



Bioassay-guided isolation of antifungal amides against *Sclerotinia sclerotiorum* from the seeds of *Clausena lansium*

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ABSTRACT

Clausena lansium (Lour.) Skeels is an attractive shrub or small tree, a member of the Rutaceae family. The seeds, leaves and roots of this plant have been used as herbal medicines for a long time. In this study, seven amides were isolated from the seeds of *Clausena lansium*. The antifungal assay indicated that lansiumamide B (4) and lansiumamide C (2) displayed the highest antifungal activity against *Sclerotinia sclerotiorum* with EC₅₀ values of 4.95 and 13.24 µg/mL, which were both lower than that of carbendazim (EC₅₀ = 0.64 µg/mL). The structure-activity relationships of these amides were investigated using assays to measure their *in vitro* antifungal activities against *Sclerotinia sclerotiorum*. The effect of the most potent compound lansiumamide B on morphology and physiology of *S. sclerotiorum* were also evaluated. Lansiumamide B caused cell rupture and mycelial abnormalities of *S. sclerotiorum*, and its curative efficacy (75.17%) against *S. sclerotiorum* infection was better than that of carbendazim (56.57%). This study demonstrated that amides isolated from *C. lansium* possessed the potential to be exploited as botanical fungicides for commercial application or can be used as templates for designing new fungicides with novel action modes.

1. Introduction

Sclerotinia sclerotiorum (Lib.) de Bary is a worldwide distributed plant pathogen with a broad host range. More than 400 species of 270 genera, including many economically important crops and vegetables, can be infected by this pathogen (Bardin and Huang, 2001; Firoz et al., 2016; Ronicke et al., 2005). The disease caused by this pathogen was called sclerotinia stem rot (SSR) or sudden death syndrome (SDS), one of the most disastrous diseases in oilseed rape (Boland and Hall, 1994; Clarkson et al., 2004; Huang et al., 2006; Kora et al., 2005; Letham et al., 1976; Purdy, 1979). In China, SSR has been widespread almost in all oilseed rape planted areas especially in Sichuan and Jiangsu province. (Duan et al., 2012).

Generally, chemical control remains the main strategy for controlling SSR in practice for lacking completely disease-resistant oilseed rape strains (Firoz et al., 2016). The benzimidazole and dicarboximide fungicides were the most efficient fungicides in controlling SSR. However, unscientific applications with repeated high doses of these fungicides have caused a widespread high level of resistance (Kuang et al., 2011; Liang et al., 2015; Ma et al., 2009; Wang et al., 2016a). Therefore, there

is a great and urgent demand for developing new fungicides with different modes of action as alternatives for chemical synthetic pesticides being used vastly now.

Botanical secondary metabolites provide a series of promising alternatives for synthetic fungicides, due to their easy biodegradation which could have low residual toxicity on mammals and other non-target organisms and potential different mode of actions compared to current commercial fungicides (Dubey et al., 2008; Gerwick and Sparks, 2014; Ziv and Cilia, 2007). The bioactive phytochemicals with unique structures, such as alkaloids, terpenoids, quinonoids and coumarins are far better than those synthetic compounds and mainly exert defensive functions to protect plants from pathogenic microorganism, which is the result of millions of year's coevolution of plant with their predators (Berenbaum, 2002). That's the reason why many botanical phytochemicals have long been playing a prominent role in the development of new fungicides and being the templates for a wide array of current commercial synthetic fungicides (Gerwick and Sparks, 2014).

Clausena lansium (Lour.) Skeels is an attractive shrub or small tree, a member of the Rutaceae family, widely distributed in Southern China and Southeast Asia. The leaves and roots of this plant have been used as

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herbal medicine for treating respiratory disease, skin disease, viral hepatitis and digestive system disease for a long time (Adebajo et al., 2009; Huei, 1989). The seeds are mainly involved in treating acute and chronic gastritis and ulcers (Maneerat et al., 2011). In addition, the fruit of *C. lansium* receives large amounts of popularity among customers for its fresh flavor, and it can be made into fruit cups, gelatins and other desserts (Campbell, 1974; Chokeyprasert et al., 2007). Previous phytochemical studies on *C. lansium* have reported the isolation of amides (Kumar et al., 1995; Maneerat et al., 2011), alkaloids (Du et al., 2015a; Du et al., 2015b; Liu et al., 2015; Tatsimo et al., 2015) and coumarins (Liu et al., 2014; Maneerat et al., 2012).

During the period of screening bioactive materials for new agrochemicals from Chinese medicinal herbs, the significant antifungal activity against *S. sclerotiorum* of the crude extract from *C. lansium* seeds was found. In the present study, we followed the bioassay-guided method to isolate active ingredients from the seeds of *C. lansium* and verified their antifungal activity against *S. sclerotiorum* *in vitro* and in greenhouse. We also evaluated the effect of the main effective component on morphology and physiology of *S. sclerotiorum*.

2. Materials and methods

2.1. General experimental procedures

The 1D NMR spectra were recorded in CDCl₃ on Bruker DRX-500 (Bruker, Bremerhaven, Germany) spectrometer with tetramethylsilane as the internal standard. The column chromatography was performed with silica gel (100–200 and 200–300 mesh, Qingdao Jiyida silica reagent Co. Ltd., Qingdao, China), FUJI RP-18 gel (40–75 mm, FUJI, Tokyo, Japan) and Sephadex LH-20 (GE healthcare, Connecticut, America). Thin-layer chromatography (TLC) was carried out on silica gel GF₂₅₄ glass plates (Qingdao Jiyida silica reagent Co. Ltd., Qingdao, China). Spots were observed under 275 nm UV light followed by spraying with 3% bismuth potassium iodide solution. Preparative reversed-phase HPLC was carried out on a Waters 600 apparatus (Waters, America) equipped with a UV detector (Waters 486) and an Agilent PrepHT C-18 column (Agilent, 250 × 21.2 mm) at a flow rate of 4 mL/min. Conductivity was measured with CON510 conductivity meter (Eutech/Oakton, Singapore). The mycelial morphology were observed by JSM-6360LV scanning electron microscope (SEM, Japan). UV Absorbance were detected by Multiskan MK3 micro plate reader (Thermo, Germany).

2.2. Plant materials

The seeds of *C. lansium* were collected from Guangzhou, Guangdong Province, China, in August 2014, and authenticated by Prof. Li Y (College of science, Northwest A&F University). A voucher specimen (No. ZX201415) has been deposited in the Research and Development Center of Biorational Pesticides in Northwest A&F University, Shaanxi Province, China.

2.3. Extraction, bio-guided fractionation and isolation

The dried seeds of *C. lansium* (2.7 kg) were crushed and then extracted with 80% acetone (3 × 20 L, 24 h each time) at room temperature, and the extract was concentrated by rotary evaporation in vacuum at 45 °C to obtain a bright yellow residue (301 g). The crude acetone extract was dissolved in water and then partitioned with CHCl₃ and *n*-BuOH. The CHCl₃ extraction section (154 g), which was the active part (Table 1), was subjected to silica column (10 × 120 cm, 1500 g), eluted with a gradient of PE-CH₃COCH₃ (99: 1, 98: 2, 97: 3, 95: 5, 90: 10, 80: 20, 50: 50, 1: 99, each 1.7 L) to give eight fractions (Fr.1–Fr.8) on the basis of the TLC profiles. Fr.2 and Fr.3 with antifungal activity were selected for further isolation (Table 1). Fr.2 (11.7 g) was chromatographed on the C-18 silica and eluted with

Table 1

The results of fungicidal activity of crude acetone extract, extraction sections, and fractions against *S. sclerotiorum*.

Sample	EC ₅₀ (μg/mL)	Toxicity regression equation (Y = a + bx)	χ ²	df	P
acetone extract	153.24 ± 37.51	Y = 1.044 + 1.271x	2.17	3	0.53
CHCl ₃ extraction section	42.96 ± 7.29	Y = 1.816 + 1.330x	0.28	3	0.96
BuOH and the remaining H ₂ O extraction sections	> 1000	–	–	–	–
fraction Fr. 2	217.60 ± 61.33	Y = 0.875 + 1.338x	1.67	3	0.64
fraction Fr.3	10.67 ± 2.03	Y = 1.201 + 0.640x	1.35	3	0.71
fraction Fr. 1, 4–8	> 1000	–	–	–	–
Compound 1	54.66 ± 8.36	Y = –0.309 + 0.015x	1.81	3	0.30
Compound 2	13.24 ± 2.65	Y = 1.284 + 0.705x	1.66	3	0.27
Compound 3	46.46 ± 7.24	Y = –0.104 + 0.013x	2.11	3	0.31
Compound 4	4.95 ± 0.98	Y = –1.035 + 1.474x	2.53	3	0.42
Compound 5-7	> 100	–	–	–	–
Carbendazim ^a	0.64 ± 0.20	Y = 0.014 + 1.264x	0.78	3	0.85

^a Commercial fungicide carbendazim as positive control.

CH₃OH/water mixtures with increasing polarity (30:70, 50:50, 70:30 and 90:10, v/v) to obtain six subfractions (Fr.2-1–Fr.2-6). For the subfraction Fr.2-3, the preparative HPLC was used for further purification, eluted with CH₃OH/H₂O mixture (80:20, v/v) to give compounds 1 (27 mg) and 2 (369 mg). Further purification of subfraction Fr.2-4 was carried out on the Sephadex LH-20 chromatography eluted with CHCl₃/CH₃OH mixture (1:1, v/v) to give compound 3 (64 mg). Fr.3 (13.4 g) was separated by column chromatography (CC) of silica gel (5 × 80 cm, 120 g), eluted with a gradient of CHCl₃-MeOH (100:0, 98: 2, 97: 3, 95: 5, 90: 10, 1: 99, each 1.7 L) to give seven subfractions (Fr.3-1–Fr.3-7). Compound 4 was crystallized as yellow crystal (> 98%, 10.2 g) in Fr.3-2. For the subfraction Fr.3-3, the preparative HPLC was used for further purification, eluted with CH₃OH/H₂O mixture (60:40, v/v) to give compounds 5 (41 mg), 6 (16 mg) and 7 (13 mg).

2.4. Media, pathogen and fungicide

S. sclerotiorum strain ACC01 used in bio-guided fractionation and isolation of the seeds of *C. lansium* was purchased from Agricultural Culture Collection of China, maintained on slants of potato sucrose agar (PSA) at 4 °C. Five wild-type strains of *S. sclerotiorum* isolates JK19, JY11, CS51, ZJ62 and TZ5 used in this study were collected from different fields of planting rape in Jiangsu Province of China between 2010 and 2012. In addition, CZ138R and LYG82R were laboratory-induced dimethachlon-resistant mutants. HA53R, YX36R and YX77R were laboratory-induced carbendazim-resistant mutants. All the isolated and mutants strains were provided by Jiangsu Key Laboratory of Pesticide, College of Plant Protection, Nanjing Agricultural University and maintained on PSA slants at 4 °C. Analytical reagent carbendazim (> 99%) was purchased from Shanghai Aladdin Biochemical Polytron Technologies Inc. (Shanghai, China).

2.5. Antifungal assay *in vitro*

The sensitivity of *S. sclerotiorum* to acetone extracts, extraction sections, fractions Fr.1-8 and compounds 1-7 from the seeds *C. lansium* were measured as following steps. A mycelial plug (5 mm in diameter) cut from the margin of medium area was placed with its mycelia-side down on the center of each plate with definite concentrations of tested samples, and the plate with the same amount of acetone was used as a solvent control. Plates were inoculated in a thermostatic chamber at

25 °C. After 3–5 days, the average diameter of each colony was measured according to the method reported in a previous study (Liang et al., 2015). The inhibition was calculated with the formula: Inhibition (%) = (average diameter of the control – average diameter of the treatment)/average diameter of the control × 100 (Wang et al., 2016a). There were three replicates for each treatment, and the whole experiment was repeated at least twice. Carbendazim was used as a positive control.

As for the measurement of minimal inhibitory concentration (MIC) of lansiumamide B, the mycelial plug (5 mm in diameter) was prepared as described above placed on the center of plate with definite concentrations of lansiumamide B. The final concentration which just inhibit the growth of mycelium is MIC.

2.6. Effect of lansiumamide B on mycelial morphology

A mycelial plug (5 mm in diameter) of *S. sclerotiorum* cut from the periphery of 2 days old colonies was placed with mycelia-side down on the center of each PDA plate containing 4.95 µg/mL of lansiumamide B (10 mL). Plates without lansiumamide B were used as blank controls. Plates were then incubated in a thermostatic chamber at 25 °C for 48 h. Samples of *S. sclerotiorum* mycelium were cut from the margin of medium area, and then were placed on the slide glass.

For SEM analysis (Soylu et al., 2007), mycelial discs (1 cm in diameter) of sclerotia exposed to the EC₅₀ concentration of test compounds were fixed with 2.5% glutaraldehyde in 0.1 mol/L phosphate-buffer (pH = 7.2) for 2 h at room temperature. They were washed twice with the same buffer, each time for 10 min. The samples were dehydrated in a graded ethanol series (70%, 80%, 90% and three times at 100%) each series for 30 min. The fixed material was then mounted on stubs using double-sided carbon tape and coated with gold/palladium in a sputter coater system in a high vacuum chamber for 150 s at 9 mA. The samples were examined and digital images were captured using a JSM-6360LV scanning electron microscope (SEM, Japan). There were three replicates for each treatment, and the experiment was repeated at least twice.

2.7. Effect of lansiumamide B on cell membrane permeability

The impact of lansiumamide B on the cell membrane permeability of *S. sclerotiorum* was determined according to the method of previous studies (Wang et al., 2016a; Duan et al., 2013). Mycelial plugs of *S. sclerotiorum* cut from the periphery of 2 days old colonies were transferred to a 250 mL triangular flask containing 100 mL of PDB which was shaken for 36 h at 180 rpm and 25 °C, then lansiumamide B was added into the flask at the final concentration of 4.95 µg/mL. The flasks were shaken for additional 36 h, then collecting 0.5 g mycelia for each treatment. The collected mycelia was washed with distilled water three times and re-suspended in 20 mL distilled water. Flasks without lansiumamide B were used as blank controls. After 0, 2, 4, 6, 8, 10 and 12 h, the conductivity of different groups was measured using CON510 conductivity meter (Eutech/Oakton, Singapore). After 12 h, the mycelia were boiled for 5 min, and final conductivity was measured. The experiments were repeated twice and each treatment had three repetitions. The relative conductivity was calculated with the formula: Conductivity/Final conductivity × 100.

2.8. Intracellular glycerol assays

Cupric glycerinate colorimetry method was used to determine the accumulation of intracellular glycerol (Duan et al., 2013). For preparing the standard curve, 0, 2.5, 3, 4, 8, and 10 mL of glycerol solution (dissolved in H₂O, 0.1 g/mL) was added to a 100 mL volumetric flask containing 10 mL CuSO₄ solution (0.035 g/L) and 35 mL NaOH solution (0.035 g/mL) respectively, and then distilled water was added to a final volume of 100 mL. After the treated flasks were incubated at 25 °C for

30 min, the absorbance (Abs) of different concentrations of glycerol was detected at 630 nm. Double distilled water was used as a blank control and then a standard curve of glycerol was established by plotting Abs against oxalate concentrations

For accumulation of intracellular glycerol assay, after treated with 4.95 µg/mL lansiumamide B for 2 h, 0.5 g mycelia (treatment of mycelia with lansiumamide B was done same as in Section 2.7) of each sample was collected and fixed in liquid nitrogen. Then, the mycelial powder was suspended in 20 mL distilled water. After heated for 20 min at 80 °C, the slurry was centrifuged at 9000 rpm for 15 min. The Abs of supernatants detected at 630 nm was used to calculate the concentration of glycerol based on the standard curve. There were three replicates for each treatment, and the experiment was repeated at least twice.

2.9. Oxalic acid content

Oxalic acid content was measured according to the method of previous studies (Davidson et al., 2016; Duan et al., 2013). For preparing the standard curve, 0, 1, 2, 3, 4, 5 mL oxalate solution (dissolved in H₂O, 0.4 mg/mL) was added to a 25 mL volumetric flask containing HCl-KCl buffer (20 mL, pH 2, 50 mM), FeCl₃ (2 mL, 0.5 mg/mL), and sulphosalicylic acid (1.2 mL, 5 mg/mL), and then distilled water was added into each flask to a final volume of 25 mL. After the treated flasks were incubated at 25 °C for 30 min, the absorbance of different concentrations of oxalate was detected at 510 nm. Double distilled water was used as blank controls and then a standard curve of oxalate was established by plotting absorbance against oxalate concentrations (Wang et al., 2017).

Mycelial plugs of *S. sclerotiorum* cut from the periphery of 2 days old colonies were transferred to a 250 mL triangular flask containing 100 mL of PDB. Then lansiumamide B was added into the flask at a final concentration of 4.95 µg/mL. After shaking the flasks for 6 days at 180 rpm, 25 °C, the broth were filtered and centrifuged at 2000 rpm for 15 min. The absorbance of supernatants detected at 510 nm was used to calculate the concentration of oxalic acid based on the standard curve. There were three replicates for each treatment, and the experiment was repeated at least twice.

2.10. Peroxidase (POD) and phenylalanine ammonia-lyase (PAL) activities

The activities of POD and PAL of oilseed rape's leaves were measured according to the method of a previous study (Wang et al., 2016b). Oilseed rapes cultivated for 60 days were used to detect the enzyme activities. The plants grew in a greenhouse at 25 °C with 50% relative humidity. Leaves collected from the plants were treated with lansiumamide B at concentrations of 20, 200 and 1000 µg/mL and water for 24 h before leaves were washed twice and then grated on ice. Three leaves were collected from each treatment. Then POD and PAL activity were measured according to the operating instructions of a commercial kit (Nanjing jiancheng of bioengineering institute). One unit of POD and PAL activity was defined as a change of one unit of absorbance per minute and per hour (Wang et al., 2016a,b), respectively. The experiments were repeated twice.

2.11. Control efficacy of lansiumamide B against *S. sclerotiorum*

The control efficacy (protective and curative activity) of lansiumamide B against *S. sclerotiorum* in leaves of oilseed rape was assessed with pot experiments according to the method of previous studies (Kuang et al., 2011; Wang et al., 2017). First, leaves of 60 day old oilseed rapes were washed twice with distilled water. For protective effect assay, Lansiumamide B solution (containing 0.1% Tween 80 as surfactant) with different concentrations (100, 300 and 900 µg/mL) were sprayed to the whole plant, respectively. Then the mycelial plugs were inoculated to the leaves after one day. Plants sprayed with water

(plus 0.1% Tween 80) and 300 µg/mL carbendazim (plus 0.1% Tween 80) were served as negative control and positive controls, respectively. Then the plants were placed in a greenhouse at 25 °C with 100% relative humidity. After 3 days, the lesion diameter was measured and the control efficacy of lansiumamide B was calculated according to the following formula: (diameter of lesion in negative control – diameter of lesion in the treatment)/diameter of lesion in negative control. There were three replicates for each treatment, and the experiment was repeated at least twice.

For curative assay, the mycelial plugs were inoculated to the leaves for one day before the leaves were sprayed with test sample solutions. The rest steps were the same as the above.

2.12. Statistical analysis

Inhibition ratio of the strains were corrected using Abbott's formula. Calculation of EC₅₀ values of the strains and analysis of variance (ANOVA) with respect to least significant difference test (LSD), $P < 0.05$ were performed via SPSS software, version 18.0.

3. Results

3.1. Fungicidal activity of the crude acetone extract, extraction sections, and fractions against *S. sclerotiorum*

In the process of the bioassay-guided isolation, the antifungal activity against *S. sclerotiorum* of the acetone crude extract, CHCl₃, BuOH and H₂O extraction sections, and fractions 1–8 were evaluated. The results were shown in Table 1. As can be seen, acetone crude extract and CHCl₃ extraction section showed antifungal activity with EC₅₀ values of 153.24 and 42.96 µg/mL, respectively, while BuOH and H₂O extraction sections had no antifungal potency. For the fractions, only Fr. 2 and Fr. 3 had an obvious antifungal activity (217.60 and 10.67 µg/mL, respectively). For the other fractions of Fr.1 and Fr. 4–8, no antifungal activities had been observed (data not shown in Table 1).

3.2. Structural elucidation of isolated alkaloids

The fraction Fr. 2 and 3 were repeatedly chromatographed on the silica gel, Sephadex LH-20, and ODS HPLC following the bioassay-guided isolation method to give seven known compounds (Fig. 1). The structures of seven known compounds were established as phenethyl cinnamide (1) (Que et al., 2008), lansiumamide C (2) (Huei, 1989), lansiumamide A (3) (Lin, 1989), lansiumamide B (4) (Lin, 1989),

Table 2

The results of fungicidal activity and MIC measurement of lansiumamide B and carbendazim against wild-type and fungicidal resistant strains of *S. sclerotiorum*.

Strains ^a	EC ₅₀ ^b (µg/mL)		MIC ^b (µg/mL)	
	lansiumamide B	carbendazim	lansiumamide B	carbendazim
ACC01	4.95 ± 1.21bc	0.64 ± 0.05a	28.00c	2.80a
JK19	4.19 ± 0.78d	0.42 ± 0.11bc	24.00c	1.30b
JY11	5.31 ± 1.35b	0.31 ± 0.08c	31.00b	1.20b
CS51	3.62 ± 1.11e	0.19 ± 0.02d	17.00d	1.00c
ZJ62	4.11 ± 0.98d	0.44 ± 0.04b	22.00	1.20b
TZ5	4.62 ± 1.47bc	0.70 ± 0.09a	25.00c	2.90a
CZ138R	3.86 ± 0.48de	0.43 ± 0.12b	20.00c	1.40b
LYG82R	4.12 ± 1.60c	0.48 ± 0.14b	30.00bc	1.30b
HA53R	7.33 ± 1.89a	> 100	41.00a	–
YX36R	3.38 ± 1.12e	> 100	19.00d	–
YX77R	4.27 ± 1.03c	> 100	24.00c	–

^a JK19, JY11, CS51, and ZJ62 were wild-type strains. CZ138R, LYG82R were laboratory-induced dimethachlon-resistant mutants. TZ5, HA53R, YX36R and YX77R were laboratory-induced carbendazim-resistant mutants.

^b Mean values followed by the same letter within the same column were not significantly different in LSD (least significant difference) tests at $P = 0.05$.

lansiumamide I (5) (Bayer and Maier, 2004), SB-204900 (6) (Milner et al., 1996) and clausenalansamide A (7) (Maneerat et al., 2011) by comparing their ¹H and ¹³C NMR spectroscopic data with literatures.

3.3. Fungicidal activity of compounds 1–7 against *S. sclerotiorum*

The fungicidal activity of isolated compounds were assessed according to the reported mycelium growth rate method and the results were given in Table 1. The results displayed that compounds 5, 6 and 7 showed absence of fungicidal activity at the concentration of 100 µg/mL. Compounds 2 and 4 displayed a high fungicidal activity with EC₅₀ values of 13.24 and 4.95 µg/mL, respectively, which were lower than carbendazim (EC₅₀ = 0.64 µg/mL), a commercial systemic fungicide. Compounds 1 and 3 exhibited a moderate activity with EC₅₀ values of 56.66 and 46.46 µg/mL, respectively.

This study applied other eleven species of *S. sclerotiorum* to further evaluate the antifungal activity of lansiumamide B (Table 2). The EC₅₀ values of lansiumamide B against the tested strains ranged from 3.38 to 7.33 µg/mL. The MIC values of lansiumamide B against the tested strains ranged from 17.00 to 21.00 µg/mL. This indicated that lansiumamide B had a high fungicidal activity against *S. sclerotiorum*. Two of

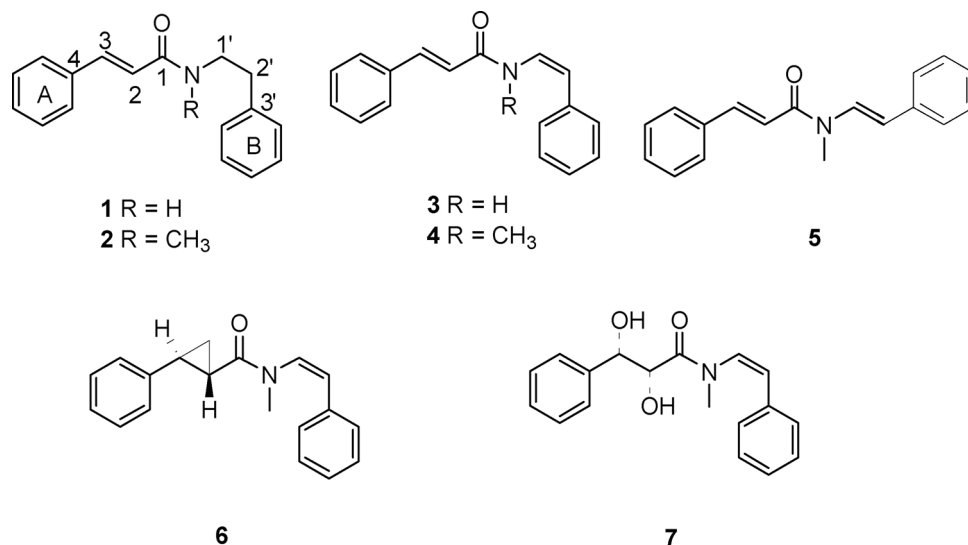


Fig. 1. Chemical structures of isolated compounds 1–7 from *Clausena lansium*.

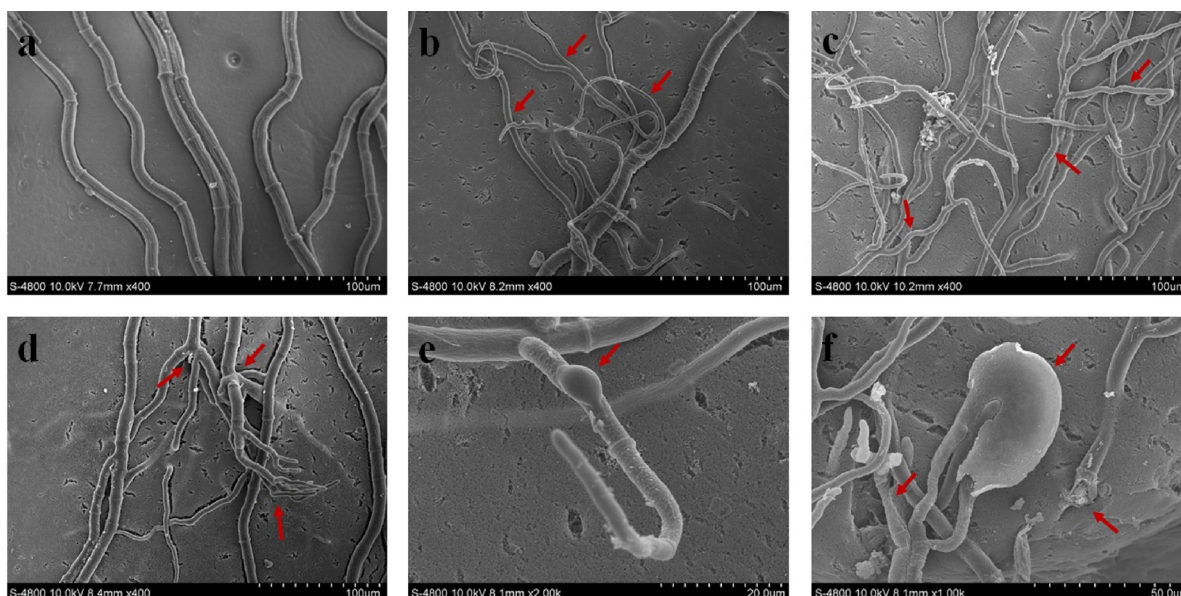


Fig. 2. Effect of lansiumamide B (4) on hyphal morphology of *Sclerotinia sclerotiorum*. (a) not treated by lansiumamide B (CK); (b) slimmer; (c) contorted; (d) offshoot of top increasing; (e) hyphal plasma extravasated; (f) death of cells for rupture.

the strains were resistant to dimethachlon. Three of the strains were resistant to carbendazim. However, the sensitivity of strains to lansiumamide B has no correlation with that to dimethachlon or carbendazim, which means that lansiumamide B has no cross resistance problem with carbendazim and dimethachlon. The solvent control had no effect on mycelium.

3.4. Effect of lansiumamide B on mycelial morphology

The mycelial morphology of *S. sclerotiorum* was observed by SEM. According to the figures, lansiumamide B (4) had an obvious inhibitory effect on mycelial growth of *S. sclerotiorum*. The mycelia without treatment was in normal growing (Fig. 2a), while after treated with lansiumamide B (4) at the concentration of 4.95 $\mu\text{g}/\text{mL}$, the mycelia was contorted (Fig. 2b) and thinner (Fig. 2c), with offshoot increasing (Fig. 2d). Meanwhile, it can also be observed that the cytochylema extravasated (Fig. 2e) and cells ruptured (Fig. 2f).

3.5. Effect of lansiumamide B on cell membrane permeability

The electrical conductivity of *S. sclerotiorum* was showed in Fig. 3. After the mycelia of *S. sclerotiorum* was treated with lansiumamide B (4)

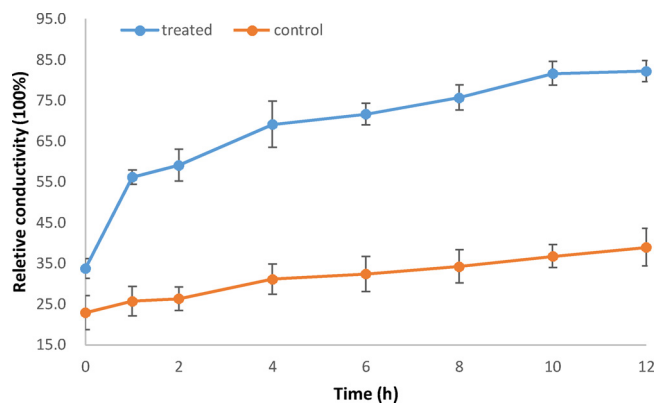


Fig. 3. Conductivity values (relative to maximum conductivity obtained with boiled mycelia) of distilled water containing mycelia of *Sclerotinia sclerotiorum* with lansiumamide B (4) treatment and the control.

at the concentration of 4.95 $\mu\text{g}/\text{mL}$, the relative conductivity of *S. sclerotiorum* was always significantly higher than that of untreated control. These results indicated that lansiumamide B could induce the increase of the cell membrane permeability of *S. sclerotiorum*.

3.6. Glycerol content

Intercellular glycerol accumulation of *S. sclerotiorum* was determined by the cupric glycerinate colorimetry method using a standard curve of glycerol. The intercellular glycerol accumulation of *S. sclerotiorum* reached to 97.32 mg/g after the mycelia of *S. sclerotiorum* was treated with lansiumamide B at the concentration of 4.95 $\mu\text{g}/\text{mL}$ for 2 h, which was considerably different from the blank control untreated with lansiumamide B (27.9 mg/g).

3.7. Oxalic acid content

The content of oxalic acid was calculated by the absorbance at 510 nm using a standard curve. The results showed that the content of oxalic acid was significantly greater after the mycelium was treated with lansiumamide B at the concentration of 4.95 $\mu\text{g}/\text{mL}$ (0.41 mg/mL) than the control (0.68 mg/mL).

3.8. Activity of defense-related enzymes (POD and PAL)

The activities of POD and PAL of oilseed rape's leaves were measured, after the leaves were treated with lansiumamide B at different concentrations of 20, 200 and 1000 $\mu\text{g}/\text{mL}$ at 24 h, and the results were shown in Fig. 4. The treated groups expressed a significant elevation in POD and PAL activities at the concentrations of 200 and 1000 $\mu\text{g}/\text{mL}$. While at the concentration of 20 $\mu\text{g}/\text{mL}$, the treated groups and the blank controls exhibited no significant difference in POD and PAL activities, respectively. Meanwhile, positive correlations were observed between lansiumamide B (4) concentration and POD and PAL activities of oilseed rape's leaves.

3.9. Protective and curative activity

The protective and curative activities of the most potent compound were preliminarily assessed by the reported method (Wang et al., 2016a,b). The results of the pot experiments showed that lansiumamide

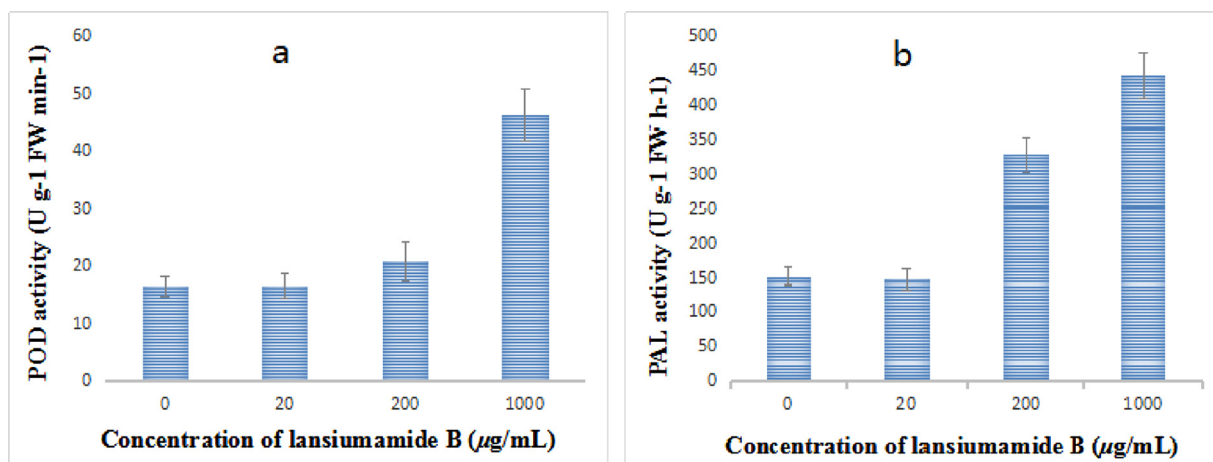


Fig. 4. POD (a) and PAL (b) activity of the rape leaves treated with lansiumamide B (4). Values are means and standard errors.

Table 3
Protective and curative activity of lansiumamide B in pot experiments.

Treatment	Protective activity		Curative activity	
	Disease Index	Control efficacy (%)	Disease Index	Control efficacy (%)
lansiumamide B (100 μg/mL)	62.30b	32.55d	44.15b	51.25c
lansiumamide B 300 μg/mL)	46.36c	49.81c	22.49c	75.17b
lansiumamide B (900 μg/mL)	30.26c	67.24b	5.92d	93.46a
Carbendazim (300 μg/mL)	29.44c	68.13b	39.33c	56.57c
Control	92.36a	–	90.58a	–

Note: Values are means of three oilseed rape seedlings and from two independent experiments. Values in each column followed by the same letter were not significant differences according to LSD ($P = 0.05$).

B exhibited a moderate protective activity and an excellent curative activity against *S. sclerotiorum* on the leaves of oilseed rape (Table 3). As shown in Table 3, apparent dose-dependent efficacies were observed both in protective curative activity and curative activity assay. And obviously, regardless of the leaves were treated in low or high concentrations of lansiumamide B, the curative efficacy of lansiumamide B was always better than protective efficacy. There was no significant difference in protective efficacy between lansiumamide B at the concentration of 900 μg/mL and carbendazim at the concentration of 300 μg/mL. For curative activity, at the concentration 300 μg/mL, the efficacy of lansiumamide B reached to 75.17%, which was significantly better than carbendazim at the same concentration. At the

concentration of 900 μg/mL, curative effect of lansiumamide B reached to 93.46%.

4. Discussion

This study has confirmed the significant antifungal activity against *S. sclerotiorum* of the crude extract from the seeds of *C. lansium*. In view of its uses in traditional Chinese herbal medicine, the crude extract of *C. lansium* should be safe to humanity and environment. Thus, *C. lansium* has the potential to be developed as a botanical fungicide for the control of *S. sclerotiorum* and other pathogens.

Bioassay-guided fractionation and isolation of active ingredient from 80% acetone extract of *C. lansium* leads to the discoveries of seven amide alkaloids. Among these compounds, lansiumamide B (4) and lansiumamide C (2) display the highest antifungal activity against *S. sclerotiorum* with EC_{50} values of 4.95 and 13.24 μg/mL, which were both lower than the carbendazim ($EC_{50} = 0.64$ μg/mL), a commercial fungicide. Other two active compounds phenethyl cinnamide (1) and lansiumamide A (3) show a moderate antifungal activity ($EC_{50} = 56.66$ and 46.46 μg/mL, respectively) in comparison with carbendazim. While compounds 5–7 have no potency at the concentration of 100 μg/mL.

Observing and investigating the structures between bioactive and inactive compounds, some interesting findings of structure-activity relationships could be concluded (Fig. 5). Firstly, olefin group $\Delta^{2,3}$ is necessary to the activity, having been revealed by comparing compounds 5 with 6. Secondly, the *cis* configuration of olefin group $\Delta^{1',2'}$ is also critical to the activity, as could be seen from the inactive compound 5, whose configuration of olefin group $\Delta^{1,2}$ was *trans*. However, the existence of olefin $\Delta^{1',2'}$ group is not necessary for antifungal activity. This could be illustrated by the high activity of compound 1 with

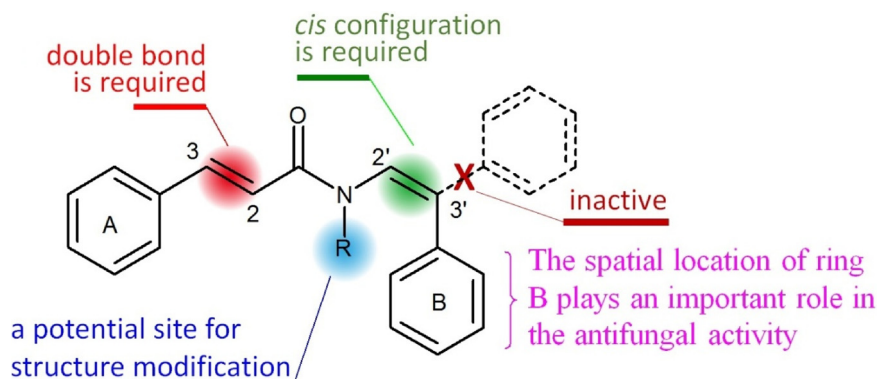


Fig. 5. The structure-activity analysis of the isolated amides.

a saturated bond at the same position. We hypothesize that the spatial location of benzene ring B may be key to the antifungal activity. Additionally, the substitution of nitrogen-atom could affect the antifungal activities of the amides, which could be highlighted by the compound **1** whose antifungal activity was significantly stronger than compound **3**. Taking all together, the unique substructures of olefin groups $\Delta^{2,3}$ and $\Delta^{1,2}$ play essential parts in the antifungal activity of amide alkaloids, while the nitrogen-atom could serve as the modified site for the improvement of the potency of primitive structures.

Electric conductivity is a characteristic parameter measuring the ionic strength of a solution, related to the ionic concentration of the solution. This research manifests that the relative electric conductivity of *S. sclerotiorum* is significantly increased after being treated with lansiumamide B, indicating that the treatment of lansiumamide B on *S. sclerotiorum* leads to the exosmosis of intracellular electrolyte and the increase of cell membrane permeability. These results are consistent with the change of mycelial morphology (Fig. 2e), which is a direct reflect for the increase of cell membrane permeability by micro-examination.

Many studies show that glycerol is the main factor for micro-organism to make osmotic adjustment (Gadd et al., 1984; Hocking and Norton, 1983; Lewis and Smith, 2010; Luard, 1982). Generally, glycerol and other osmotic stabilizers are synthesized and accumulated excessively in wild strains via activating the relevant signal transduction pathway during environmental stress, and thereby causing the massive influxes of external water that further results in the rupture and death of cells. The content of intracellular glycerol increased after the mycelium was treated with lansiumamide B, illustrating that it may be the mechanism of the antifungal activity of lansiumamide B on *S. sclerotiorum*.

Oxalate secretion is a main factor for the pathogenicity of *S. sclerotiorum* (Godoy et al., 1990; Kim et al., 2008; Lumsden, 1979), and it could enhance the pathogenicity of *Sclerotinia* in several ways (Dutton and Evans, 1996). Oxalate-deficient mutants of *S. sclerotiorum* are nonpathogenic and unable to produce sclerotia⁴². Here, the content of oxalic acid decreases significantly after the mycelium *S. sclerotiorum* is treated with lansiumamide B, which will be conducive to the decrease of pathogenicity and result in the failure of infection.

Plants are dependent on their antioxidant enzymes for defending themselves from infection, including peroxidase (POD) and phenylalanine ammonia-lyase (PAL), being used as the signal to respond the invasion of the pathogens (Bucheli and Robinson, 1994; Duan et al., 2013). In this study, the activities of POD and PAL are significantly increased after the strain was treated with lansiumamide B at the concentrations of 200 and 1000 $\mu\text{g}/\text{mL}$. These results demonstrate that lansiumamide B can also enhance the defense capacity of plants besides a direct action on pathogen.

The greenhouse experiment under a controlled experimental condition is routinely used to simulate the efficacy of fungicide in field. The results of the greenhouse experiment show that the most potent antifungal amide, lansiumamide B, exhibits a moderate protective activity and a remarkable curative activity against *S. sclerotiorum* on the leaves of oilseed rape and the curative activity of lansiumamide B is better than protective activity. Interestingly, although the antifungal activity of lansiumamide B *in vitro* ($\text{EC}_{50} = 4.95 \mu\text{g}/\text{mL}$) is nearly tenfold lower than carbendazim ($\text{EC}_{50} = 0.64 \mu\text{g}/\text{mL}$), the curative activity of lansiumamide B *in vivo* is significantly superior to carbendazim at the same concentration. These data suggest that lansiumamide B has a great potential to be exploited as a curative fungicide, and might have a novel mode of action different from carbendazim.

5. Conclusion

In conclusion, by means of bioassay guided method, seven amides were isolated and identified. Among the isolated compounds, compounds phenethyl cinnamide (**1**), lansiumamide C (**2**), lansiumamide A

(**3**) and lansiumamide B (**4**) showed different levels of *in vitro* antifungal activity against *S. sclerotiorum*. Based on the results of antifungal trials *in vitro*, several structure-activity relationships were discussed, which may be helpful in the further exploration of these bioactive compounds. The most potent compound lansiumamide B (**4**) *in vitro* led to the successive further study. The results of pot experiments showed that lansiumamide B possessed excellent control efficacy on leaves of oilseed rape, and the curative activity of lansiumamide B was significantly better than carbendazim at the same concentration. The results of physiological and biochemical experiment showed that the treatment with lansiumamide B led to glycerol accumulation and the enlargement of cell membrane permeability, and thereby causing the rupture and death of cells of *S. sclerotiorum*. In addition, lansiumamide B could enhance the defensive capacity of oilseed rape through increasing POD and PAL activity. And we are conducting further experiments to investigate the relevant mechanisms about this observed antifungal activity of bioactive compounds and promote the likely of those bioactive compounds for commercial applications.

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References

- Adebajo, A.C., Iwalewa, E.O., Obuotor, E.M., Ibikunle, G.F., Omisore, N.O., Adewunmi, C.O., Obaparusi, O.O., Klaes, M., Adetogun, G.E., Schmidt, T.J., Verspohl, E.J., 2009. Pharmacological properties of the extract and some isolated compounds of *Clausena lansium* stem bark: anti-trichomonal, antidiabetic, anti-inflammatory, hepatoprotective and antioxidant effects. *J. Ethnopharmacol.* 122, 10–19.
- Bardin, S.D., Huang, H.C., 2001. Research on biology and control of *Sclerotinia* diseases in Canada. *Can. J. Plant. Pathol.* 23, 88–98.
- Bayer, A., Maier, M.E., 2004. Synthesis of enamides from aldehydes and amides. *Tetrahedron* 60, 6665–6677.
- Berenbaum, M.R., 2002. Postgenomic chemical ecology: from genetic code to ecological interactions. *J. Chem. Ecol.* 28, 873–896.
- Boland, G.J., Hall, R., 1994. Index of plant hosts of *sclerotinia-sclerotiorum*. *Can. J. Plant. Pathol.* 16, 93–108.
- Bucheli, C.S., Robinson, S.P., 1994. Contribution of enzymatic browning to color in sugarcane juice. *J. Agric. Food Chem.* 42, 257–261.
- Campbell, C.W., 1974. Wampee, a fruit well adapted to southern Florida. *Proc. Florida State Hort. Soc.* 87, 390–392.
- Chokeprasert, P., Charles, A.L., Sue, K.H., Huang, T.C., 2007. Volatile components of the leaves: fruits and seeds of wampee *Clausena lansium* (Lour.) Skeels. *J. Food Compos. Anal.* 20, 52–56.
- Clarkson, J.P., Phelps, K., Whipps, J.M., Young, C.S., Smith, J.A., Watling, M., 2004. Forecasting *Sclerotinia* disease on lettuce: toward developing a prediction model for carpogenic germination of sclerotia. *Phytopathology* 94, 268–279.
- Davidson, A.L., Blahut-Beatty, L., Itaya, A., Zhang, Y., Zheng, S., Simmonds, D., 2016. Histopathology of *Sclerotinia sclerotiorum* infection and oxalic acid function in susceptible and resistant soybean. *Plant Pathol.* 65, 878–887.
- Du, Y.Q., Liu, H., Li, C.J., Ma, J., Zhang, D., Li, L., Sun, H., Bao, X.Q., Zhang, D.M., 2015a. Bioactive carbazole alkaloids from the stems of *Clausena lansium*. *Fitoterapia* 17, 1048–1053.
- Du, Y.Q., Liu, H., Li, C.J., Yang, J.Z., Ma, J., Zhang, D., Sun, H., Zhang, D.M., 2015b. Carbazole and amide alkaloids from the stems of *Clausena lansium*. *J. Asian Nat. Prod. Res.* 17, 1048–1053.
- Duan, Y., Liu, S., Ge, C., Feng, X., Chen, C., Zhou, M., 2012. *In vitro* inhibition of *Sclerotinia sclerotiorum* by mixtures of azoxystrobin, SHAM, and thiram. *Pestic. Biochem. Phys.* 103, 101–107.
- Duan, Y., Ge, C., Liu, S., Chen, C., Zhou, M., 2013. Effect of phenylpyrrole fungicide fludioxonil on morphological and physiological characteristics of *Sclerotinia sclerotiorum*. *Pestic. Biochem. Phys.* 106, 61–67.
- Dubey, N.K., Srivastava, B., Kumar, A., 2008. Current status of plant products as botanical pesticides in storage pest management. *J. Biopestic.* 1, 182–186.
- Dutton, M.V., Evans, C.S., 1996. Oxalate production by fungi: its role in pathogenicity and ecology in the soil environment. *Can. J. Microbiol.* 42, 881–895.
- Firoz, M.J., Xiao, X., Zhu, F.X., Fu, Y.P., Jiang, D.H., Schnabel, G., Luo, C.X., 2016. Exploring mechanisms of resistance to dimethachlone in *Sclerotinia sclerotiorum*. *Pest Manag. Sci.* 72, 770–779.
- Gadd, G.M., Chudek, J.A., Foster, R., Reed, R.H., 1984. The osmotic responses of penicillium ochro-chloron: changes in internal solute levels in response to copper and salt stress. *Microbiology* 130, 1969–1975.
- Gerwick, B.C., Sparks, T.C., 2014. Natural products for pest control: an analysis of their

- role, value and future. *Pest Manag. Sci.* 70, 1169–1185.
- Godoy, G., Steadman, J.R., Dickman, M.B., Dam, R., 1990. Use of mutants to demonstrate the role of oxalic acid in pathogenicity of *Sclerotinia sclerotiorum* on *Phaseolus vulgaris*. *Physiol. Mol. Plant Pathol.* 37, 179–191.
- Hocking, A.D., Norton, R.S., 1983. Natural-abundance ¹³C nuclear magnetic resonance studies on the internal solutes of xerophilic fungi. *Microbiology* 129, 2915–2925.
- Huang, H.C., Erickson, R.S., Phillippe, L.M., Mueller, C.A., Sun, S.K., Huang, J.W., 2006. Control of apothecia of *Sclerotinia sclerotiorum* by soil amendment with S-H mixture or Perlka (R) in bean, canola and wheat fields. *Soil Biol. Biochem.* 38, 1348–1352.
- Huei, L.J., 1989. Cinnamamide derivatives from *Clausena lansium*. *Phytochemistry* 28, 621–622.
- Kim, K.S., Min, J.Y., Dickman, M.B., 2008. Oxalic acid is an elicitor of plant programmed cell death during *Sclerotinia sclerotiorum* disease development. *Mol. Plant Microbe Interact.* 21, 605–612.
- Kora, C., McDonald, M.R., Boland, G.J., 2005. Epidemiology of *sclerotinia* rot of carrot caused by *Sclerotinia sclerotiorum*. *Can. J. Plant. Pathol.* 27, 245–258.
- Kuang, J., Hou, Y.P., Wang, J.X., Zhou, M.G., 2011. Sensitivity of *Sclerotinia sclerotiorum* to fludioxonil: in vitro determination of baseline sensitivity and resistance risk. *Crop Prot.* 30, 876–882.
- Kumar, V., Vallipuram, K., Adebajo, A.C., Reisch, J., 1995. 2,7-Dihydroxy-3-formyl-1-(3'-methyl-2'-butenyl)carbazole from *Clausena lansium*. *Phytochemistry* 40, 1563–1565.
- Letham, D., Huett, D., Trimboli, D., 1976. Biology and control of *Sclerotinia sclerotiorum* in cauliflower and tomato crops in coastal New South Wales. *Plant Dis. Rep.* 60, 286–289.
- Lewis, D.H., Smith, D.C., 2010. Sugar alcohols (polyols) in fungi and green plants: i. distribution physiology and metabolism. *New Phytol.* 66, 185–204.
- Liang, H.J., Di, Y.L., Li, J., Zhu, F.X., 2015. Baseline sensitivity and control efficacy of fluazinam against *Sclerotinia sclerotiorum*. *Eur. J. Plant Pathol.* 142, 691–699.
- Lin, J.H., 1989. Cinnamamide derivatives from *Clausena lansium*. *Phytochemistry* 28, 621–622.
- Liu, H., Li, F., Li, C.J., Yang, J.Z., Li, L., Chen, N.H., Zhang, D.M., 2014. Bioactive furanocoumarins from stems of *Clausena lansium*. *Phytochemistry* 107, 141–147.
- Liu, J., Li, C.J., Ni, L., Yang, J.Z., Li, L., Zang, C.X., Bao, X.Q., Zhang, D., Zhang, D.M., 2015. Anti-inflammatory alkaloid glycoside and quinoline alkaloid derivatives from the stems of *Clausena lansium*. *RSC Adv.* 5, 80553–80560.
- Luard, E.J., 1982. Effect of osmotic shock on some intracellular solutes in two filamentous fungi. *Microbiology* 128, 2575–2581.
- Lumsden, R.D., 1979. Histology and physiology of pathogenesis in plant disease caused by *Sclerotinia* species. *Phytopathology* 69, 890–896.
- Ma, H.X., Feng, X.J., Chen, Y., Chen, C.J., Zhou, M.G., 2009. Occurrence and characterization of dimethachlon insensitivity in *Sclerotinia sclerotiorum* in jiangsu province of China. *Plant Dis.* 93, 36–42.
- Maneerat, W., Tha-in, S., Cheenpracha, S., Prawat, U., Laphookhieo, S., 2011. New amides from the seeds of *Clausena lansium*. *J. Med. Plants Res.* 5, 2812–2815.
- Maneerat, W., Ritthiwigrom, T., Cheenpracha, S., Laphookhieo, S., 2012. Carbazole alkaloids and coumarins from *Clausena lansium* roots. *Phytochem. Lett.* 5, 26–28.
- Milner, P., Coates, N., And, M., Spear, S., Eggleston, D., 1996. SB-204900, a novel oxirane carboxamide from *Clausena lansium*. *J. Nat. Prod.* 59, 400–402.
- Purdy, L.H., 1979. *Sclerotinia sclerotiorum*: history, diseases and symptomatology, host range, geographic distribution and impact. *Phytopathology* 69, 875–880.
- Que, S., Zhao, Y.Y., Zhang, Q.Y., 2008. Studies on chemical constituents of Tibetan medicine *Caryopteris toroetii*. *China J. Chin. Mater. Med.* 33, 2639–2641.
- Ronicke, S., Hahn, V., Friedt, W., 2005. Resistance to *Sclerotinia sclerotiorum* of 'high oleic' sunflower inbred lines. *Plant Breed.* 124, 376–381.
- Soylu, S., Yigitbas, H., Soyulu, E.M., Kurt, S., 2007. Antifungal effects of essential oils from oregano and fennel on *Sclerotinia sclerotiorum*. *J. Appl. Microbiol.* 103 (4), 1021–1030.
- Tatsimo, S.J.N., Tamokou, J.D.D., Lamshoef, M., Mouafo, F.T., Lannang, A.M., Sarkar, P., Bag, P.K., Spitteller, M., 2015. LC-MS guided isolation of antibacterial and cytotoxic constituents from *Clausena anisata*. *Med. Chem. Res.* 24, 1468–1479.
- Wang, Y., Duan, Y.B., Zhou, M.G., 2016a. Baseline sensitivity and efficacy of fluazinam in controlling *Sclerotinia* stem rot of rapeseed. *Eur. J. Plant Pathol.* 144, 337–343.
- Wang, Y., Sun, Y., Zhang, Y., Zhang, X., Feng, J., 2016b. Antifungal activity and biochemical response of cuminic acid against *Phytophthora capsici* leoniana. *Molecules* 21, 755–767.
- Wang, Y., Sun, Y., Zhang, Y., Zhang, Y., Han, L., Zhang, X., Feng, J., 2017. Sensitivity and biochemical characteristics of *Sclerotinia sclerotiorum* to propamidine. *Pestic. Biochem. Phys.* 135, 82–88.
- Ziv, A., Cilia, L.F., 2007. Accelerated biodegradation of pesticides: an overview of the phenomenon, its basis and possible solutions; and a discussion on the tropical dimension. *Crop Prot.* 26, 1733–1746.