Activity in vitro and in vivo against Plant Pathogenic Fungi of Grifolin Isolated from the Basidiomycete Albatrellus dispansus

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In the course of screening for novel naturally occurring fungicides from mushrooms in Yunnan province, China, the ethanol extract of the fruiting bodies of Albatrellus dispansus was found to show antifungal activity against plant pathogenic fungi. The active compound was isolated from the fruiting bodies of A. dispansus by bioassay-guided fractionation of the extract and identified as grifolin by IR, 1H and 13C NMR and mass spectral analysis. Its antifungal activities were evaluated in vitro against 9 plant pathogenic fungi and in vivo against the plant disease of Erysiphe graminis. In vitro, Sclerotinia sclerotiorum and Fusarium graminearum were the most sensitive fungi to grifolin, and their mycelial growth inhibition were 86.4 and 80.9% at 304.9 µm, respectively. Spore germination of F. graminearum, Gloeosporium fructigenum and Pyricularia oryzae was almost completely inhibited by 38.1 µm grifolin. In vivo, the curative effect of grifolin against E. graminis was 65.5% at 304.9 µm after 8 days.

Key words: Grifolin, Albatrellus dispansus, Antifungal Activity, Plant Pathogenic Fungi

Introduction

Research during the last decade has convincingly shown that natural products play an important role, not only in pharmacology, but also in agriculture as a rich source of bioactive components that can be used in crop protection (Wink, 1993). Some successful examples could be given in the past investigations, such as, pyrrolnitrin, produced by Pseudomonas pyrrocinia, and strobilurin A, produced by Strobilurus tenacellus. From pyrrolnitrin, two synthetic phenylpyrroles, fenpiclonil (Nevill et al., 1988) and fludioxonil (Gehmann et al., 1990), have been developed as seed-dressing agents and fumigants against numerous fungal pathogens. Strobilurin A and oudemansin A are fungicidal natural products found in the basidiomycete fungus S. tenacellus (Pers. ex Fr.) Singer (Anke et al., 1977) and Oudemansiella mucida Hoehn (Musilek et al., 1969), respectively. Several fungicides, such as azoxystrobin (Godwin et al., 1992), kresoxim-methyl (Ammerman et al., 1992), metominostrobin (Mizutani et al., 1995) have been synthesized by using the mentioned two fungal metabolites as indicator and were recently commercialized. The mushrooms, as one of the main biological resources, are still an unexplored source of new agricultural chemicals.

Yunnan province, in the southwest of China, is one of the areas with the richest and diverse bio-resources in the world based on its unique geoenvironment, diverse geomorphology and three differentiations of climate. About 1500 species of mushrooms are now known from Yunnan. Edible and inedible are about 600 and 900 species, including 10 poisonous species. Mushrooms in bio-resources belong to the very productive biological sources which produce a large and diverse variety of secondary metabolites. We have been interested in the biologically active substances present in the untapped and diverse source of mushrooms from Yunnan province. In our previous paper, we reviewed the new results of investigations on some basidiomycetes and ascomycetes of Yunnan including the structures of novel terpenoids, phenolics and nitrogen-containing compounds and their biological activities (Liu, 2002). Several prenylphenols from basidiocarps of European and Chinese Albatrellus spp., namely grifolin, neogrifolin, confluentin, scutigeral, and albaconol, were investigated concerning their activities in test models for vanilloid receptor modulation (Hellwig et al.,...
As part of a search for antifungal compounds from mushrooms in Yunnan province, in the southwest of China, grifolin was isolated from the fruit bodies of *A. dispansus* by bioassay-guided fractionation. Its activity against plant pathogenic fungi has been described in this report.

**Results and Discussion**

The antifungal activities of crude ethanol extracts of 20 mushroom species of Yunnan were tested. The antifungal activities were assayed against 9 plant pathogenic fungi. The growth inhibitory activities of the ethanol extracts of the different mushrooms against plant pathogenic fungi are summarized in Table I. The data indicated that ethanol extract from fruit bodies of *A. dispansus* has considerable activities in vitro against all plant pathogenic fungi. It was found that the extract from *A. dispansus* almost completely inhibited the mycelial growth of *Sclerotinia sclerotiorum* (Lib.) de Bary and *Fusarium graminearum* Schw.

The EtOH extract of *A. dispansus* was decanted and evaporated and the residue was extracted with CH$_2$Cl$_2$/MeOH 1:1 v/v. Antifungal activity was detected in the extract of CH$_2$Cl$_2$/MeOH 1:1. The antifungal active extract was further chromatographed on a silica gel column. Fractions 1–4 eluted with CHCl$_3$/AcOEt (8:2, v/v) showed a high level of antifungal activity against 9 plant pathogenic fungi. The antifungal active fractions were rechromatographed on a silica gel column using petroleum ether/ethyl acetate (19:1, 9:1, v/v). The active compound was obtained as colorless needles after recrystallization from hexane. Comparison of the physicochemical properties with the reported data allowed to identifying this active

<table>
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<tr>
<th>Table I. Radial growth inhibition of the ethanol extracts of 20 different mushrooms against 9 plant pathogenic fungi*</th>
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<tr>
<td><strong>Ethanol extract</strong></td>
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*The extract concentration (dry matter) in the culture medium was 1.0 mg/ml in all cases. The inhibition was reported as (−) <10%, (+) between 10 and 20%, (+) between 20 and 40%, (+++) between 40 and 80%, (++) between 40 and 80%, (++) between 40 and 80%, (++) between 40 and 80%, (++) between 40 and 80%, (++) between 40 and 80%, (++) between 40 and 80%, (++) between 40 and 80% growth inhibition.*


Grifolin isolated from *A. dispansus* inhibited the mycelial growth of some of the plant pathogenic fungi tested (Fig. 2). However, the fungal sensitivity varied with the species. *S. sclerotiorum* and *F. graminearum* were the most sensitive fungi to grifolin, and their mycelial growth inhibition was 86.4 and 80.9% at 304.9 µM. The growth of the other fungi, including *Botrytis cinerea* Per. ex Tris., *Alternaria alternata* (Fries) Keissler, *Gloeosporium fructigenum* Berk., and *Venturia nashicola* Tanaka et Yamamoto, were also obviously inhibited at the same dose. To determine the fungicidal sensitivity of grifolin, this compound and the commercially available fungicide carbendazim were compared under the same assay conditions at 304.9 µM. We found that carbendazim was more effective to inhibit mycelial growth of test fungi except *A. alternata* and *V. nashicola*. The growth of *A. alternata* and *V. nashicola* was not affected by carbendazim, in contrast, while 304.9 µM grifolin inhibited the growth by 70.2 and 74.3%, respectively.

The dose response curves of antifungal activity for grifolin against spore germination of *Pyricularia oryzae* Br. et Cav., *F. graminearum*, *G. cingulata* and *B. cinerea* are shown in Fig. 3. Grifolin showed inhibitory activities to various phytopathogenic fungi. Spore germination of *F. graminearum*, *G. fructigenum* and *P. oryzae* was almost completely inhibited by 38.1 µM grifolin. At this dose, *B. cinerea* was only slightly inhibited. The spore germination of *B. cinerea* was completely inhibited at 76.2 µM grifolin. Its inhibition of spore germination to *P. oryzae* was 81.9% at 19.1 µM, the inhibition of spore germination to the other fungi was less than 50% at the same dose.

Based on the above results, the activity of grifolin was tested for its control effects on wheat powdery mildew in greenhouse. The fungicidal activities of isolated grifolin from *A. dispansus* against *Erysiphe graminis* when treated with 609.8, 304.9 and 152.4 µM were determined in vivo,
respectively (Table II). The data indicated that the preventive effect of grifolin was obviously inferior to triadimefon. The curative effect of grifolin was 65.5% at 304.1 µm after 8 days.

The present work showed that grifolin isolated from A. dispansus exhibited significant inhibitory effects to mycelial growth of S. sclerotiorum, F. graminearum and spore germination of P. oryzae in vitro. In particular, it had a high level of curative activity against plant disease of E. graminis 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, the active compounds isolated from A. dispansus may play an important role as a potential candidate for development of a new fungicide. At the same time grifolin was firstly reported that it shows antifungal activity against plant phytopgenic fungi.

**Experimental**

**General**

$^{1}H$ NMR (400 MHz, CDCl$_3$) and $^{13}C$ NMR (100 MHz, CDCl$_3$) were recorded on a Bruker AM-400 spectrometer with TMS as internal standard. MS were measured with a VG Autospec 3000 mass spectrometer. Silica gel (200–300 mesh) was used for column chromatography (CC). Thin layer chromatography was done on pre-coated silica gel plates (GF$_{254}$). Spots were detected on TLC under UV or by heating after spraying with 5% H$_2$SO$_4$ in C$_2$H$_5$OH.

**Materials**

The mushrooms were collected at Wuding in Yunnan province of China, in July 2001. The voucher specimen was deposited at the herbarium of Kunming Institute of Botany, the Chinese Academy of Sciences. Carbendazim and triadimefon were used as positive controls and purchased from Hebei Shuangji Chemical Co. Ltd., China.

**Preparation of ethanol extracts of mushrooms**

10 g air-dried fruit bodies of 20 mushroom species were shaken in 100 ml EtOH at 40 °C for 48 h (40 cycles/min). The insoluble material was filtered and the filtrate evaporated to dryness under reduced pressure at 40 °C. The extracts were weighed and dissolved in DMSO to 1 mg/ml final concentration for antifungal test against mycelia growth.

**Isolation of active principle**

The entire freshly collected fruiting bodies of A. dispansus (dry weight 0.7 kg) were immersed in 95% EtOH and left at room temperature for several days. Then the EtOH extract was decanted and evaporated and the residue was extracted with CH$_2$Cl$_2$/MeOH 1:1 (6 times). The extract (285.0 g) was fractionated by column chromatography (silica gel, CHCl$_3$/AcOEt 8:2). The combined fractions 1–4 were purified by column chromatography (silica gel, petroleum ether/AcOEt 19:1, 9:1, v/v) to give the active compound (4.8 g) after recrystallization from hexane.

**Table II. In vivo control of Erysiphe graminis with preventive and curative spray applications of grifolin**

<table>
<thead>
<tr>
<th>Compound</th>
<th>Dose [µm]</th>
<th>1 d prior to inoculation</th>
<th>1 d after inoculation</th>
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<tr>
<td></td>
<td></td>
<td>Preventive effect (%)</td>
<td>Curative effect (%)</td>
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<tr>
<td></td>
<td></td>
<td>(±SD)$^b$</td>
<td>(±SD)$^b$</td>
</tr>
<tr>
<td>Grifolin</td>
<td>609.8</td>
<td>23.0 (±4.4) a</td>
<td>70.3 (±4.8) A</td>
</tr>
<tr>
<td></td>
<td>304.9</td>
<td>22.6 (±3.5) a</td>
<td>62.5 (±4.6) A</td>
</tr>
<tr>
<td></td>
<td>152.4</td>
<td>22.0 (±3.3) a</td>
<td>51.0 (±2.4) B</td>
</tr>
<tr>
<td>Triadimefon</td>
<td>340.7</td>
<td>87.5 (±2.6) b</td>
<td>64.1 (±4.6) A</td>
</tr>
</tbody>
</table>

$^a$ Disease incidence observed in untreated control was about 100%. Disease incidence was determined 8 d after inoculation.

$^b$ Means followed by the same letter are not significantly different ($P = 0.05$) according to the least significant difference test.
Mycelial growth inhibition test

The isolated compound dissolved in acetone was tested for antifungal activity in vitro by a Poison Food Technique (Agarwal et al., 2001). Potato dextrose agar (PDA) medium was used as the medium for all test fungi. The media incorporating test compound at 304.9 µM was inoculated at the centre of agar discs of the test fungi (4 mm diameter). Three replicate plates for each fungus were incubated at 26 °C. The mycelial growth of fungi (mm) in both treated (T) and control (C) Petri dishes was measured diametrically in three different directions till the fungal growth in the control dishes was almost complete. The percentage of growth inhibition (I) was calculated using the formula (Sztejnberg et al., 1983):

\[ I(\%) = \left(\frac{(C - T)}{C}\right) \times 100 \]

The corrected inhibition (IC) was then calculated as follows (Agarwal et al., 2001):

\[ IC = \left(\frac{(I - CF)}{(100 - CF)}\right) \times 100, \]

where CF = [(90 - C₀)/C₀] × 100; 90 is the diameter (mm) of the Petri dish, and C₀ is the growth (mm) of the fungus in the control.

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.

Spore germination test

B. cinerea was cultured on potato sucrose agar (PSA; 20% potato extract, 2% sucrose, 2% agar) at 22.5 °C in the darkness. After incubation for 5 d, 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 d at the same temperature under continuous near ultraviolet irradiation of an intensive of 360 Mw/cm² that promoted synchronous spore formation. Spore suspensions were prepared by adjusting to \( 2 \times 10^5 \) spores ml⁻¹ with a haemocytometer.

P. oryzae was grown on oatmeal-agar medium at 25 °C in the dark for 7–10 d. Spores were collected by agitating with distilled water from cultures 2 d older fluorescent light after removal of spores from the culture plate. The spore suspension was filtered through tissue paper and adjusted to a density of about \( 1 \times 10^5 \) spores ml⁻¹ by addition of distilled water with a haemocytometer.

Glomerella cingulata (Stonem.) Spauling et Schrenk were obtained from a culture provided by Prof. Sun Guangyu, the Pathogenic Institute, College of Protection, Northwest Sci-Tech University of Agriculture and Forestry, Yangling, Shaanxi, P. R. China. The isolate was grown on potato dextrose agar for 5 d at 28 °C. The culture was overlaid with 10 ml of sterile distilled water, after that conidia were obtained by filtration of the water extract through four layers of sterile cheese-cloth to remove mycelia debris. Conidia were washed three times in sterile distilled water by centrifugation and adjusted to \( 1 \times 10^8 \) conidia ml⁻¹ with a haemocytometer.

F. graminearum obtained from corn was used for test. Colonies of F. graminearum were applied to oatmeal-agar medium at 25 °C in the dark for 5 d. 5 ml of sterile distilled water were added to each slant of 5-day-old cultures, gently scraping the agar surface to give a turbid suspension, corresponding to \( 1 \times 10^8 \) spores ml⁻¹ with a haemocytometer.

The tests for inhibition of B. cinerea, P. oryzae, F. graminearum and G. cingulata spore germination were done on micro slides (Dhiangra and Sinclair, 1986). 30 µl of the spores suspension were added to 30 µl of the test compounds (0.3, 3.1, 19.1, 38.1, 76.2 µM acetone solutions), well mixed with a toothpick and incubated at 25 °C in the dark. 100 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 with standard deviation of the mean.

In vivo assay

In order to investigate further in vivo antifungal activities of the isolated compound such as the duration of preventive and curative activity, the plant disease of wheat powdery mildew (Erysiphe graminis DC f. sp. tritici Marchal graminis) was used in the test. The effects of the test compound on disease development and spread were determined using potted plants in a greenhouse. The potted plants were randomly arranged in two groups and watered twice daily with tap water. The potted plant seedlings were sprayed with the test compounds dissolved in water/acetone (95:5 v:v) con-
taining Tween 20 (250 µg/ml) as wetter and allowed to stand for 24 h.

For the test of preventive effects, the plants in the first group were inoculated with the pathogen of the plant disease, 1 d after spraying with either the test compound or a standard fungicide at a dose of 609.8, 304.9 and 152.4 µM. For the test of curative effects, the plants in the second group were firstly inoculated with the pathogen, 1 d before the application of the test compounds and a standard fungicide at a dose of 609.8, 304.9 and 152.4 µM. Control plants within each group were similarly inoculated with sterile distilled water/acetone containing Tween 20.

For the development of wheat powdery mildew, the treated wheat seedlings at the first stage were inoculated by spraying with a spore suspension (4 mg/ml) of E. graminis. The inoculated wheat seedlings were incubated for 8 d at 20 (±1) °C and 60% RH (relative humidity) during the day and 18 (±1) °C and 60% RH during the night with 16 h of daylight per day in artificial climate chambers (RP-300, R. P. China), and then the disease severity was determined. The disease severity was recorded on a 0–5 scale, where 0 = no colonies visible to the unaided eye; 1 = few scattered, small discrete colonies; 2 = larger, but still 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 (Hickey, 1986).

Pots were arranged as a randomized complete block with three replicates per treatment. The experiment was conducted three times and values are expressed as percentage control (±SD) compared with the control (Song et al., 2004).

The percentage disease incidence was determined using the formulas:

disease incidence (%) = [(Σ scale × number of plants infected)/(highest scale × total number of plants)] × 100 and preventive or curative effects (%) = [(disease incidence of the control – disease incidence of the treatment)/disease incidence of the control] × 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.

Grifolin, C_{22}H_{32}O_{2}: M.p. 38–40 °C (lit. 40–41 °C); colorless needles. – 1R (KBr): $\nu_{\text{max}}$ = 3620 (OH), 2920 (CH), 1635, 1585 cm$^{-1}$ (C=C). – HRMS: $m/z$ = 328.2396. – 1H NMR (CDCl$_3$), δ in ppm: δ = 1.56 (6H, s, CH$_3$-13′ and CH$_3$-14′), 1.63 (3H, s, CH$_3$-12′), 1.76 (3H, s, CH$_3$-15′), 1.87–2.07 (8H, m, H-4′, 5′, 8′, and 9′), 2.10 (3H, s, CH$_3$-7), 3.33 (2H, d, $J = 7$ Hz, H-1′), 5.06–5.09 (2H, m, H-6′ and 10′), 5.30 (1H, br t, $J = 7$ Hz, H-2′), 6.19 (2H, s, H-4 and 6), 7.80 (2H, s, OH-1 and 3). – 13C NMR (DEPT): δ = 15.6 (C-14′), 15.8 (C-15′), 17.3 (C-13′), 20.8 (CH$_3$-7), 22.1 (C-1′), 25.4 (C-12′), 26.8 (C-5′ and C-9′), 39.8 and 40.0 (C-4′ and 8′), 107.8 (C-4 and 6), 112.0 (C-2), 123.9 (C-2′), 124.5 and 124.6 (C-6′ and C-10′), 130.8 (C-11′), 133.7 (C-7′), 134.6 (C-3′), 136.0 (C-5), 156.0 (C-1 and C-3) (Goto et al., 1963; Mahiiou et al., 1995).

Acknowledgements

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