Antifungal Activity of Compounds Extracted from Cortex Pseudolaricis against Colletotrichum gloeosporioides

Jing Zhang, Li-Ting Yan, En-Lin Yuan, Hai-Xin Ding, Huo-Chun Ye, Zheng-Ke Zhang, Chao Yan, Ying-Qian Liu, and Gang Feng

Abstract: Cortex Pseudolaricis is the root bark of Pseudolarix amabilis Rehder, found only in China, and has been widely used in folk antifungal remedies in traditional Chinese medicine. In order to find the natural antifungal agents against mango anthracnose, eight compounds, namely pseudolaric acid A (1), ethyl pseudolaric acid B (2), pseudolaric acid B (3), pseudolaric acid B-O-β-D-glucoside (4), piperonylic acid (5), propionic acid (6), 3-hydroxy-4-methoxybenzoic acid (7), and 4-(3-formyl-5-methoxyphenyl) butanoic acid (8) were isolated from the ethanol extracts of Cortex Pseudolaricis by bioassay-guided fractionation and evaluated for in vitro antifungal activity against Colletotrichum gloeosporioides Penz. Results demonstrated that all of the eight compounds inhibited the mycelial growth of C. gloeosporioides at 5 μg/mL. Among them, pseudolaric acid B and pseudolaric acid A showed the strongest inhibition with the EC50 values of 1.07 and 1.62 μg/mL, respectively. Accordingly, both Pseudolaric acid B and Pseudolaric acid A highly inhibited spore germination and germ tube elongation of C. gloeosporioides. Dipping 100 μg/mL pseudolaric acid B treatment exhibited more effective suppression on postharvest anthracnose in mango fruit when compared to the same concentration of carbendazim. Scanning electron microscopy observations revealed that pseudolaric acid B caused alterations in the hyphal morphology of C. gloeosporioides, including distortion, swelling, and collapse. Pseudolaric acid B caused the mycelial apexes to show an abnormal growth in dimensions with multiple ramifications in subapical expanded areas with irregular shape. These findings warrant further investigation into optimization of pseudolaric acid B to explore a potential antifungal agent for crop protection.

Keywords: Pseudolarix amabilis, antifungal activity, Colletotrichum gloeosporioides, pseudolaric acid B, mango

Introduction

Mango (Mangifera indica L. Anacardiaceae) fruit is an important tropical and subtropical crop with high commercial value due to its bright color, favorable flavor and taste, and rich nutrition. However, mango is highly susceptible to various fungal pathogens, resulting in huge economic losses. Anthracnose disease, caused by Colletotrichum gloeosporioides Penz., is one of the most common and serious diseases affecting mango. The pathogen causes severe symptoms of black lesions on leaves, twigs, and blossoms and may attack immature fruit as a latent infection. The disease appears progressively until fruit ripening during storage. In general, control of mango anthracnose disease is achieved by applications of fungicides, such as benomyl and prochloraz. However, repeated and exclusive application of chemical-based fungicides often results in increased chemical resistance in pathogens, undesirable effects on nontarget organisms, and the potential risks to human health and environmental pollution. Therefore, identification and exploitation of natural products as an alternative strategy to control anthracnose diseases has been proposed instead of synthetic-based fungicides. Previous studies have reported a number of natural compounds isolated from medicinal plants, herbs, and antagonistic microorganisms which have exerted antifungal activity against C. gloeosporioides in vitro and controlled mango anthracnose disease.

Pseudolarix amabilis Rehder (Pseudolarix kaempferi Goeden) is a rare endemic species of Pinaceae found only in south China. The root and trunk bark of P. amabilis, named Cortex Pseudolaricis (Tu-Jin-Pi) in traditional Chinese medicine, have been used traditionally to treat skin diseases caused by fungal infections. Increasing evidence demonstrates that some constituents of Cortex Pseudolaricis possess biological activity such as antifungal activity, anticancer activity, and peroxisome proliferator-activated receptor activity. Ethanol extracts of Cortex Pseudolaricis showed strong in vitro antifungal activity against Rhizoctonia cerealis, the main pathogen causing wheat sharp eyespot. However, the

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antifungal effects of Cortex Pseudolaricis on mango anthracnose and other horticultural diseases has not been previously investigated. Our preliminarily experiments showed that ethanol extract from Cortex Pseudolaricis had a significant inhibitory activity against *C. gloeosporioides* (data not shown). Therefore, we further conducted a phytochemical study on the extract using a bioactivity-guided purification process, isolated and identified the main metabolites, and then evaluated their antifungal activity.

**MATERIALS AND METHODS**

**General.** The Cortex Pseudolaricis was collected in Yinzhou District, Ningbo City, Zhejiang Province of the People’s Republic of China (29°46′6″ E, 121°19′5″ N) and identified by Associate Professor Jianyin Li from the Pharmacy Institute of Lanzhou University. The commercial fungicide carbendazim (formulated analytical grade, 98% purity) (Jiangsu Bailing Agrochemical Co., Ltd., China) was used as a positive control. *C. gloeosporioides* was obtained from Postharvest Protection Institute of Chinese Academy of Tropical Agricultural Science. It is carbadenazim-sensitive and isolated from mango fruit. Analytical thin-layer chromatography (TLC) and preparative thin-layer chromatography (PTLC) were performed with silica gel plates using ethyl acetate to give a neutral ethyl acetate-soluble fraction. The aqueous-soluble fraction was subjected to silica gel chromatography using a gradient solvent system of petroleum ether–acetone (10:1), and three major fractions, 1, 2, and 3, were collected. Fraction 1 was then chromatographed sequentially over silica gel column using a solvent system of petroleum–ethyl acetate (4:1) to afford Pseudolaric Acid A (1, PAA), a silica gel column petroleum–ethyl acetate (5:1), and then chloroform–methanol (10:1), to afford Pseudolaric Acid B (2, EPAB). Fraction 2 was recrystallized from petroleumether–ethyl acetate (4:1) to afford Pseudolaric Acid B (3, PABG) (2.5 g). Fraction 3 was chromatographed sequentially over silica gel column using a solvent system of chloroform–methanol (20:1) to yield Pseudolaric Acid B--O-β-D-glucoside (4, PAGB) (2.5 g).

**Isolation of Other Compounds.** The dried Cortex Pseudolaricis (20 kg) was ground and percolated with 95% ethanol. After filtration and removal of the solvent, the ethanol extract was dissolved in 5 L of 5% NaHCO₃ solution to make a suspension and extracted with ethyl acetate to give a neutral ethyl acetate-soluble fraction. The aqueous-soluble fraction was subjected to silica gel column chromatography using a gradient solvent system of petroleum–ethyl acetate (4:1), and then chloroform–methanol (10:1), and three major fractions, 1–3, were collected. Fraction 1 was then chromatographed sequentially over silica gel column using a solvent system of petroleum–ethyl acetate (4:1) to afford Pseudolaric Acid A (1, PAA), a silica gel column petroleum–ethyl acetate (5:1), and then chloroform–methanol (3:1) to afford compound 5. Fraction 2 was chromatographed sequentially over silica gel column using a gradient solvent system of petroleum–chloroform–acetone (5:2:2), and then petroleum–chloroform–acetone (5:1:2), and then petroleum–chloroform (1:1), to afford compounds 6, 7, and 8, respectively.
3-Hydroxy-4-methoxybenzoic Acid (7). White amorphous powder; mp 155–157 °C; 1H NMR (400 MHz, DMSO-d_6): δ: 12.6 (1H, s, COOH), 7.54 (1H, dd, J = 1.6, 6.8 Hz, H-6), 7.43 (1H, d, J = 0.8 Hz, H-2), 7.01 (1H, d, J = 8.4 Hz, H-5), 6.10 (1H, d, J = 8.0 Hz, C-3=O). 13C NMR (400 MHz, DMSO-d_6): δ: 167.2 (C=O), 152.7 (C-4), 148.4 (C-3), 125.0 (C-6), 123.2 (C-1), 111.9 (C-2), 108.9 (C-5), 55.7 (−O−CH_2−); ESI-MS m/z: 169 [M + H]^+; 191 [M + Na]^+.

4-(3-Formyl-5-methoxyphenyl)butanoic Acid (8). White amorphous powder; mp: 99–102 °C; 1H NMR (400 MHz, DMSO-d_6): δ: 12.3 (1H, s, COOH), 9.82 (1H, s, CHO), 7.41 (2H, t, J = 7.2 Hz, H-2, H-6), 6.83 (1H, d, J = 8.8 Hz, H-4), 3.79 (3H, s, O−CH_3), 3.34 (1H, s, H-9), 2.17 (1H, t, J = 7.6 Hz, H-7), 1.46 (1H, t, J = 6.8 Hz, H-7), 1.23 (2H, m, H-8), 1.15 (1H, dd, J = 6.4, 16.4 Hz, H-9). 13C NMR (400 MHz, DMSO-d_6): δ: 174.5 (C=O), 167.3 (CHO), 151.1 (C-5), 147.3 (C-3), 123.5 (C-1), 121.7 (C-6), 115.1 (C-4), 112.7 (C-2), 55.6 (O−C(H)_2), 33.7 (C-7), 28.5 (C-8), 24.5 (C-9); ESI-MS m/z: 223 [M + H]^+; 245 [M + Na]^+.

**In Vivo Effect on Mycelial Growth of C. gloeosporioides.** The effects of the compounds from Cortex Pseudolaricis on the mycelial growth against C. gloeosporioides were assessed using Poison Food Technique in solid media. First, the compounds were dissolved in acetone and water containing Tween-80, then mixed with sterile distilled water to obtain six different concentrations of compounds. The acetone solution of the test compounds was diluted with conidial suspension to obtain six different concentrations of C. gloeosporioides growth against each compound. After incubation at 28 °C for 9 d. Disease developments were assessed by measuring the diameter of the anthracnose lesions on the mango fruit. Each treatment consisted of 3 replicates with 10 fruits per replicate, and the experiment was repeated twice.

**Scanning Electron Microscopy (SEM).** SEM observations on the hyphae of C. gloeosporioides was conducted according to the method described by Feng et al. A mycelial disk (4 mm diameter) was taken from the periphery of the colony grown on PDA medium containing 2.5 µg/mL (EC_{50}) of test compounds and incubated for 24–72 h at 28 °C. The youngest C. gloeosporioides hyphae were chosen from the margin of the mycelia, and the samples were routinely fixed in 2.5% glutaraldehyde (prepared previously with 0.1 mol/L phosphate buffer, pH 7.2) at 4 °C overnight, briefly postfixed for 2 h at 4 °C in 1% OsO_4 in the same buffer, and then dehydrated in a graded ethanol series, critical point dried, and gold coated. SEM observations were performed with an FEI Quanta 400 Thermal FE Environment Scanning Electron Microscope at an accelerating voltage of 20 kV.

**Statistical Analyses.** Data were presented as means and standard deviations. ANOVA was used to determine whether there were significant (P < 0.05) treatment effects between groups, and the means were compared using the Duncan’s multiple range test. Differences among different treatments were analyzed using SAS version 9.13. Dose–response curves were analyzed using a four-parameter log–logistic model using R software (version 2.15.2, R Foundation for Statistical Computing, Vienna, Austria) with the drc module. Means and standard deviations were obtained using the raw data, and the EC_{50} values were obtained from the parameters in the regression curves. The graphs were generated with Sigma Plot (version 11, Systat Software Inc, San Jose, CA, U.S.A.).

## RESULTS

### Structure Elucidation of the Bioactive Constituents.

Bioassay-guided separation of the components in the ethanol extract of the root bark of Cortex Pseudolaricis afforded eight major bioactive metabolites (Figure 1). All these compounds were purified by column chromatography, and their structures were characterized by 1H NMR, 13C NMR, and ESI-MS data.

![Chemical structures of compounds 1–8.](image-url)
and their structures also were confirmed by direct comparison with previously reported spectroscopic data.\(^{26,27}\) On the basis of the following data, the constituents were identified as pseudolaric acid A (1, PAA), ethyl pseudolaric acid B (2, EPAB), pseudolaric acid B (3, PAB), pseudolaric acid B—O—β-D-glucoside (4, PABG), piperonylic acid (5), propionic acid (6), 3-hydroxy-4-methoxybenzoic acid (7), and 4-(3-formyl-5-methoxyphenyl)butanoic acid (8).

Effects of Compounds on Mycelial Growth In Vitro. To evaluate the antifungal activity, the effects of the eight compounds on \(C.\) \(gloeosporioides\) mycelial growth on solid media were determined (Figure 2). The results showed that the PAB, PAA, EPAB, and PABG had high dose-dependent activities against the \(C.\) \(gloeosporioides\) mycelial growth with the EC\(_{50}\) value of 1.62, 3.7, 1.07, and 11.30 \(\mu\)g/mL, respectively. In particular, PAB and PAA showed a greater mycelial growth inhibition than that of carbendazim (2.37 \(\mu\)g/mL) (Figure 2). By contrast, compounds piperonylic acid, propionic acid, 3-hydroxy-4-methoxybenzoic acid, and 4-(3-formyl-5-methoxyphenyl) butanoic acid showed weaker inhibitory activity, whose mycelial growth inhibitions were all less than 30% at 40 \(\mu\)g/mL.

Effects of Compounds on Spore Germination and Germtube Elongation. PAB and PAA exhibited greater inhibition of spore germination and germ tube elongation than did the carbendazim standard (Table 1). PAB had an effective concentration of 50% (EC\(_{50}\)) value of 3.80 \(\mu\)g/mL on spore germination and 0.75 \(\mu\)g/mL on germ tube elongation.

Effects of PAB on Control of \(C.\) \(gloeosporioides\) in Mango Fruit. The effects of four active compounds, including PAB, PAA, EPAB, and PABG, on the postharvest anthracnose of mango fruit were demonstrated in Figure 3. Results indicated that all the compounds tested at 50 and 100 \(\mu\)g/mL significantly suppressed lesion diameter of anthracnose in mango fruit inoculated with spore suspension of \(C.\) \(gloeosporioides\). Smaller lesion development was achieved at higher concentrations of test compounds (\(P < 0.05\)). PAB treatment showed the greatest efficiency in controlling mango anthracnose, where the lesion diameters were 87.61% and 46.67% lower than those in control and carbendazim-treated fruit.

Table 1. EC\(_{50}\) Values (\(\mu\)g/mL) of Active Components from Cortex Pseudolaricis against Spore Germination and Germ Tube Elongation of \(C.\) \(gloeosporioides\)

<table>
<thead>
<tr>
<th>treatments</th>
<th>spore germination</th>
<th>germ tube elongation</th>
</tr>
</thead>
<tbody>
<tr>
<td>Pseudolaric acid A</td>
<td>5.69</td>
<td>1.14</td>
</tr>
<tr>
<td>ethyl Pseudolaric acid B</td>
<td>11.42</td>
<td>2.78</td>
</tr>
<tr>
<td>Pseudolaric acid B</td>
<td>3.80</td>
<td>0.75</td>
</tr>
<tr>
<td>Pseudolaric acid B—O—β-D-glucoside</td>
<td>23.57</td>
<td>6.35</td>
</tr>
<tr>
<td>piperonylic acid</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>propionic acid</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>3-hydroxy-4-methoxybenzoic acid</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>4-(3-formyl-5-methoxyphenyl)butanoic acid</td>
<td>&gt;40</td>
<td>&gt;40</td>
</tr>
<tr>
<td>carbendazim (98%)</td>
<td>21.34</td>
<td>2.04</td>
</tr>
</tbody>
</table>

\(^{a}\)EC\(_{50}\) was determined by probit-log analysis. FL, fiducial limits.

Changes in the Hyphae Morphology of \(C.\) \(gloeosporioides\). To reveal the antifungal mechanism of PAB, the hyphae morphology of \(C.\) \(gloeosporioides\) treated with 2.4 \(\mu\)g/mL (EC\(_{50}\)) PAB was observed by scanning electron microscopy. \(C.\) \(gloeosporioides\) mycelium grown in PDA with 1% acetone (control) displayed characteristic morphology with lengthened, regular, homogeneous hyphae of constant diameter with smooth external surfaces and rounded apexes (Figure 4A,B). In the PAB treatment at 24 h, the hyphae morphology showed undulations along the hyphal border, a little swelling localized along the hyphae and at their extremities, and the formation of anomalous apex bifurcations (Figure 4C,D). Macroscopic morphologic alterations of \(C.\) \(gloeosporioides\) were more profound when the PAB treatment time was extended to 48 h. The apexes of \(C.\) \(gloeosporioides\) treated with PAB showed an irregular growth in dimension, with multiple ramifications in subapical expanded areas with an irregular shape (Figure 4E); several swellings of ovoidal or spherical shape were evident and were localized in the subterminal position near the apexes, or in the middle position along the hyphae (Figure 4F). Seventy-two hours after PAB treatment, hyphae were distorted and collapsed (Figure 4G).

![Figure 2. Dose–response curves of the test compounds on inhibition of \(C.\) \(gloeosporioides\) mycelial growth. ○ = pseudolaric acid A; □ = ethyl pseudolaric acid B; ▽ = pseudolaric acid B—O—β-D-glucoside; △ = pseudolaric acid-O-β-D-glucoside; and ■ = commercial standard carbendazim.](image)

![Figure 3. Efficacy of active components from Cortex Pseudolaricis in controlling anthracnose disease on mango fruits (Disease incidence was determined 9 days after inoculation). Each bar represents the mean of three independent experiments ± standard deviation. Different letters indicate that the means are significantly different at \(P < 0.05\).](image)
DISCUSSION

In recent years, consumer demand for fresh fruits and vegetables without chemical residues has increased.26 Susceptibility of anthracnose disease caused by C. gloeosporioides is one of the most serious problems affecting the mango industry worldwide. As alternatives to synthetic fungicides, the use of natural antifungal compounds isolated from plants to control mango anthracnose disease has been investigated, but few if any satisfactory compounds have been found.9−13 The root bark of P. kaempferi has been successfully used in traditional Chinese medicine as an antifungal remedy and more recently, its major constituents have been isolated and characterized.10,23,27 Although the antifungal activity and possible mechanisms of PAB against medical fungi have been well demonstrated, the effects of PAB and the pseudolaric acid analogues on plant phytopathogenic fungi were not documented. This is the first report on the potential agricultural use of these compounds isolated from Cortex Pseudolaricis against C. gloeosporioides in vitro and in vivo.

The present study evaluated the in vitro antifungal activity of eight compounds from the ethanol extracts of Cortex Pseudolaricis and their capability in controlling postharvest anthracnose. It was previously reported that pseudolaric acid analogues from Cortex Pseudolaricis were the active components.27−29 Such observation was also supported by our study, in which pseudolaric acid analogues PAA, PAB, EPAB, and PABG, effectively suppressed C. gloeosporioides in mango fruit. PAB showed the strongest efficacy and had greater inhibitory activity than fungicide carbendazim. These results indicate that PAB has great potential to control postharvest mango anthracnose as a natural product based alternative to synthetic fungicides. Currently, natural PAA and PAB can only be obtained from the root bark of P. kaempferi. Recently, PAB was successfully achieved by manual synthesis in laboratory,18 which could provide an ample supply of this compound and introduce a variety of novel structural modifications to the PAB molecule based on synthesis for structure activity relationships. Further studies need to be expanded to other important plant pathogenic fungi, and the indicated SAR need to be conducted on these compounds.

Research on the fungicidal mechanism of action for PAB is being carried out. Zhang et al. proposed that the mechanism of PAB could be unique and different from antibiotics with the known mechanisms of amphotericin B, nystatin, ketoconazole, and clotrimazole. It was been reported that PAB resulted in DNA damage and inhibited cell division.30,31 Recently, Wong et al. showed that the antitumor activity of PAB could be associated with its ability to directly interact with tubulin and identified the microtubules as the molecular target of PAB.18 In agricultural fungi, tubulin is one kind of important fungicide target. Both benimidazole and oxamido fungicides inhibit phytopathogenic fungus by binding to β-tubulin.32,33 In our present study, PAB showed stronger inhibition of mycelial growth and germ tube elongation than spore germination. SEM images revealed that PAB treatments caused hyphal tip swelling, malformed branches, and swollen, distorted, concave-collapsed hyphae in C. gloeosporioides. Morphological changes induced by PAB are similar to those caused by tubulin inhibitors, such as benimidazole fungicides.34 Therefore, these findings suggest that PAB may have a similar function in inhibiting mycelial growth and germ-tube elongation in C. gloeosporioides.

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Notes
The authors declare no competing financial interest.

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