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Antifungal properties of pristimerin and celastrol isolated from *Celastrus hypoleucus*

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Abstract: Pristimerin and celastrol isolated from the roots of *Celastrus hypoleucus* (Oliv) Warb f *argutior* Loes exhibited inhibitory effects against diverse phytopathogenic fungi. Pristimerin and celastrol were found to inhibit the mycelial growth of *Rhizoctonia solani* Kühn and *Glomerella cingulata* (Stonem) Spauld & Schrenk *in vitro* by 83.6 and 62.6%, respectively, at 10 μg ml^{-1}. Pristimerin showed good preventive effect (96.7% at 100 μg ml^{-1}) and curative effect (66.5% at 100 μg ml^{-1}) against wheat powdery mildew *in vivo*. For celastrol, the preventive and curative effects against wheat powdery mildew were 80.5 and 45.4%, respectively, at 100 μg ml^{-1}.

Keywords: pristimerin; celastrol; *Celastrus hypoleucus*; antifungal activity; phytopathogenic fungi

1 INTRODUCTION

New agrochemicals developed from natural products are considered likely to be more environmentally acceptable than those presently used.[1] Natural products have been the source of many pesticides, used either directly as crude preparations or as pure compounds. They have also been used as structural leads for the discovery and development of natural product-based pesticides.[2] Plants, one of main biological resources, are still a largely unexplored source of new agricultural chemicals.

The plants of Celastraceae family have been used as important folk medicines in China for a long time and also as natural insecticides.[3–5] *Celastrus hypoleucus* (Oliv) Warb f *argutior* Loes, which is widely distributed in China, has been used in the treatment of inflammation and detumescence.[6] Although there are reports in the literature of the isolation of many components, such as terpenoids, alkaloids and others, from Celastraceae,[7] phytochemical examination of *C hypoleucus* has been little reported. In the our previous paper,[8] we reported the structures of the compounds β-amyrin, 12-oleanaen-3β-caffeate, 9(11),12-oleanadien-3β-ol, 9(11),12-oleanadien-3-one, 9(11),12-oleanadien-3β-caffeate, friedelin, friedela-3-one-29-ol, β-sitosterol, octadecadienoic acid, *n*-octadecyl 3,4-dihydroxycinnamate and *n*-octadecyl caffeate isolated from the aerial part of *C hypoleucus*.

In the course of our screening for antifungal agents from plants, pristimerin and celastrol were isolated from the roots of *C hypoleucus* by bioassay-guided fractionation. Although pristimerin and celastrol had been isolated and characterized previously,[9–11] and their insecticidal and antifeedant effects against *Sitophilus zeamais* (Motsch) and *Cydia pomonella* L,[12,13] antibacterial activity,[14] cytotoxic properties[15] and antimalarial activity[16] were reported, there are no data reported concerning activity against plant pathogenic fungi. In this paper the isolation, identification and antifungal activities against phytopathogenic fungi *in vitro* and *in vivo* of pristimerin and celastrol are described.

2 MATERIALS AND METHODS

2.1 General

^{1}H NMR (400 MHz, deuterochloroform) and ^{13}C NMR (100 MHz, deuterochloroform) were recorded on a Bruker AM-400 spectrometer with TMS as internal standard. Mass spectra were obtained on an Ahp-5988 MS spectrometer. Silica gel (200–300 mesh) was used for column chromatography. Thin layer chromatography (TLC) was done on precoated silica gel plates (GF_{254}). Spots were detected on TLC under UV or by heating after spraying with 5% sulfuric acid in ethanol.

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2.2 Materials

The roots of *C hypoleucus* f *argutior* Loes were collected in Xiaolong Mountain, Gansu Province of China in October 2000, and identified by Professor J Zhi, Lanzhou University, China. A voucher specimen (No 2001001) was deposited in the Department of Biology, Lanzhou University, China. Commercial mancozeb 700 g kg^{-1} WP and triadimefon 150 g kg^{-1} WP (both from Hebei Shuangji Chemical Co Ltd, China) were used as positive controls at 100 and 500 μg AI ml^{-1}, respectively.

2.3 Extraction and isolation

Antifungal compounds were isolated from the roots of *C hypoleucus* by bioassay-guided fractionation as outlined in Fig 1. The antifungal activity of each fraction obtained in the course of isolation was measured by the mycelial growth inhibition test described in Section 2.4.1. To measure the antifungal activity of each fraction, the solvent of each fraction was removed by evaporation under vacuum. The residues were dissolved in dimethyl sulfoxide (DMSO; 20–40 mg ml^{-1}) as stock solutions. These stock solutions were diluted with potato dextrose agar (PDA) medium (Difco) to prepare the prescribed concentrations before use.

Dried, powdered roots (1.2 kg) of *C hypoleucus* were extracted with acetone under reflux for 12 h using a Soxhlet extractor. The solution was evaporated under vacuum, and the residue (100 g) was subjected to column chromatography over 2000 g of silica gel (200–300 mesh), eluting with petroleum ether + acetone from 20 + 1 to 0 + 1 by volume to give 20 fractions. Fractions 2 and 8 which showed antifungal activity were selected for further study for the active compounds. Fraction 2 (2.0 g), eluted with petroleum ether + acetone (10 + 1 by volume), was crystallized from petroleum ether to give active compound **1** (113 mg, $R_f = 0.45$, petroleum ether + acetone, 5 + 1 by volume). Fraction 8 (3.0 g), eluted with petroleum ether + acetone (3 + 1 by volume), was subjected to column chromatography (30 × 3 cm column) using 30 g silica gel and eluted with petroleum ether + acetone (15 + 1 to 0 + 1 by volume, 300 ml for each fraction) to give active compound **2** (42 mg, $R_f = 0.26$, petroleum ether + acetone, 4 + 1 by volume). All the experiments were monitored by bioassay against *Rhizoctona solani*.

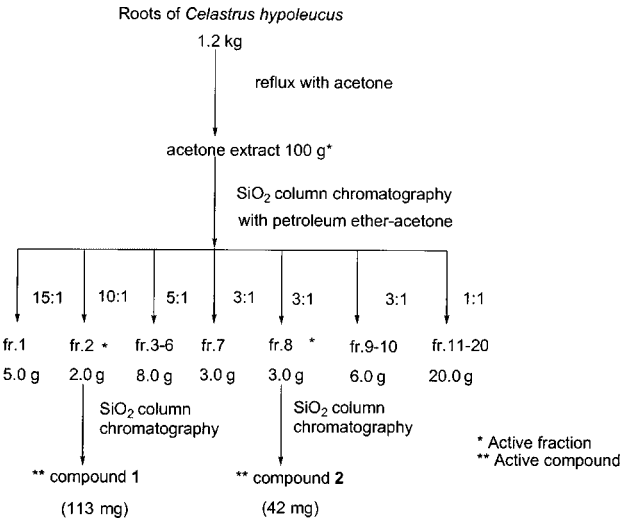

Figure 1. Scheme for the isolation of the antifungal compounds from *Celastrus hypoleucus*.

2.4 *In vitro* assay

2.4.1 *Mycelial growth inhibition test*

The isolated compounds dissolved in acetone were tested for antifungal activity *in vitro* by a Poison Food Technique.[17] Potato dextrose agar (PDA) medium was used as the medium for all test fungi. The pathogenic fungi were *Pyricularia oryzae* Br & Cav, *Phytophthora capsici* Leonian, *Alternaria brassicae* (Berk) Sacc, *Botrytis cinerea* Pers, *Collectotrichum lagenarium* (Pass) Ell & Halst, *Rhizoctonia ceralis* Vander Hoeven, *Rhizoctonia solani* Kühn, *Fusarium oxysporum* f sp *vasinfectum* (Atk) Snyder & Hansen, *Fusarium graminearum* Schw, *Glomerella cingulata* (Stonem) Schr & Spauld, respectively.

The medium incorporating the test compounds at a concentration of 10 μg ml^{-1} was inoculated at the centre with agar discs of the test fungi (5 mm diameter). Three replicate plates of each compound for each fungus were incubated at 26 (±2) °C. Control plates containing media mixed with acetone (10 ml liter^{-1}) were included. The treated and control dishes were kept in laboratory at 26 (±2) °C until the fungal growth in the control dishes was almost complete, and the mycelial growth of fungi in both treated (T) and control (C) Petri dishes was measured diametrically (mm) in three different directions and the percentage growth inhibition (I) was calculated using the formula:[18]

$$I\ (\%) = [(C - T)/C] \times 100$$

Analysis of variance was performed on the data with the PROCGLM procedure (SAS Institute, Cary, NC, USA). If $P > F$ was less than 0.01, means were separated with the least significant different (LSD) test at the $P = 0.05$ level.

2.4.2 *Spore germination test*

Botrytis cinerea was cultured on potato sucrose agar (PSA; 20% potato extract, 2% sucrose, 2% agar) at 22.5 °C in darkness. After incubation for 5 days, the aerial hyphae were removed by washing with a brush and sterilized distilled water. The culture surfaces were allowed to dry for 30 min and re-incubated for 3 days at the same temperature under continuous near ultraviolet irradiation of 360 MW cm^{-2} that promoted synchronous spore formation. Spore suspensions were prepared and adjusted to 2 × 10^5 spores ml^{-1} with a haemocytometer.

Pyricularia oryzae was grown on oatmeal-agar medium at 25 °C in the dark for 7–10 days. Spores

were collected by agitating with distilled water from 2-day-old cultures under fluorescent light after removal of spores from the culture plate. The spore suspension was filtered through tissue paper and adjusted with a haemocytometer to a density of about 1×10^5 spores ml^{-1} by addition of distilled water.

Tests for inhibition of *B cinerea* and *P oryzae* spore germination were done on microslides.[17] Spore suspension (30 µl) was added to 30 µl of the test compound in acetone (50, 100, 300, 600, 800 µg ml^{-1}), mixed well with a toothpick and incubated at 25 °C in the dark for 5 h. One hundred spores from each of five replicates were examined under a light microscope to determine the percentage of germinated spores. The experiment was repeated three times and results presented as mean values ± standard deviation.

2.5 *In vivo* assay

In order to investigate further the *in vivo* antifungal activities of the isolated compounds such as the duration of protection and curative activity, two plant diseases of wheat powdery mildew (*Erysiphe graminis* DC f sp *tritici* Marchal) and cucumber downy mildew (*Pseudoperonospora cubensis* (Berk & MA Curtis) Rostovzev) were used in the test. Wheat (*Triticum aestivum* L, cv Chokwang) and cucumber (*Cucumis sativus* L, cv Hausbackdadagi) were grown in vinyl pots (4.5 cm diameter) in a greenhouse for one to four weeks. The potted plants were randomly arranged in two groups in a greenhouse and watered twice daily with tap water. The potted plant seedlings were sprayed with the test compounds dissolved water + acetone (95 + 5 by volume) containing Tween 20 (250 µg ml^{-1}) as wetter and allowed to stand for 24 h.

For the test for preventative effects, the plants in the first group were inoculated with the pathogen of the plant disease 1 day after spraying with pristimerin, celastrol or commercial fungicides at doses of 500 and 100 µg ml^{-1}. For the test for curative effects, the plants in the second group were inoculated with the pathogen of the plant disease 1 day before the application of pristimerin, celastrol or commercial fungicides at doses of 500 and 100 µg ml^{-1}. Control plants within each group were similarly inoculated with sterile distilled water + acetone containing Tween 20 (250 µg ml^{-1}).

For wheat powdery mildew, the wheat seedlings at the first stage treated with either pristimerin, celastrol or triadimefon as above were inoculated with *E graminis* by dusting with dry inoculum from diseased plants. The inoculated wheat seedlings were incubated for 7 days at 20 (±1) °C and 60% RH during the day and 18 (±1) °C at night with 16 h of daylight per day in a artificial climate chamber (RP-300, China), and the disease severity was then determined. The disease severity was recorded on a 0–5 scale, with 0: no colonies visible to the unaided eye; 2: a few scattered, small discrete colonies; 3: colonies merging to form larger mildew lesions; 4: mildew covering half the total leaf surface and 5: mildew covering the total leaf surface.[19]

For cucumber downy mildew, the cucumber plants grown for four weeks and treated with either pristimerin, celastrol or mancozeb (positive control) as above were inoculated with *P cubensis* sporangia (5×10^4 sporangia per ml). The inoculated cucumber plants were kept in the dark for 1 day at 25 (±2) °C and 100% RH and then transferred to an artificial climate chamber at 25 (±2) °C and 70–80% RH with 12 h of daylight per day. Disease severity was recorded 5 days after inoculation. Cucumber downy mildew was on a scale of 0–9 with 0: no necrosis, leaf area is was completely healthy; 1:0–5% of the leaf area shows symptoms; 3:6–10% of the leaf area shows symptoms; 5:11–25% of the leaf area shows symptoms; 7:26–50% of the leaf area shows symptoms; 9: more than 50% of the leaf area shows symptoms.[20]

Pots were arranged as a randomized complete block with three replicates per treatment. The experiment was conducted twice and values are expressed as percentage control (±SD) compared with the control.[21]

The percentage disease incidence was determined using the formulae:

$$\%\ \text{disease incidence} = [(\Sigma\ \text{scale} \times \text{number of plants infected})/(\text{highest scale} \times \text{total number of plants})] \times 100$$

$$\text{preventive or curative effects}\ (\%) = [(\text{disease incidence of the control} - \text{disease incidence of treatment})/\text{disease incidence of the control}] \times 100$$

Analysis of variance was performed on the data with the PROC GLM procedure (SAS Institute, Cary, NC, USA). If $P > F$ less than 0.01, means were separated with the least significant different (LSD) test at the $P = 0.05$ level.

3 RESULTS AND DISCUSSION

3.1 Structure elucidation

The active compounds **1** and **2** isolated from the roots of *C hypoleucus* were identified by means of their IR, MS and NMR spectral data as pristimerin and celastrol, respectively: (Fig 2).

*3.1.1 Pristimerin (**1**; $C_{30}H_{40}O_4$)*

Amorphous orange–red solid; mp: 216–218 °C (lit 219–220 °C); IR (potassium bromide) cm^{-1}: 3346, 2941, 1723, 1630, 1589, 1518, 1439, 1374, 863; UV (ethanol) nm: 206, 422; EIMS (70 ev) *m/z* (%): 464 [M^+] (15), 241 (100), 203 (16), 202 (25); FABMS:

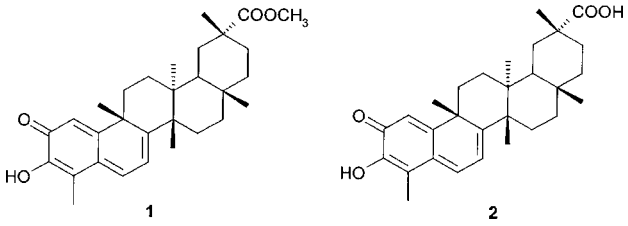

Figure 2. Structures of pristimerin (**1**) and celastrol (**2**).

465[M + 1$^+$]. The ^{1}H and ^{13}C spectral data are identical with the data previously reported.[9]

*3.1.2 Celastrol (**2**; $C_{29}H_{38}O_4$)*

Yellow needle crystals; mp: 198–201 °C (lit 205 °C); IR (potassium bromide) cm^{-1}: 3352, 2944, 1704, 1636, 1586, 1548, 1510, 870; UV (ethanol) nm: 207, 425; EIMS (70 ev) *m/z* (%): 450 [M$^+$] (22), 253 (15), 241 (36), 215 (15), 214 (18), 203 (19), 202 (58), 201 (100), 162 (10); FABMS: 451 [M$^+$ + 1].

The ^{1}H and ^{13}C NMR spectral data were in agreement with the data reported in literature.[11]

3.2 Effect on mycelial growth of phytopathogenic fungi *in vitro*

Pristimerin (**1**) and celastrol (**2**) were found to inhibit the mycelial growth of some plant pathogenic fungi *in vitro* (Fig 3). Pristimerin inhibited the mycelial growth of *Rhizoctonia solani* by 83.6% at 10 μg ml^{-1} *Glomerella cingulata* was the most sensitive to celastrol, with 62.2% inhibition of mycelial growth 10 μg ml^{-1}.

3.3 Effect on spore germination of *Botrytis cinerea* and *Pyricularia oryzae in vitro*

The germination of *B cinerea* and *P oryzae* spores on slides after 5 h showed that pristimerin and celastrol

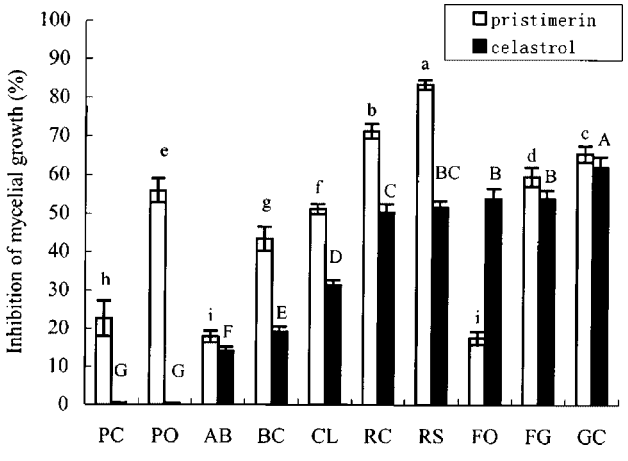

Figure 3. Antifungal activity spectrum of pristimerin (**1**) and celastrol (**2**) at 10 μg ml^{-1} against ten pathogenic fungi. PO: *Pyricularia oryzae*; PC: *Phytophthora capsici*; AB: *Alternaria brassicae*; BC: *Botrytis cinerea*; CL: *Collectotrichum lagenarium*; RC: *Rhizoctonia ceralis*; RS: *Rhizoctonia solani*; FO: *Fusarium oxysporum* f sp *vasinfectum*; FG: *Fusarium graminearum*; GC: *Glomerella cingulata.* Error bars represent the standard error of the mean of five replicates. Means followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

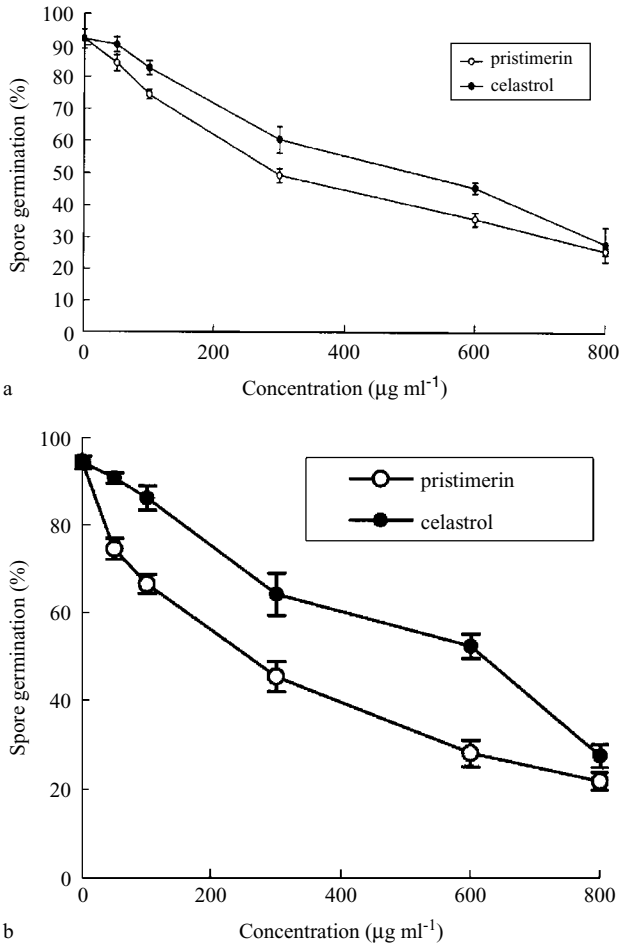

Figure 4. Inhibition of spore germination of (a) *Botrytis cinerea* and (b) *Pyricularia oryzae* by pristimerin and celastrol. Error bars represent the standard error of the mean of three replicates. The activity of each compound was determined by measuring of the percentage spore germination (considered inhibited when length was less than 1.5 times the length of the spore).

had similar inhibitory effects on the germination of *B cinerea* and *P oryzae* (Fig 4). The inhibition rates against *B cinerea* were 25.7 and 27.9%, and of *P oryzae* were 22.0 and 44.0% for pristimerin and celastrol, respectively, at 800 μg ml^{-1}.

3.4 *In vivo* fungitoxic activity

Based on the above results *in vitro*, pristimerin and celastrol were tested for their preventive and curative effects on wheat powdery mildew and cucumber downy mildew *in vivo* in the greenhouse (Table 1 and Table 2). Pristimerin inhibited the development of wheat powdery mildew more effectively than that of cucumber downy mildew. Against wheat powdery mildew, pristimerin caused 96.7 and 100% inhibition after 7 days at 100 and 500 μg ml^{-1}, respectively, the same as triadimefon at 100 μg ml^{-1}. The preventive effect of celastrol was 80.5% at 100 μg ml^{-1} and 88.5% at 500 μg ml^{-1}.

Both pristimerin and celastrol showed strong curative effects against *E graminis*. The curative effects of pristimerin and celastrol were 66.5 and 45.5% at 100 μg ml^{-1} and 74.2 and 62.0% at 500 μg ml^{-1},

Table 1. *In vivo* control of *Erysiphe graminis* with protective and curative spray applications of pristimerin and celastrol[a]

Compound	Dose ($\mu g\,ml^{-1}$)	One day prior to inoculation: Protective effect (%) (±SD)[b]	One day after inoculation: Curative effect (%) (±SD)[b]
Pristimerin	100	98.7 (±2.3)a	66.5 (±3.3)C
	500	100 a	74.2 (±2.8)B
Celastrol	100	80.5 (±1.8)b	45.5 (±3.3)D
	500	88.5 (±1.4)b	61.9 (±3.7)C
Triadimefon	150	97.3 (±1.2)a	82.7 (±3.0)A

[a] Disease incidence observed in untreated control was about 100%. Disease incidence was determined 7 days after inoculation. Disease severity is based on a 0–5 scale, where 0 = no colonies visible to the unaided eye; 2 = few scattered, small discrete colonies; 3 = colonies merging to form larger mildew lesions; 4 = mildew covering half the total leaf surface and 5 = mildew covering the total leaf surface. [b] Means followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.

Table 2. *In vivo* control of *Pseudoperonospora cubensis* with protective and curative spray applications of pristimerin and celastrol[a]

Compound	Dose ($\mu g\,ml^{-1}$)	One day prior to inoculation: Protective effect (%) (±SD)[b]	One day after inoculation: Curative effect (%) (±SD)[b]
Pristimerin	100	56.5 (±2.5)a	21.0 (±2.0)D
	500	92.2 (±3.2)a	36.4 (±4.3)B
Celastrol	100	29.7 (±1.0)b	6.7 (±2.9)D
	500	46.5 (±4.4)b	27.5 (±1.9)C
Mancozeb	150	100 a	42.6 (±1.7)A

[a] Disease incidence observed in untreated control was about 60%. Disease incidence was determined 5 days after inoculation. Disease severity is based on a 0–9 scale, where 0 = no visible symptom and 9 = lesion number per leaf ≥50%. [b] Means followed by the same letter are significantly different ($P = 0.05$) according to the least significant difference test.

respectively. The curative effect of pristimerin was superior to that of celastrol. Against cucumber downy mildew, pristimerin exhibited good preventive activity at $500\,\mu g\,ml^{-1}$ but 1-day curative treatment provided no obvious control of visible symptoms at 100 or $500\,\mu g\,ml^{-1}$.

4 CONCLUSIONS

The present work is the first report that pristimerin (**1**) and celastrol (**2**) isolated from *C hypoleucus* exhibit significant antifungal activity against plant pathogenic fungi *in vitro* and *in vivo*. Modern fungicides are generally selective, systemic and curative, achieving control with limited numbers of applications and at low rates. However, the development of fungicide resistance is often a problem, leading to a requirement for new fungicides with different modes of action. In this respect, pristimerin and celastrol could be considered as potential candidates for the development as new fungicides.

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