Preliminary Study on the Antifungal Activity of Helicteres angustifolia Extract Against 10 Species of Plant Pathogenic Fungi (Postprint)

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Abstract

The present study employed the mycelial growth rate method to investigate the inhibitory activity of different solvent extracts from the roots, stems, and leaves of Helicteres angustifolia against the mycelial growth of ten plant pathogenic fungi at a concentration of 1.5 mg · mL ^{1}, utilized the spore germination method to test the inhibitory effects of petroleum ether and ethyl acetate phase extracts from H. angustifolia roots on the conidial germination of Colletotrichum musae, assessed the control efficacy of petroleum ether and ethyl acetate phase extracts from H. angustifolia roots against banana anthracnose using an in vitro method, and analyzed the main components of petroleum ether and ethyl acetate phase extracts from H. angustifolia roots by gas chromatography-mass spectrometry (GC-MS), and evaluated the inhibitory activity of eight main compounds against the mycelial growth of C. musae. The results demonstrated that extracts from all parts of H. angustifolia exhibited varying degrees of inhibition against the mycelial growth of the ten plant pathogenic fungi. Specifically, at 1.5 mg · mL ^{1}, the petroleum ether and ethyl acetate phase extracts from H. angustifolia roots showed inhibition rates of 87.00% and 86.14% against the mycelial growth of C. musae, with EC values of 0.062 mg · mL ^{1} and 0.052 mg · mL ^{1}, respectively. At concentrations of 2 mg · mL ^{1}, 4 mg · mL ^{1}, and 8 mg · mL ^{1}, the relative inhibition rates of petroleum ether and ethyl acetate phase extracts from H. angustifolia roots against conidial germination of C. musae were all above 70%. At 10 mg · mL ^{1}, the control efficacy of petroleum ether and ethyl acetate phase extracts from H. angustifolia roots against banana anthracnose was 72.32% and 59.77%, respectively. GC-MS analysis of petroleum ether and ethyl acetate phase extracts from H. angustifolia roots identified 36 main chemical components in the petroleum ether phase extract and 17 main chemical components in the ethyl acetate phase extract. Among the eight selected main compounds, diisobutyl phthalate and dibutyl phthalate exhibited relatively high inhibitory

activity against the mycelial growth of C. musae at 100 g · mL ^{1}, with inhibition rates of 65.12% and 68.07%, and EC values of 56.66 g · mL ^{1} and 37.04 g · mL ^{1}, respectively.

Full Text

Preamble

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Title: Preliminary Study on the Antifungal Activity of *Helicteres angustifolia* Extracts Against Ten Phytopathogenic Fungi

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Abstract

This study investigated the antifungal activity of different solvent extracts from the roots, stems, and leaves of *Helicteres angustifolia* against ten plant pathogenic fungi. The mycelial growth rate method was used to evaluate inhibitory activity at 1.5 mg · mL ^{1}. The spore germination method tested the inhibitory effects of root petroleum ether and ethyl acetate extracts on conidial germination of *Colletotrichum musae*. In vitro assays assessed the control efficacy of these extracts against banana anthracnose. Gas chromatography-mass spectrometry (GC-MS) analyzed the main components of the root petroleum ether and ethyl acetate extracts, and eight major compounds were tested for mycelial growth inhibition against *C. musae*.

Results showed that all extracts exhibited varying degrees of mycelial growth inhibition against the ten pathogens. At 1.5 mg · mL ^{1}, the root petroleum ether and ethyl acetate extracts achieved 87.00% and 86.14% inhibition against *C. musae*, with EC values of 0.062 mg · mL ^{1} and 0.052 mg · mL ^{1}, respectively. At concentrations of 2, 4, and 8 mg · mL ^{1}, both extracts showed >70% relative inhibition of conidial germination. At 10 mg · mL ^{1}, the control efficacy against banana anthracnose reached 72.32% and 59.77% for the petroleum ether and ethyl acetate extracts, respectively. GC-MS identified 36 major compounds in the root petroleum ether extract and 17 in the ethyl acetate extract. Among eight selected compounds tested at 100 g · mL ^{1}, diisobutyl phthalate and dibutyl phthalate showed the highest activity against *C. musae* with inhibition rates of 65.12% and 68.07%, and EC values of 56.66 g · mL ^{1} and 37.04 g · mL ^{1}, respectively.

Keywords: *Helicteres angustifolia*; plant pathogenic fungi; antifungal activity; GC-MS; diisobutyl phthalate; dibutyl phthalate

Introduction

Helicteres angustifolia is a dwarf shrub belonging to the family Sterculiaceae, genus *Helicteres*, widely distributed in Australia, Japan, Laos, China, and other Southeast Asian countries. Its dried roots are used in traditional Chinese medicine to treat influenza (Wang & Liu, 1987), fever, inflammation, and diabetes (Chang et al., 2001). Previous phytochemical studies have identified diverse compounds including quinones, sesquiterpenoids (Guo et al., 2005), esters (Wei et al., 2011), triterpenoids (Chen et al., 1990), phenols, flavonoids (Li et al., 2015), coumarins (Chang et al., 2001), cucurbitacins (Chen et al., 2006a), polysaccharides (Liu et al., 2018), steroids (Chen et al., 2006b), lignans (Chin et al., 2006), and alkaloids (Pan et al., 2008; Wang et al., 2012).

Biological studies have demonstrated that *H. angustifolia* extracts possess antimicrobial, antidiabetic, antioxidant, immunomodulatory, and antitumor activities (Lin et al., 2012; Hu et al., 2016; Li et al., 2016). The aqueous extract can balance inflammatory cytokine levels in rats with ulcerative colitis and improve pathological tissue damage (Gao et al., 2012). Huang (2013) isolated methyl helicterate from *H. angustifolia*, which significantly alleviated liver injury induced by hepatitis B virus in rats. Sun (2018, 2019) extracted polysaccharides that inhibited tumor diffusion and metastasis in mice, and further isolated an acidic heteropolysaccharide compound SPF3-1 that significantly enhanced macrophage proliferation, stimulated phagocytosis, and induced immunomodulatory cytokine production. Yang (2019) conducted suspension culture of *H. angustifolia* callus and found the ethanol extract rich in phenols, flavonoids, terpenoids, saponins, and triterpenoids with strong antioxidant activity and sucrase/maltase inhibition, plus enhanced macrophage proliferation and phagocytic activity.

While most research has focused on medicinal applications for human diseases, few studies have investigated the agricultural antifungal activity of *H. angustifolia*. Our research group previously screened Hainan plants for antifungal activity and found that *H. angustifolia* extracts showed promising effects against plant pathogenic fungi. To further investigate, we prepared 95% ethanol extracts from roots, stems, and leaves, then fractionated them with different organic solvents. We evaluated the antifungal activity of each fraction against ten plant pathogenic fungi, tested control efficacy against banana anthracnose using in vitro fruit assays, and analyzed the main components of the most active fractions using GC-MS.

Materials and Methods

1.1.1 Plant Material

Helicteres angustifolia plants were collected from Songgui Town, Yunan County, Yunfu City, Guangdong Province. Healthy, whole plants were harvested and transported back to the laboratory.

1.1.2 Chemicals, Reagents, and Instruments

Diisobutyl phthalate, dibutyl phthalate, ethyl palmitate, 2,6-dimethoxyphenol, linoleic acid, methyl linoleate, ethyl linoleate, and syringaldehyde were purchased from Aladdin. 95% ethanol, petroleum ether, ethyl acetate, n-butanol, DMF (N,N-dimethylformamide), and Tween-80 were obtained from Xilong Scientific Co., Ltd. A 0.22 m syringe filter was produced by Shanghai Chuding Analytical Instrument Co., Ltd. An Agilent Technologies 7000 GC-MS system was used for analysis. A rotary evaporator (RV 8) was from IKA (China) Co., Ltd., and a super-clean bench (SW-CJ-1D) was from Suzhou Purification Equipment Co., Ltd.

1.1.3 Test Plant Pathogenic Fungi

The test strains included *Neoscytalidium dimidiatum* (dragon fruit canker), *Botryosphaeria dothidea* (apple ring rot), *Colletotrichum musae* (banana anthracnose), *Gibberella zeae* (maize scab), *Pestalotiopsis guepinii* (camellia gray spot), *Fusarium moniliforme* (rice bakanae), *Botrytis cinerea* (grape gray mold), *Sclerotinia sclerotiorum* (rapeseed sclerotinia rot), *Alternaria solani* (tomato early blight), and *Botryodiplodia theobromae* (mango stem-end rot). These strains were obtained from the Agricultural Culture Collection of China and the College of Plant Protection, Hainan University, and maintained at 4°C before use.

1.1.4 Culture Media

Potato dextrose agar (PDA) medium: 200 g potato, 20 g glucose, 20 g agar, and 1 L distilled water, used for cultivating plant pathogenic fungi.

1.2.1 Preparation of Crude Extracts

Fresh *H. angustifolia* plants were separated into roots, stems, and leaves, washed with distilled water, air-dried, then oven-dried at 50°C and cut into 3 cm segments before pulverization. Each plant part powder (1.5 kg) was soaked in 15 L of 95% ethanol for 24, 48, and 72 h. The organic phases were combined and concentrated to a paste using a rotary evaporator. The paste was diluted with water and sequentially extracted with petroleum ether, ethyl acetate, and n-butanol. The remaining liquid constituted the aqueous phase. All fractions were concentrated to paste and vacuum-dried to obtain petroleum ether, ethyl acetate, n-butanol, and water extracts, which were stored at 4°C.

1.2.2 GC-MS Analysis Conditions

Gas chromatography conditions: HP-5MS capillary column (30 m × 250 m × 0.25 m); initial column temperature 60°C held for 1 min, then increased at 6°C/min to 300°C held for 17 min; injector temperature 250°C; carrier gas helium at 1.0 mL/min; splitless injection mode.

Mass spectrometry conditions: Electron impact ion source at 70 eV; ion source temperature 250°C; MS quadrupole temperature 150°C; transfer line temperature 280°C; mass scan range (m/z) 20-450.

1.2.3 Mycelial Growth Inhibition Assay

The mycelial growth rate method (Wu, 1987) was used for in vitro toxicity determination against ten plant pathogenic fungi. Each extract (75 mg) was dissolved in 0.1 mL DMF, filtered through a 0.22 m syringe filter, and mixed with 49.9 mL molten PDA medium to achieve a final concentration of 1.5 mg · mL ^{1}. The medium was poured into 9 cm diameter Petri dishes. Five-day-old cultures were used to prepare 6 mm mycelial plugs, which were placed at the center of each plate (one plug per plate) with the mycelial side down. Plates were incubated at 28°C. DMF alone served as the blank control, with three replicates per treatment. When colonies reached >5 cm diameter, colony diameter was measured using the cross method and inhibition rate was calculated as:

Colony diameter (cm) = measured diameter - plug diameter
Mycelial growth inhibition rate = [(control colony diameter - treatment colony diameter) / control colony diameter] × 100%

1.2.4 Mycelial Growth Inhibition by Eight Compounds

Each of the eight compounds from section 1.1.2 (5 mg) was dissolved in 0.1 mL DMF, filtered, and mixed with 49.9 mL molten PDA to achieve 100 g · mL ^{1}. The procedure followed section 1.2.3.

1.2.5 EC Determination for Plant Extracts

Extracts showing >70% mycelial growth inhibition were selected for EC determination. Test extracts were dissolved in DMF and diluted with 0.5% Tween-80 to prepare serial concentrations of 1000, 500, 250, 125, and 62.5 mg · mL ^{1}. After filtration, 0.1 mL of each solution was mixed with 49.9 mL molten PDA to obtain final concentrations of 2, 1, 0.5, 0.25, and 0.125 mg · mL ^{1}. The procedure followed section 1.2.3.

1.2.6 EC Determination for Eight Compounds

Test compounds were dissolved in DMF and diluted with 0.5% Tween-80 to prepare serial concentrations of 7.5, 5, 2.5, 1.25, and 0.625 mg · mL ^{1}. After

filtration, 0.1 mL was mixed with 49.9 mL molten PDA to obtain final concentrations of 150, 100, 50, 25, and 12.5 g · mL ^{1}. The procedure followed section 1.2.3.

1.2.7 Spore Germination Inhibition Assay

The agar plate surface germination method (Fang, 2007) was used. Five-day-old *C. musae* cultures were washed with 10 mL sterile water, and conidia and mycelia were scraped off with a spreader. The suspension was filtered through three layers of gauze to remove mycelia, yielding a conidial suspension of 1×10 cfu/mL. Extracts were dissolved in DMF, filtered, and mixed with 1 mL PDA to obtain final concentrations of 8, 4, 2, 1, 0.5, and 0.25 mg · mL ^{1}. The extract-containing medium was dropped onto microscope slides. After solidification, 20 L of conidial suspension was applied and spread evenly. Slides were incubated at 28°C in darkness for 6 h. DMF in sterile water served as the blank control with three replicates. Spore germination rate and relative inhibition rate were calculated as:

Spore germination rate = (germinated spores / total spores) × 100%
Relative spore germination inhibition rate = [(blank germination rate - treatment germination rate) / blank germination rate] × 100%

1.2.8 In Vitro Control Efficacy on Banana Fruit

Root petroleum ether and ethyl acetate extracts were dissolved in DMF and diluted with 0.5% Tween-80 to 10 mg · mL ^{1}. Mature, uniform, undamaged bananas were selected, washed, and air-dried. Each banana was sprayed with 10 mL of extract solution. Carbendazim (0.2 mg · mL ^{1}) served as positive control and sterile water as blank control. After 24 h, bananas were sprayed with an equal volume of *C. musae* conidial suspension (1×10 cfu/mL), placed in plastic boxes, sealed with plastic wrap, and incubated at 28°C. Disease severity was observed and graded after 7 days. The experiment was conducted twice with nine replicates each time.

Disease grading scale: - 0 level: No disease spots - 1 level: Disease spots covering <5% of fruit surface - 3 level: Disease spots covering 5–15% of fruit surface - 5 level: Disease spots covering 16–25% of fruit surface - 7 level: Disease spots covering 26–50% of fruit surface - 9 level: Disease spots covering >50% of fruit surface

Disease index = Σ(level × number of fruits at that level) / (total fruits × highest level) × 100
Control effect = (control disease index - treatment disease index) / control disease index × 100%

1.2.9 Data Analysis

Excel and SPSS 19.0 software were used to calculate R^2, EC values, and toxicity regression equations.

Results

2.1 Preliminary Screening of Antifungal Activity

The mycelial growth inhibition effects of *H. angustifolia* extracts at 1.5 mg·mL ^{1} against ten common agricultural pathogens are shown in Table 1 . All extracts exhibited varying degrees of inhibition.

Leaf petroleum ether extract showed moderate activity (~50% inhibition) against *N. dimidiatum*, *B. dothidea*, *C. musae*, *B. cinerea*, *A. solani*, and *B. theobromae*, with the strongest effect against *S. sclerotiorum* (83.33% inhibition). Leaf n-butanol extract inhibited *N. dimidiatum* and *B. theobromae* by 51.85% and 55.69%, respectively. Stem petroleum ether extract inhibited *S. sclerotiorum*, *A. solani*, and *B. theobromae* by 51.17%, 46.57%, and 52.61%, respectively. Stem ethyl acetate extract showed >55% inhibition against *N. dimidiatum*, *B. dothidea*, *C. musae*, *S. sclerotiorum*, and *B. theobromae*, with particularly strong effects against *B. dothidea* (71.60%), *C. musae* (78.91%), and *S. sclerotiorum* (70.19%). Root petroleum ether extract inhibited *N. dimidiatum* and *C. musae* most effectively (74.59% and 87.00%, respectively). Root ethyl acetate extract showed notable inhibition against *C. musae* (86.14%).

2.2 EC Values Against Four Pathogens

EC values for four active extracts were calculated using SPSS 19.0 (Table 2). Leaf petroleum ether extract had an EC of 0.703 mg·mL ^{1} against *S. sclerotiorum*. Stem ethyl acetate extract showed EC values of 1.067, 0.635, and 1.178 mg·mL ^{1} against *B. dothidea*, *C. musae*, and *S. sclerotiorum*, respectively. Root petroleum ether extract had EC values of 0.945, 1.149, and 0.062 mg·mL ^{1} against *N. dimidiatum*, *B. dothidea*, and *C. musae*, respectively. Root ethyl acetate extract showed an EC of 0.052 mg·mL ^{1} against *C. musae*.

2.3 Spore Germination Inhibition

The spore germination inhibition results are shown in Figure 1 [Figure 1: see original paper] and Figure 2 [Figure 2: see original paper]. Root petroleum ether extract exhibited dose-dependent inhibition of *C. musae* conidial germination, with relative inhibition rates of 63.59%, 73.13%, 87.78%, and 92.13% at 1, 2, 4, and 8 mg·mL ^{1}, respectively. Activity decreased significantly at 0.5 and 0.25 mg·mL ^{1} (10.50% and 9.50% inhibition, respectively). Root ethyl acetate extract also showed dose-dependent inhibition, though slightly less potent than

the petroleum ether extract. At 2, 4, and 8 mg · mL ^{1}, inhibition rates were 71.42%, 81.58%, and 90.75%, respectively, decreasing to 31.86%, 11.73%, and 2.23% at 1, 0.5, and 0.25 mg · mL ^{1}, respectively.

2.4 In Vitro Control Efficacy on Banana Fruit

Bananas treated with root petroleum ether or ethyl acetate extracts showed only mild disease symptoms after one week, while control bananas developed extensive lesions and rotting (Figure 3 [Figure 3: see original paper]). At 10 mg · mL ^{1}, the petroleum ether and ethyl acetate extracts reduced disease indices to 24.69 and 35.80, respectively, compared to 88.89 in the blank control, corresponding to control efficacies of 72.32% and 59.77% (Table 3). Both extracts outperformed carbendazim (0.2 mg · mL ^{1}).

2.5 GC-MS Analysis of Root Extracts

GC-MS analysis identified 36 major compounds in the root petroleum ether extract (Figure 4 [Figure 4: see original paper]) and 17 in the ethyl acetate extract (Figure 5 [Figure 5: see original paper]), with relative quantification by area normalization.

Petroleum ether extract contained 14 esters (21.07%), 6 ketones (6.41%), 5 alcohols (5.54%), 5 acids (4.11%), 4 alkenes (6.76%), plus 1 alkane (0.04%) and 1 amide (0.74%). Major components included ethyl palmitate (10.14%), ethyl oleate (2.37%), dibutyl phthalate (1.50%), diisobutyl phthalate (1.39%), and ethyl linoleate (1.15%). Prominent ketones included 1-(2-(3-isopropylfuran-2-yl)-3-methylcyclopentyl)ethenone (2.02%), 2' -isopropyl-5' ,6-dimethyl-7-oxaspiro[bicyclo[4.1.0]heptane-3,1' -cyclopentan]-5-one (1.58%), and benzophenone (1.55%). Major alcohols were 8-isopropyl-2,5-dimethyl-5,6,7,8-tetrahydronaphthalen-1-ol (2.45%) and 4' ,5-dimethoxy-[1,1' -biphenyl]-3-ol (1.30%). Linoleic acid was the predominant acid (3.04%), while o-xylene (3.83%) and squalene (2.52%) were the main alkenes.

Ethyl acetate extract contained 8 esters (19.99%), 5 phenols (11.31%), 2 aldehydes (3.38%), 1 acid (1.26%), and 1 alkene (1.11%). Major components were phenol (6.91%), ethyl palmitate (5.52%), methyl linoleate (3.91%), ethyl linoleate (3.62%), syringaldehyde (2.29%), 2,6-dimethoxyphenol (2.06%), palmitic acid (1.26%), and 4-vinyl-2-methoxyphenol (1.20%).

2.6 Antifungal Activity of Eight Major Compounds

Based on GC-MS analysis, eight compounds with >1% relative content were selected: dibutyl phthalate, diisobutyl phthalate, ethyl palmitate, 2,6-dimethoxyphenol, linoleic acid, methyl linoleate, ethyl linoleate, and syringaldehyde. These were tested against *C. musae* (Table 6). At 100 g · mL ^{1}, diisobutyl phthalate and dibutyl phthalate showed the highest activity with inhibition rates of 65.12% and 68.07%, and EC values of 56.66 g · mL ^{1} and 37.04 g · mL ^{1}, respectively.

Discussion and Conclusion

While numerous studies have reported on the chemical constituents and medicinal value of *H. angustifolia*, confirming its antimicrobial, antidiabetic, antioxidant, immunomodulatory, and antitumor activities, research on its agricultural antifungal properties remains limited. This study prepared 95% ethanol extracts from roots, stems, and leaves, fractionated them with petroleum ether, ethyl acetate, and n-butanol, and evaluated their antifungal activity against ten plant pathogens. The root petroleum ether and ethyl acetate fractions showed the strongest inhibition against *C. musae* and were further tested for spore germination inhibition and in vivo control efficacy, with GC-MS analysis of their chemical composition.

Root extracts demonstrated higher antifungal activity than stem and leaf extracts, suggesting differential distribution of bioactive compounds among plant parts. Root petroleum ether and ethyl acetate extracts achieved >80% mycelial growth inhibition against *C. musae* with EC values of 0.062 and 0.052 mg·mL ^{1}, respectively. At 2–8 mg · mL ^{1}, both extracts inhibited conidial germination by >70%, indicating dual action against mycelial growth and spore germination. In in vitro fruit assays, the extracts provided 72.32% and 59.77% control at 10 mg · mL ^{1}, outperforming carbendazim (0.2 mg · mL ^{1}), suggesting potential as fruit preservatives.

GC-MS analysis identified 36 compounds in the root petroleum ether extract (mainly ethyl palmitate, o-xylene, linoleic acid, squalene, dibutyl phthalate, diisobutyl phthalate, and ethyl linoleate) and 17 in the ethyl acetate extract (mainly phenol, ethyl palmitate, methyl linoleate, ethyl linoleate, syringaldehyde, palmitic acid, and 4-vinyl-2-methoxyphenol). Among eight selected major compounds, diisobutyl phthalate and dibutyl phthalate showed the highest activity against *C. musae* (EC = 56.66 and 37.04 g · mL ^{1}, respectively), suggesting they may contribute to the extracts' antifungal activity. These findings provide a theoretical basis for discovering lead compounds and developing botanical fungicides from *H. angustifolia*.

Further research should focus on isolation and identification of additional antifungal compounds, elucidation of mechanisms and modes of action, and evaluation of field efficacy against crop diseases.

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Note: Figure translations are in progress. See original paper for figures.