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The Phytochemicals with Antagonistic Activities Toward Pathogens of a Disease Complex Caused by *Meloidogyne incognita* and *Ralstonia solanacearum*

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Application of phytochemical compounds with dual functional activities against pathogens is a novel strategy to control the disease complexes. Targeting a complex disease of tobacco caused by *Meloidogyne incognita* and *Ralstonia solanacearum*, the present study identified four active compounds from stem of a medical plant *Daphne acutiloba* which with bioactivities against the both pathogens. At a concentration of 25 μg ml^{-1}, daphneone 2 (1), daphneolon (2), daphnodorin A (3) and daphnodorin B (4) displayed nematicidal activities of 40.23-70.6%, and showed antibacterial circle diameters of 4.04-8.09 mm.

Key words: *Daphne acutiloba*; Disease complexes; Phytochemicals; Soilborne pathogens.

Plant-parasitic nematodes are one of the most damaging pathogens in agriculture. Only the root-knot nematodes (*Meloidogyne* spp.) cause an estimated US$100 billion loss annually to a wide variety of cultivated crops worldwide [1]. These phytopathogens infect crop roots and induce gall formation, which impedes normal uptake of water and nutrients, and facilitates the infection of some soil-borne phytopathogens [2,3]. Currently, at least 20 cases of disease complexes involving plant-parasitic nematodes and soilborne pathogens have been reported [4-6]. *Ralstonia solanacearum* is a destructive bacterial phytopathogen which can infect the hosts more 200 species plants [7]. The mechanisms underlying synergistic interactions and the significant role of nematodes in the development of disease complexes have been demonstrated which including [5]: utilization of nematode-induced wounds by soilborne pathogens; nematode-induced physiological changes to the host plant; modifications within the rhizosphere; reduction of host resistance; pathogen- induced changes to the host plant.

Management of the disease complexes involving nematodes appears to be less straightforward than one might anticipate. Current

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solution still relies on the use chemical pesticides respectively against each of the pathogens. However, the reduction of one pathogen may not resolve the problem of the interaction, as even low densities of each pathogen can also result in the disease complex [8]. The dose of chemical nematicide used to control the disease complex caused by *M. incognita* and *Thielaviopsis basicola* in cotton were 30-50% higher than where the disease infected by nematode alone [9]. A number of alternative strategies have been investigated for the management of disease complexes, including plant breeding, cultural practices such as multiyear cropping regimes and soil solarization, and application of biocontrol microorganisms [5]. These integrated approaches targeting both pathogens appear to be the most promising way of defeating disease complexes involving nematodes.

Application of phytochemical compounds with dual functional activities against pathogens is a novel strategy to control the disease complexes. However, none of these phytochemical compounds has been identified. In the southwest of China, disease complex caused by *M. incognita* and *R. solanacearum* in tobacco is popular [4]. By targeting pathogens of this disease complex, this study firstly reported those functional compounds from a medical plant *Daphne acutiloba* Rehd. (Thymelaeceae).

MATERIALS AND METHODS

Plant material

The stems of *D. acutiloba* were collected in Hexi country Yunnan Province, China, and identified by Prof. H. Sun and Dr. Yue L.L. of Kunming Institute of Botany, Chinese Academy of Sciences. Voucher specimen (HUANG0004) was deposited at the State Key Laboratory of Phytochemistry and Plant Resources in West China, Kunming Institute of Botany, Chinese Academy of Sciences, P. R. China.

Extraction and isolation of compounds

The stems of *D. acutiloba* (7 kg dry weight) were crushed and extracted with EtOH (50 L). The EtOH extract was concentrated to a gum, dissolved in water and extracted thoroughly with petrol ether (3 × 5 L). The petrol ether-soluble portion was concentrated to a gum (304 g) which was chromatographed on a silica gel column (Qingdao Marine, 200–300 mesh, 7.0 Kg). The elution of the column was initiated with $CHCl_3$ to afford sub-fractions A1-A8. The sub-fraction A1 (56.22 g) was again subjected to chromatographic column (CC) using RP-18 (Merck, 40-75 mM, MeOH/H_2O, 2:3, 5:5, 3:2, 4:1, 9:1, v/v), and Sephadex LH-20 ($CHCl_3$/MeOH 1:1) to afford **1** (28.3 g), **2** (5.6 g). The aqueous layer of fraction A8 (26.2 g) was extracted with methanol and dried as a crude mixture which was chromatographed on a Si-gel column (Qingdao Marine, 200-300 mesh, 300 g, $CHCl_3$/MeOH, 8: 1, 4: 1, 2:1, v/v) to yielded sub-fractions B1-B3. B2 (13.60 g) was chromatographed on a RP-18 (Merck, 40-75 mM, MeOH/H_2O, 2:3, 5:5, 3:2, v/v) and preparative TLC ($CHCl_3$/MeOH 6 : 1) to afford **3** (8.2 g). B3 (7.23 g) was chromatographed on a RP-18 (Merck, 40-75 mM, MeOH/H_2O, 2:3, 5:5, 3:2, v/v) and Sephadex LH-20 (MeOH) to afford **4** (4.2 g).

General experimental procedures for identification of compounds. NMR spectra were acquired on a Bruker AV-400, a DRX-500, or AVANCE III-600 spectrometer with TMS as an internal standard, whereas ESIMS was obtained using an API QSTAR Pulsar 1 spectrometer. EIMS was recorded with a Waters Autospec Premier. Silica gel (200–300 mesh, Qingdao Marine Chemical Inc., People's Republic of China), RP-18 (40–70 mm, Merck, Germany) and Sephadex LH-20 (Amersham Biosciences, Sweden) were used for column chromatography (CC). Semipreparative HPLC was performed on an Agilent 1100 liquid chromatograph with a Zorbax SB-C18, 9.4 mm × 25 cm, column. Fractions were monitored by TLC and spots were visualized by heating after spraying with 10% H_2SO_4 in ethanol (B.P. 77–79°C).

Bioassay of compounds towards phytopathogens. Juveniles of *M. incognita* were collected from the infected tobacco roots by Baermann-funnel method [10]. Chemical samples were dissolved in dimethylmethane, and then diluted with sterilized water containing 0.3% (v/v) Tween-20 to obtain a stock solution with concentration of 25 mg/ml. In a Petri dish (6 cm diameter) containing 300 µl nematode suspension (about 200 juveniles), a suitable volume of sample solution was added and gently mixed to obtain the final concentration of 25µg ml^{-1}. The same amount of dimethylmethane dissolved in water containing 0.3% (v/v) Tween-20 was established as control. All treatments were

conducted in triplicate and replicated three times. Nematicidal activity (NA) was assessed and calculated using the formula after incubation at 28ºC for 24 h: NA = DN/SN × 100% (DN: number of dead nematodes, SN: sum of all counted nematodes, SN > 100).

Tobacco pathogen *R. solanacearum*, kindly provided by Dr. DH Fang of Yunnan Tobacco Agriculture Science Research Institute, was severed as target for antagonistic screening. The pathogen was inoculated into NA without agar medium (beef extract 3 g, peptone 10 g, NaCl 5 g, water 1000 ml, pH 7.2). After incubation at 120 rpm, 32°C for 48h, a volume of 200 µl culture with cell concentration about 10^8 cfu ml^{-1} was mixed with 250 mL melt NA and poured equably into 10 Petri dishes. A volume of 5 µl chemical solution with concentration of 25 µg ml^{-1} was added on the surface of plate after solidification. The same amount of dimethylmethane dissolved in water containing 0.3% (v/v) Tween-20 was established as control. All treatments were conducted in triplicate. After incubation 48 h at 37°C, antibacterial diameters (Ads) was measured and used to express the antibacterial efficiency of compounds.

RESULTS AND DISCUSSION

Totally, 41 compounds were isolated from stem of *D. acutiloba* and used for bioassay using *M. incognita* and *R. solanacearum* as targets. Of them, 4 compounds (**1**-**4**) displayed antagonistic activities towards the both pathogens. These active compounds were identified as daphneone 2 (**1**) [11], daphneolon (**2**) [12], daphnodorin A (**3**) [13] and daphnodorin B (**4**) [13] by NMR and MS data and comparison of their data with those in the literatures (Figure 1).

At the tested concentration of 25 µg×ml^{-1}, the four active compounds showed antibacterial activity towards *R. solanacearum* with Ad values of 4.04-8.09 mm. Aaphnodorin A (**3**) exhibited the highest antibacterial activity (Ad = 8.09 mm), followed by daphneone 2 (**1**) and daphneolon (**2**), which showed similar activities with Ads of 6 mm and 6.72 mm, respectively. At same tested concentration, the chemicals 1-4 displayed nematicidal activity of 49.15%, 70.6%, 40.23% and 45.63%, respectively. Of the four active compounds, daphneolon (**2**) showed the relatively stronger bioactivities to the both pathogens (Ad = 6.72 mm, NA = 70.6%).

Higher plants had been reported to yield a broad spectrum of nematocidal compounds, including polythienyls, isothiocyanates, glucosinolates, cyanogenic glycosides, polyacetylenes, alkaloids, lipids, terpenoids, sesquiterpenoids, diterpenoids, quassinoids, steroids, triterpenoids, simple and complex phenolics, and several other classes [14]. Some extracts from plants were reported to exhibit antibacterial activity against the phytopathogen *R. solanacearum*, but few compound has been identified, such as *Boerhaavia diffusa* [15], pomegranate fruit peel and beleric myrobalan fruit [16].

D. acutiloba is a traditional folk medical plant widely distributed in the southwest of China, and was used as biotic pesticide in Lisu minority communities for disinfecting and desinsection [17]. The Thymelaeceae plants were claimed to be a good medicine against tumor [12], inflammation [18], antihyperglycemics [19] and neurotrophics [13].

Table 1. Compounds from *D. acutiloba* with antagonistic activities both towards *M. incognita* and *R. solanacearum* at texted concentration of 25 µg×ml^{-1}

Compound (No.)	Ads to *R. solanacearum* (mm)	NAs to *M. incognita* (%)
Daphneone 2 (**1**)	6.00±0.23 b	49.15±2.51 c
Daphneolon (**2**)	6.72±0.18 b	70.60±3.28 b
Daphnodorin A (**3**)	8.09±0.64 a	40.23±1.85 e
Daphnodorin B (**4**)	4.04±0.31 d	45.63±2.36 d

Results followed by the same letter in the columns are not significantly different ($P < 0.05$) according to ANOVA.

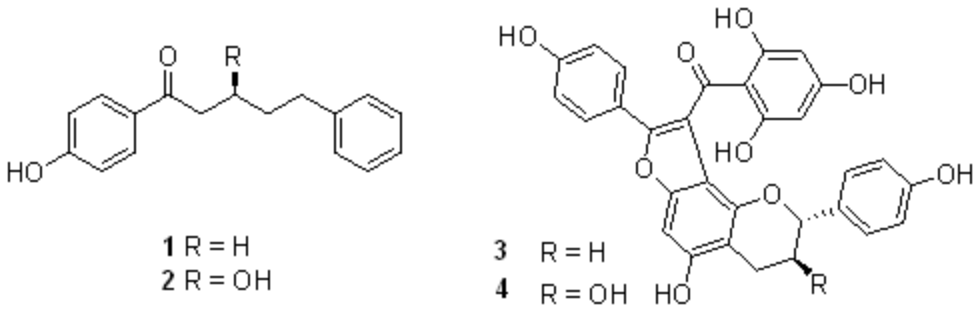

Fig. 1. Structures of active compounds **1**-**4**

Previous studies focused on the common species of *Daphne*, such as *D. genkwa* and *D. odora*, had reported a series of lignans [20,21] and biflavonoids [22]. At present, some compounds have been identified from *D. acutiloba*. Some of them, such as daphnenin and caffeic acid *n*-octadecyl ester, showed anti-HIV activity [23-25]. Previously, only two daphane diterpenoids (odoracin and odoratrin) from *D. odora* were reported to show nematicidal activities toward phytoparasitic nematode *Aphelenchoides besseyi* [26]. In this investigation, two lignan compounds (**1**, **2**) and two biflavonoid compounds (**3**, **4**) from *D. acutiloba* were found to possess inhibition activities against phytopathogens *M. incognita* and *R. solanacearum*.

By targeting the complex disease of tobacco caused by *M. incognita* and *R. solanacearum*, four compounds exhibiting nematicidal and antibacterial activities have been identifies from medical plant *D. acutiloba*, which including daphneone 2, daphneolon, daphnodorin A and daphnodorin B. These compounds showed the potential to be developed for use as bioagents, or they could serve as model compounds for the development of chemically synthesized derivatives with enhanced activity or environmental friendliness. This was the first report on the phytochemical compounds with dual antagonistic activities on the pathogens involved in disease complexes.

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