 1.0	45.1 ± 0.5	47.8 ± 3.3	51.5 ± 1.7	31.4 ± 0.3
5p	34.9 ± 2.8	28.7 ± 0.1	15.0 ± 1.0	58.9 ± 1.0	27.7 ± 0.3	32.6 ± 1.2
5q	52.4 ± 6.4	94.4 ± 0.1	32.7 ± 4.4	0.9 ± 4.7	54.4 ± 0.9	28.2 ± 0.5
5r	20.8 ± 0.8	44.3 ± 2.5	50.5 ± 0.7	17.4 ± 2.8	55.8 ± 1.8	18.0 ± 0.3
5s	67.8 ± 1.4	20.6 ± 0.4	17.3 ± 4.6	67.4 ± 0.5	25.5 ± 1.4	49.4 ± 2.5
5t	**92.1 ± 0.9**	86.6 ± 0.5	**67.1 ± 1.0**	39.1 ± 2.6	70.6 ± 0.8	49.8 ± 0.6
5u	37.5 ± 1.9	36.8 ± 0.8	66.0 ± 0.3	59.1 ± 1.6	53.2 ± 1.6	44.4 ± 0.9
5v	82.6 ± 0.1	69.7 ± 3.6	57.2 ± 2.3	**87.7 ± 0.3**	45.7 ± 2.2	51.6 ± 0.2
5w	12.4 ± 1.9	34.5 ± 0.8	30.0 ± 0.9	29.5 ± 0.4	10.6 ± 1.2	17.5 ± 0.2
5x	0.0 ± 0.0	5.4 ± 0.8	9.4 ± 3.8	24.5 ± 0.4	9.5 ± 2.5	7.1 ± 0.5
5y	9.2 ± 2.7	7.3 ± 2.6	0.0 ± 0.0	9.5 ± 1.0	39.8 ± 1.1	3.9 ± 0.1
thymol	10.8 ± 1.9	37.8 ± 2.7	59.0 ± 0.7	0.0 ± 0.0	66.4 ± 1.6	13.3 ± 0.3
2	0.0 ± 0.0	11.9 ± 0.7	17.6 ± 1.9	0.0 ± 0.0	6.8 ± 2.1	11.8 ± 0.9
3	13.5 ± 0.8	20.1 ± 0.3	24.4 ± 2.6	12.6 ± 1.2	19.2 ± 1.5	13.2 ± 0.6
4	18.1 ± 0.2	32.1 ± 1.0	38.4 ± 1.8	14.3 ± 0.1	28.5 ± 3.1	16.7 ± 0.3
AZO†	63.7 ± 1.3	34.9 ± 0.2	46.7 ± 3.5	37.2 ± 1.6	88.6 ± 0.7	25.8 ± 1.4
CAB†	19.6 ± 0.2	52.1 ± 0.5	100 ± 0.0	100 ± 0.0	42.7 ± 1.4	45.3 ± 0.7

*Pc (*P. capsici*), Af (*A. flavus*), Rs (*R. solani*), Ss (*S. sclerotiorum*), Bd (*B. dothidea*), Pg (*P. grisea*); activity data with prominent are presented in bold.
†AZO (azoxystrobin), CAB (cabendazim).

(Fig. 3). The experimental findings revealed that the hyphal surface of *S. sclerotiorum* appeared smooth and normal in the control group (Fig. 3a,b). In contrast, treatment with compound **5v** at concentrations of 50 and 100 μg/mL led to observable changes in the mycelial morphology. The hyphae of *S. sclerotiorum* exhibited signs of dehydration, slight wrinkling (Fig. 3c,d), and increased roughness (Fig. 3e,f). These alterations revealed that compound **5v** exerts a discernible inhibitory effect on the mycelia of *S. sclerotiorum*.

3.5 Transmission electron microscopy (TEM)

We further conducted transmission electron microscopy (TEM) study to elucidate the influence of compound **5v** on the morphology of *S. sclerotiorum*, as depicted in Fig. 4. In the control group, the cell wall (CW), cell membrane (CM), nucleus (N), and vacuole (V) appeared intact (Fig. 4a,b). However, when *S. sclerotiorum* was subjected to treatment with compound **5v** at the concentration of 13 μg/mL, notable changes were observed (Fig. 4c,d): (a) the cell wall exhibited signs of breakage and separation from the cell membrane; (b) the vacuoles showed enlargement; (c) most organelles appeared to be absent. These findings strongly suggested that compound **5v** induced substantial toxicological damage to the cellular structure of *S. sclerotiorum*.

3.6 Enzyme activity assays

Peroxidase (POD), superoxide dismutase (SOD), and catalase (CAT) stand as pivotal antioxidant enzymes within living organisms. Intracellular antioxidant enzymes, along with other redox molecules are important to preserve the equilibrium of intracellular reactive oxygen species (ROS) production. When microorganisms are affected by external adversity, living cells will generate reactive oxygen species. Concurrently, the antioxidant defense system including antioxidant enzymes will work to prevent excessive levels of reactive oxygen species within cells. As shown in Fig. 5, compound **5v** increased the activities of POD, SOD and CAT in *S. sclerotiorum* across various concentration gradients (50, 25, 12.5, 6.25, and 3.125 μg/mL). In comparison to the control (CK) group, the activity of POD enzyme increased at concentrations ranging from 50 to 3.125 μg/mL for both

Table 2. The EC_{50} values of several target compounds against plant pathogens*

Compounds	Phytopathogen	Regression Equation	*r*	EC_{50} (μg/mL)	95% CI‡
5h	*Pc*	$y = 3.1112x + 1.8176$	0.999	10.943	7.83–15.29
5L	*Pc*	$y = 3.3250x + 1.2263$	0.980	23.221	13.91–38.76
5m	*Pc*	$y = 3.6262x + 1.4847$	0.985	**8.420**	5.41–13.10
5n	*Pc*	$y = 3.2032x + 1.5750$	0.990	13.831	9.64–19.84
5t	*Pc*	$y = 3.3041x + 1.8334$	0.992	**8.414**	5.76–12.28
5v	*Pc*	$y = 2.8283x + 1.8274$	0.989	15.432	11.12–21.41
AZO†	*Pc*	$y = 4.1809x + 0.6229$	0.964	20.649	8.11–52.56
CAB†	*Pc*	$y = 2.7864x + 0.9221$	0.988	251.625	237.23–263.30
5h	*Af*	$y = 2.7105x + 2.4174$	0.986	**8.853**	6.61–11.87
5q	*Af*	$y = 1.1297x + 2.8850$	0.978	21.954	16.15–29.83
5t	*Af*	$y = 2.0261x + 2.2369$	0.987	21.353	15.40–29.59
AZO	*Af*	$y = 4.8149x + 0.3326$	0.991	3.603	0.19–5.65
CAB	*Af*	$y = 4.3581x + 0.4423$	0.977	28.283	16.58–33.35
5i	*Ss*	$y = 1.9410x + 2.0532$	0.968	30.895	20.10–47.48
5v	*Ss*	$y = 3.2320x + 1.5954$	0.980	**12.829**	9.07–18.15
AZO	*Ss*	$y = 2.9203x + 1.1531$	0.986	63.629	55.84–70.12

*Average of three replicates; *Pc* (*P. capsici*), *Af* (*A. flavus*), *Ss* (*S. sclerotiorum*); activity data with prominent are presented in bold.
†AZO (azoxystrobin), CAB (cabendazim).
‡95% confidence interval.

Table 3. Curative and protective activities of target compound **5v** against *S. sclerotiorum**

		Curative effect		Protective effect	
Compounds	Concentration (μg/mL)	Lesion length (mm ± SD)	Controlling efficacy (%)	Lesion length (mm ± SD)	Controlling efficacy (%)
5v	200	5.2 ± 0.1	99.2	5.4 ± 0.2	98.3
	100	11.3 ± 0.2	78.8	11.1 ± 0.7	72.9
AZO†	100	5.0 ± 0.0	100.0	5.0 ± 0.0	100.0
Control	0	30.1 ± 0.1	-	27.5 ± 0.9	-

*Values are mean ± SD of three replicates.
†AZO (azoxystrobin).

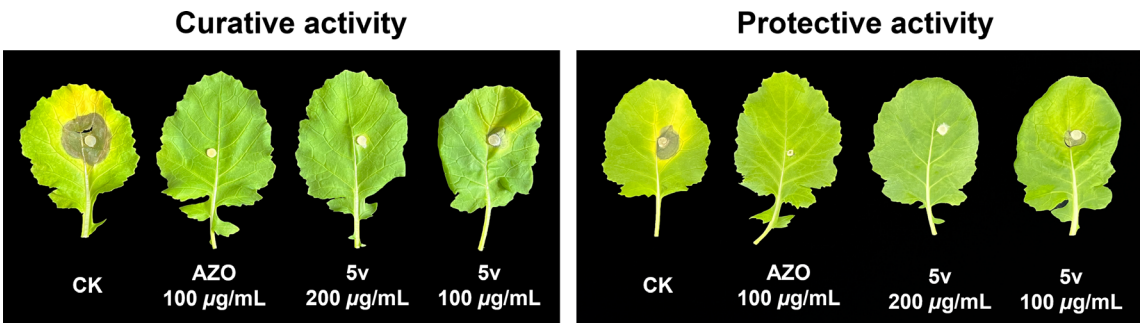


Figure 2. *In vivo* curative and protective effects of compound **5v** against *S. sclerotiorum*.

compounds **5v** and AZO. However, the activities of SOD and CAT enzymes for compound **5v** were greatly increased at lower concentrations than the CK group, followed by a decline at higher concentrations and lower than the AZO group. This phenomenon arises because the treatment with compound **5v** prompts the production of a substantial amount of antioxidant enzymes in the living cells to combat the detrimental effects, resulting in an augmented enzyme activity when compared to the CK group. Nevertheless, as the concentration of compound **5v** escalates, the living cells reach a point where they cannot

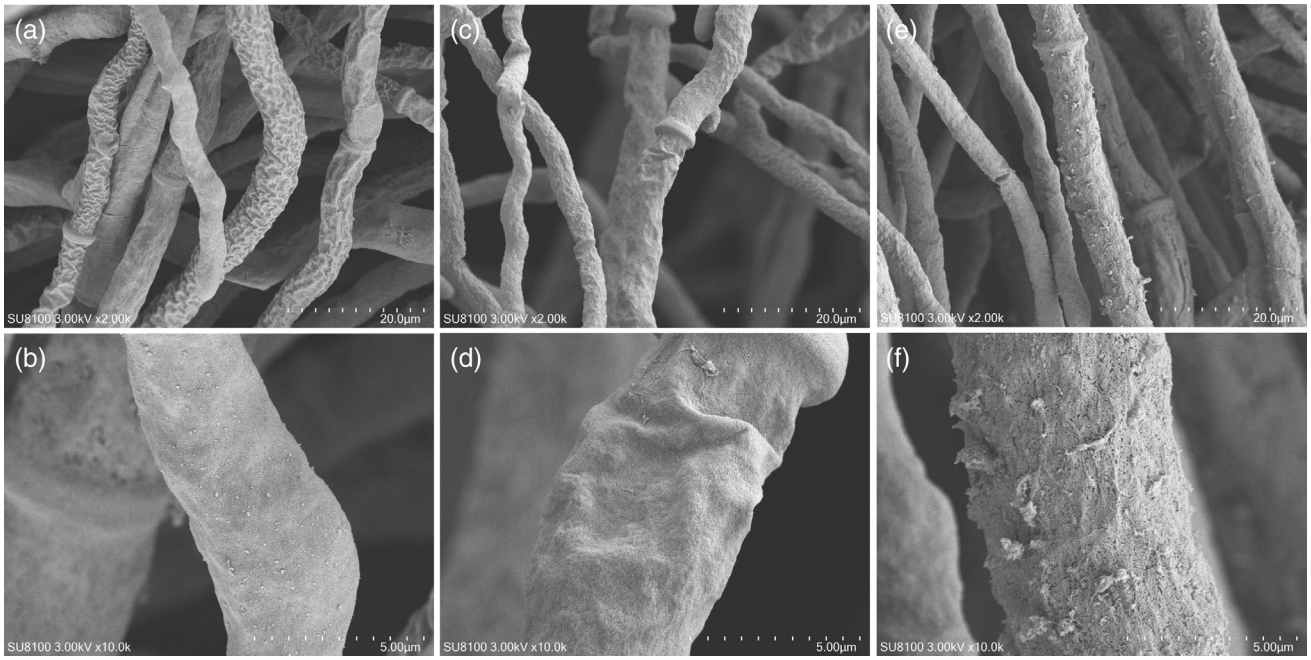


Figure 3. Effects of compound **5v** on the mycelium morphology of *S. sclerotioruma* by SEM. (a, b) Negative control. (c, d) Treatment with compound **5v** at 50 μg/mL. (e, f) Treatment with compound **5v** at 100 μg/mL.

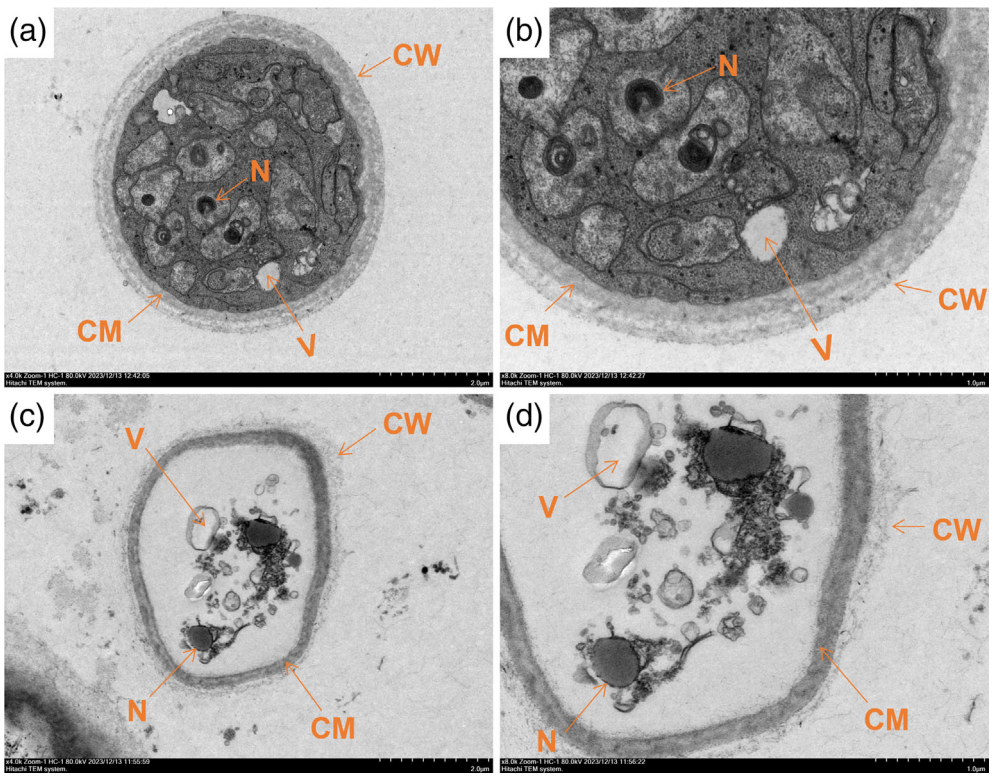


Figure 4. Effects of compound **5v** on the cellular structure of *S. sclerotioruma* by TEM. (a, b) Negative control. (c, d) Treatment with compound **5v** at 13 μg/mL. CW, cell wall; CM, cell membrane; N, nucleus; V, vacuole.

withstand excessive damage, leading to heightened harm in the high-concentration treatment group and subsequently causing a decrease in enzyme activity compared to the CK group.

4 CONCLUSION

In summary, a series of novel thymol containing derivatives were designed and synthesized for antifungal applications against six plant pathogens by using the mycelial growth inhibition method.

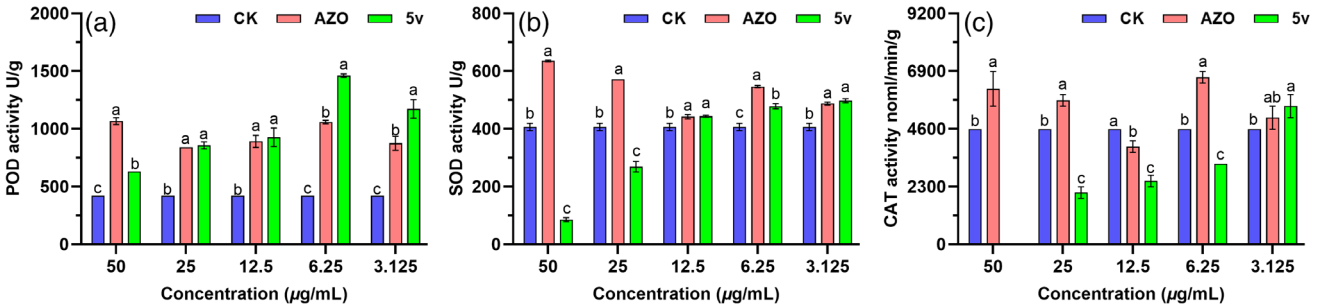


Figure 5. Effects of compound **5v** on antioxidant enzymes. (a) peroxidase (POD), (b) superoxide dismutase (SOD), (c) catalase (CAT). All experiments were executed in three replicates to obtain the mean and standard deviation. Different lowercase letters indicate values with significant differences among different treatment groups according to one-way ANOVA ($P < 0.05$).

Preliminary bioassay results revealed that several target compounds exhibited notable antifungal effects against *P. capsici*, *A. flavus*, and *S. sclerotiorum* at the concentration of 50 μg/mL. Significantly, the antifungal activities of compounds **5m** and **5t** against *P. capsici* surpassed that of the commercial fungicides AZO and CAB. Additionally, compound **5v** demonstrated superior antifungal activity against *S. sclerotiorum* compared to AZO and CAB. *In vivo* bioassays confirmed the excellent curative and protective activity of compound **5v** against *S. sclerotiorum*. Preliminary mechanism studies revealed that compound **5v** induced contraction and collapse of *S. sclerotiorum* mycelium, resulting in organelle damage and the accumulation of antioxidant enzyme activity. These findings suggested that compound **5v** is a promising candidate scaffold for development of potent antifungal agents. Further exploration to search for novel fungicides with remarkable efficiency from naturally occurring thymol derivatives could be anticipated and is currently in progress in our laboratory.

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CONFLICT OF INTEREST

The authors declare no competing financial interest.

DATA AVAILABILITY STATEMENT

The data that supports the findings of this study are available in the supplementary material of this article.

SUPPORTING INFORMATION

Supporting information may be found in the online version of this article.

REFERENCES

1 Fisher MC, Hawkins NJ, Sanglard D and Gurr SJ, Worldwide emergence of resistance to antifungal drugs challenges human health and food security. *Science* **360**:739–742 (2018).
2 Lamour KH, Stam R, Jupe J and Huitema E, The oomycete broad-host-range pathogen *Phytophthora capsici*. *Mol Plant Pathol* **13**:329–337 (2012).
3 Kamoun S, Furzer O, Jones JD, Judelson HS, Ali GS, Dalio RJ *et al.*, The top 10 oomycete pathogens in molecular plant pathology. *Mol Plant Pathol* **16**:413–434 (2015).
4 Liang X and Rollins JA, Mechanisms of broad host range necrotrophic pathogenesis in *Sclerotinia sclerotiorum*. *Phytopathology* **108**:1128–1140 (2018).
5 Derbyshire MC, Newman TE, Khentry Y and Taiwo AO, The evolutionary and molecular features of the broad-host-range plant pathogen *Sclerotinia sclerotiorum*. *Mol Plant Pathol* **23**:1075–1090 (2022).
6 Ragsdale NN, The role of pesticides in agricultural crop protection. *Ann N Y Acad Sci* **894**:199–205 (1999).
7 Azizbekyan RR, Biological preparations for the protection of agricultural plants (review). *Appl Biochem Microbiol* **55**:816–823 (2019).
8 Chen T, Xiong H, Yang JF, Zhu XL, Qu RY and Yang GF, Diaryl ether: a privileged scaffold for drug and agrochemical discovery. *J Agric Food Chem* **68**:9839–9877 (2020).
9 Hu Z, Tao Y, Tao X, Su Q, Cai J, Qin C *et al.*, Sesquiterpenes with phytopathogenic fungi inhibitory activities from fungus *Trichoderma virens* from *Litchi chinensis* Sonn. *J Agric Food Chem* **67**:10646–10652 (2019).
10 He J, Dou M, Xie J, Hou S, Liu Q, Hu Z *et al.*, Discovery of zeylenone from *Uvaria grandiflora* as a potential botanical fungicide. *Pest Manag Sci* **77**:5407–5417 (2021).
11 Wang M, Du Y, Ling C, Yang Z, Jiang B, Duan H *et al.*, Design, synthesis and antifungal/antioomycete activity of pyrazolyl oxime ethers as novel potential succinate dehydrogenase inhibitors. *Pest Manag Sci* **77**:3910–3920 (2021).
12 Dong J, Gao W, Li K, Hong Z, Tang L, Han L *et al.*, Design, synthesis, and biological evaluation of novel psoralen based 1,3,4-oxadiazoles as potent fungicide candidates targeting pyruvate kinase. *J Agric Food Chem* **70**:3435–3446 (2022).
13 Liu W, Zhang S, Xiao L, Wan Y, He L, Wang K *et al.*, Synthesis and biological activity of novel hydantoin cyclohexyl sulfonamide derivatives as potential antimicrobial agents in agriculture. *Pest Manag Sci* **78**:1438–1447 (2022).

14 Zheng Z, Dai A, Jin Z, Chi YR and Wu J, Trifluoromethylpyridine: an important active fragment for the discovery of new pesticides. *J Agric Food Chem* **70**:11019–11030 (2022).
15 Nunes CA, Biological control of postharvest diseases of fruit. *Eur J Plant Pathol* **133**:181–196 (2011).
16 Ribas ADR, Spolti P, Del Ponte EM, Donato KZ, Schrekker H and Fuentefria AM, Is the emergence of fungal resistance to medical triazoles related to their use in the agroecosystems? A mini review. *Braz J Microbiol* **47**:793–799 (2016).
17 Wang KY, Zhang X, Shao X, Wei Y, Xu F and Wang H, Flavonoids from *Sedum aizoon L*. inhibit *Botrytis cinerea* by negatively affecting cell membrane lipid metabolism. *Appl Microbiol Biot* **106**:7139–7151 (2022).
18 Dayan FE, Cantrell CL and Duke SO, Natural products in crop protection. *Bioorg Med Chem* **17**:4022–4034 (2009).
19 Wang H and Song H, Synthesis of four optical isomers of antiviral agent NK0209 and determination of their configurations and activities against a plant virus. *J Agric Food Chem* **68**:2631–2638 (2020).
20 Ren Z, Lv M, Li T, Hao M, Li S and Xu H, Construction of oxime ester derivatives of osthole from *Cnidium monnieri*, and evaluation of their agricultural activities and control efficiency. *Pest Manag Sci* **76**:3560–3567 (2020).
21 Harvey AL, Edrada-Ebel R and Quinn RJ, The reemergence of natural products for drug discovery. *Nat Rev* **14**:111 (2015).
22 Huang G, Lv M, Hu J, Huang K and Xu H, Glycosylation and activities of natural products. *Mini-rev Med Chem* **16**:1013–1016 (2016).
23 Marrone PG, Pesticidal natural products-status and future potential. *Pest Manag Sci* **75**:2325–2340 (2019).
24 Zhang M, Ding X, Kang J, Gao Y, Wang Z and Wang Q, Marine natural product for pesticide candidate: pulmonarin alkaloids as novel antiviral and anti-phytopathogenic-fungus agents. *J Agric Food Chem* **68**:11350–11357 (2020).
25 Wang Y, Mu Y, Hu X, Zhang C, Gao Y, Ma Z *et al*., Indole/tetrahydroquinoline as renewable natural resource-inspired scaffolds in the devising and preparation of potential fungicide candidates. *J Agric Food Chem* **70**:4582–4590 (2022).
26 He HW, Xu D, Wu KH, Lu ZY, Liu X and Xu G, Discovery of novel salicylaldehyde derivatives incorporating an α-methylene-γ-butyrolactone moiety as fungicidal agents. *Pest Manag Sci* **79**:5015–5028 (2023).
27 Zhang P, Duan CB, Jin B, Ali AS, Han X, Zhang H *et al*., Recent advances in the natural products-based lead discovery for new agrochemicals. *Adv Agrochem* **2**:324–339 (2023).
28 Song H, Liu Y and Wang Q, Research progress of anti-plant virus agents based on marine natural products. *Adv Agrochem* **2**:31–38 (2023).
29 Liu Y, Wang R, Zhao L, Huo S, Liu S, Zhang H *et al*., The antifungal activity of cinnamon-litsea combined essential oil against dominant fungal strains of moldy peanut kernels. *Foods* **11**:1586 (2022).
30 Salehi B, Mishra AP, Shukla I, Sharifi-Rad M, Contreras MM, Segura-Carretero A *et al*., Thymol, thyme, and other plant sources: health and potential uses. *Phytother Res* **32**:1688–1706 (2018).
31 Marchese A, Orhan IE, Daglia M, Barbieri R, Lorenzo AD, Nabavi SF *et al*., Antibacterial and antifungal activities of thymol: a brief review of the literature. *Food Chem* **210**:402–414 (2016).
32 Bishoyi AK, Mahapatra M, Paidesetty SK and Padhy RN, Design, molecular docking, and antimicrobial assessment of newly synthesized phytochemical thymol Mannich base derivatives. *J Mol Struct* **1244**:130908 (2021).
33 Wang K, Jiang S, Yang Y, Fan L, Su F and Ye M, Synthesis and antifungal activity of carvacrol and thymol esters with heteroaromatic carboxylic acids. *Nat Prod Res* **33**:1924–1930 (2019).
34 Swain SS, Paidesetty SK and Padhy RN, Synthesis of novel thymol derivatives against MRSA and ESBL producing pathogenic bacteria. *Nat Prod Res* **33**:3181–3189 (2019).
35 Alves Eloy M, Ribeiro R, Martins Meireles L, Antonio de Sousa Cutrim T, Santana Francisco C, Lirian Javarini C *et al*., Thymol as an interesting building block for promising fungicides against *Fusariumsolani*. *J Agric Food Chem* **69**:6958–6967 (2021).
36 Wang M, Du Y, Liu C, Yang X, Qin P, Qi Z *et al*., Development of novel 2-substituted acylaminoethylsulfonamide derivatives as fungicides against *Botrytis cinerea*. *Bioorg Chem* **87**:56–69 (2019).
37 Yang Y, Liu Y, Song H, Li Y and Wang Q, Design, synthesis, and insecticidal activity of novel triazone derivatives containing sulfonamide or sulfonimide moieties. *J Agric Food Chem* **69**:10790–10796 (2021).
38 Zhao W, Zheng S, Zou J, Liang Y, Zhao C and Xu H, Synthesis of novel pesticidal *N*, *N'*-disubstituted sulfamide derivatives using sulfur (VI) fluorine exchange click reaction. *J Agric Food Chem* **69**:5798–5803 (2021).
39 Wu R, Liu T, Wu S, Li H, Song R and Song B, Synthesis, antibacterial activity, and action mechanism of novel sulfonamides containing oxyacetal and pyrimidine. *J Agric Food Chem* **70**:9305–9318 (2022).
40 Yang S, Tang J, Li B, Yao G, Peng H, Pu C *et al*., Rational design of insecticidal isoxazolines containing sulfonamide or sulfinamide structure as antagonists of GABA receptors with reduced toxicities to honeybee and zebrafish. *J Agric Food Chem* **71**:14211–14220 (2023).
41 FRAC, Fungicide resistance action committee. https://www.frac.info (2021).
42 Schneider C, Broda E and Snieckus V, Directed *ortho*-metalation-cross-coupling strategies. One-pot Suzuki reaction to biaryl and heterobiaryl sulfonamides. *Org Lett* **13**:3588–3591 (2011).
43 Jiang S, Tang X, Chen M, He J, Su S, Liu L *et al*., Design, synthesis and antibacterial activities against *Xanthomonas oryzae* pv. *Oryzae*, *Xanthomonas axonopodis* pv. *Citri* and *Ralstonia solanacearum* of novel myricetin derivatives containing sulfonamide moiety. *Pest Manag Sci* **76**:853–860 (2020).
44 Wang LL, Li C, Zhang YY, Qiao CH and Ye YH, Synthesis and biological evaluation of benzofuroxan derivatives as fungicides against phytopathogenic fungi. *J Agric Food Chem* **61**:8632–8640 (2013).
45 Zhou R, Zhan W, Yuan C, Zhang T, Mao P, Sun Z *et al*., Design, synthesis and antifungal activity of novel 1,4-pentadiene-3-one containing quinazolinone. *Int J Mol Sci* **24**:2599 (2023).
46 Zhang ZJ, Jiang ZY, Zhu Q and Zhong GH, Discovery of β-carboline oxadiazole derivatives as fungicidal agents against rice sheath blight. *J Agric Food Chem* **66**:9598–9607 (2018).
47 Cheng X, Wang W, Wang Y, Xia D, Yin F, Liu Q *et al*., Novel pyrazolo[3,4-*d*]pyrimidin-4-one derivatives as potential antifungal agents: design, synthesis, and biological evaluation. *J Agric Food Chem* **69**:11395–11405 (2021).
48 Sun XP, Yu CS, Min LJ, Cantrell CL, Hua X, Sun NB *et al*., Discovery of highly efficient novel antifungal lead compounds targeting succinate dehydrogenase: pyrazole-4-carboxamide derivatives with an *N*-phenyl substituted amide fragment. *J Agric Food Chem* **71**:19312–19323 (2023).
49 Zhang M, Feng S, Chen S, Zhou Y, Gong C and Xue W, Synthesis, antibacterial and antifungal activity of myricetin derivatives containing piperidine and amide fragments. *Pest Manag Sci* **79**:4795–4808 (2023).
50 Xu R, Chen K, Han X, Lou Y, Gu S, Gao Y *et al*., Design and synthesis of antifungal candidates containing triazole scaffold from natural rosin against *Valsa Mali* for crop protection. *J Agric Food Chem* **71**:9718–9727 (2023).

