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Antimicrobial assay of endophytic fungi from *Rumex madaio* and chemical study of strain R1

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Abstract

In order to search more antimicrobial natural products for new drug discovery, bioassay and chemical investigation of endophytic strains from *Rumex madaio* were carried out. Six fungal strains (numbered as L1, L6, L9, L10, R1, R2) were successfully isolated and their antimicrobial effects on three human pathogens, *Escherichia coli*, *Staphylococcus aureus* and *Candida albicans*, were evaluated. The results indicated that ethyl acetate extracts of fermentation broth of these strains had no potent antimicrobial activity against *E. coli* and *C. albicans* while strains L1 and R1 had stronger inhibitory effects on *S. aureus*. On basis of morphological characteristics and 18S rRNA sequence analysis, strains L1 and R1 were respectively classified as *Colletotrichum* and *Fusarium* gen. The chemical study of strain R1 led to isolation of two known antimicrobial agents, dibutyl phthalate (1) and beauvericin (2).

Introduction

Endophyte, one of special microorganisms, resides in the intracellular or intercellular area of healthy plant tissues during their periods of life or the whole (Zhang et al., 2006; Zhang et al., 2018). In this symbiotic system, host plants and endophytes maintain a win-win relationship between species. Host plants offer endophytes with essential nutrients for their growth, while endophytes promote the growth and chemical defense of host plants by producing beneficial metabolites (de Siqueira et al., 2011). Endophytes can dwell in almost all vascular plants (Zheng et al., 2016). Several evidences indicate that endophytic fungi are a species of abundant sources of natural products with diverse chemical structures and bioactivities, such as antimicrobial effect, anti-insect, antioxidant, anti-cancer, anti-neoplastic, cytotoxic, and so on (Akay et al., 2014; Zhou et al., 2014a; Zhou et al., 2014b; Li et al., 2015; Wang et al., 2016; Liu et al., 2017; Rani et al., 2017; Dhankhar et al., 2013). Moreover, endophytic microbes have the

potential capability to produce the same and/or similar bioactive chemicals which are originally detected in their hosts, such as camptothecin, diosgenin, ginkgolide B, huperzine A, hypericin, podophyllotoxin, taxol, vinblastine, and vincristine (Zhang et al., 2014a). So, endophytic microbe could service as an alternative source to make valuable natural products (Yadav et al., 2014).

Rumex belongs to the family of Polygonaceae and possesses about 200 species of annual, biennial, and perennial herbs including *Rumex madaio* Makino, *R. obtusifolius* L., *R. chalapensis* Mill., *R. japonicus* Houtt., *R. patientia* L., *R. nepalensis* Spreng and so on. The root and rhizome of *R. madaio*, was a historic medicine for the treatment of hepatitis and skin diseases (Islam and Abay, 2013). Pharmacological study had shown that this medicinal plant has a broad spectrum of biological properties, including antimicrobial, hemostasis, hypoglycemic action, anti-inflammatory and cytotoxic effect (Zhang et al., 2014b). Up to now, however, no literature about biology and chemistry of its endophytic microbes



had been reported. As part of an ongoing effort to characterize medicinal plant-derived endophytes and their functional metabolites (Zhang et al., 2012; Zhang et al., 2016), isolation of endophytic strains from *R. madaio* and evaluation of antimicrobial effects of their culture extracts were carried out in this study as well as chemical investigation.

Materials and Methods

Material

The healthy plant collected from the coast of Putuo Island (Zhoushan, China) was identified by Prof. Bin Wu (Zhejiang University) and used for endophyte isolation within 48 hours after harvest. The specimen (HZNU-201606) was deposited at the College of Life and Environmental Sciences, Hangzhou Normal University. Three testing human pathogenic strains, *Escherichia coli* AB94012, *Staphylococcus aureus* AB2010021, *Candida albicans* AY204006, were obtained from the China Center for Type Culture Collection. All chemicals used in this work were of analytical grade.

Isolation of endophytic strain

Isolation of endophytic strain was immediately carried out within 24 hours after harvest. Fungal endophytes were separated from leaves and roots according to our reported procedure (Zhang et al., 2012). Firstly, leaves and roots were washed with running tap water followed by sterilizing with 75% ethanol for 1 min and 2.5% sodium hypochlorite for 10 min, then rinsed in sterile water for three times and cut into several segments with one centimeter in length and width using aseptic surgical blades. Then each segment was inserted in potato dextrose agar medium in petri plates supplemented with 200 µg/mL ampicillin and 200 µg/mL streptomycin and incubated at 28°C until the mycelium or colony originating from the newly formed surface of the segments appeared. If a lot of hypha came out, the mycelia of fungal strain were further purified and transferred to other petri plates at the same condition. Another segment of the same specimen without surface sterilization was cultured as a negative control to check the presence of contaminated microbes on the segment surface. Up to six purified endophytic strains were obtained and respectively numbered as L1, L6, L9, L10, R1, R2, and transferred to potato dextrose agar slants separately and were kept at 4°C after being cultured at 28°C for 7 days.

Identification of bioactive strain

All purified endophytic strains were subjected to antimicrobial assay. Taxonomic assignment was only deployed on bioactive endophytic strains with potent antimicrobial effects on basis of morphological properties and 18S rRNA sequence analysis according to the

methods developed (Santos et al., 2012; de Siqueira et al., 2017). 18S rRNA fragments of bioactive strains were obtained by amplification of genomic DNA using forward primer NS1-GTAGTCATATGCTTGTCTC and reverse primer NS6-GCATCACAGACCTGTTATTG-CCTC. Their sequences were analyzed using standard nucleotide BLAST software of NCBI and phylogenetic tree was built using MEGA 6.0 adjacency method (Kumar et al., 2008).

Preparation of crude extract

Six strains were separately cultured on PDA at 30°C for 5-7 days. A balanced amount of fungal colony was transferred to the culture broth in a 500 mL Erlenmeyer flask, which contained 300 mL sterilized potato dextrose broth and salted PDB (3% sea salt) followed by shaking at 200 rpm for 7-10 days at 30°C. After fermentation, the mycelium and the culture broth were separated by centrifugation at 4,000 rpm for 10 min at 4°C. The filtrate was extracted twice with ethyl acetate (Merck). The upper solvent was evaporated at 25°C *in vacuum* to yield the crude extract. The crude extract was reserved at 4°C. Each extract was kept in a vacuum drier for 3 days and dissolved in dimethyl sulfoxide (DMSO, Merck) before antimicrobial test. The final concentration of each crude extract were respectively 0.1, 1, 10 mg/mL.

Antibacterial test

The antibacterial assay was conducted on double plate according to agar dilution method (Wang, 2009). Strains *E. coli* and *S. aureus* were respectively transferred into one 250-mL Erlenmeyer flask. Each flask contained 100 mL sterilized Luria-Bertani medium and incubated at 37°C on a rotary shaker at 180 rpm for 24 hours. About 10 mL of the melted water agar medium was poured into a Petri dish ($\Phi = 90$ mm) followed by laid with five stainless cylinders (Oxford cup, $\Phi = 6$ mm) equidistantly. After that, 200 µL of cultured bacterium was mixed with 200 mL of fresh nutrition agar medium, and then add it to the solidified water agar medium. After cooling, Oxford cup was removed and 100 µL of sample was add to each hole. Ampicillin sodium (30 µg/disk, Amresco) was used as the positive control while DMSO was the negative control. Then the plates were incubated at 37°C for 24 hours. All tests were carried out in triplicate and the antibacterial activity was expressed as the average value of inhibition diameters (mm), which the sizes are measured from the edge of the disk to the end of the clear zone.

Antifungal test

Antifungal assay was also carried out using the same approach described above. Pathogenic strain *C. albicans* was transferred into one 250 mL Erlenmeyer flask. Each flasks had 100 mL sterilized PDB and incubated at 28°C on a rotary shaker at 180 rpm for 48 hours. Potato dextrose agar was used as the upper medium layer of

the testing plate. Amphotericin B (30 µg/disk, Sigma-Aldrich) and DMSO were respectively used as positive and negative controls. The diameter of inhibition zone (in mm) was measured to evaluate anti-fungal effect. All tests were performed in triplicate.

Extraction and isolation of secondary metabolite

The fresh mycelium of strain R1 grown on potato dextrose agar plates for 7 days was inoculated into 250 mL conical flasks containing 100 mL of PDB medium (Zhang et al., 2014a). After 3 days cultivation at 28°C on a rotary shaker at 200 rpm, the seed culture (10 mL for each flask) was transferred into 500 mL conical flasks containing 200 mL of Czapek's medium composed of sucrose (30 g/L), NaNO₃ (3 g/L), K₂HPO₄ (1 g/L), KCl 0.5 g/L, MgSO₄·7H₂O (0.5 g/L), FeSO₄ (0.01 g/L), and H₂O (added to 1000 mL) followed by shaking at 200 rpm at 28°C for 7 days. The fermented whole broth was filtered through gauze to remove mycelia. The filtrate (approximate 30 L) was extracted two times with ethyl acetate in the ratio of 1:1 (v/v). Evaporation of the solvent from the extract *in vacuo* yielded a crude residue (5.5 g).

The afforded extract was dissolved in methanol followed by filtration and subjected to fast separation on a HPLC apparatus (Water D600) equipped with a preparative column (Phenomenex Gemini-NX C18, 50 × 21.2 mm, 5 µm) to afford six fractions F0-F5 using the method described before (Zhang et al., 2017). Then the antimicrobial fraction F4 (0.1547 g) was further separated by semipreparative HPLC with acetonitrile/water (72:28, v/v) to give compounds **1** (35 mg, *t_R* = 13.2 min) and **2** (75 mg, *t_R* = 18.0 min) (Figure S1).

Structure analysis of compounds 1 and 2

All NMR spectra of compounds **1** and **2** were measured in CDCl₃ on a Bruker DRX-500 instrument. Their ESI-MS data were recorded on a Mariner Mass 5304 spectrometer and melting points were measured using an uncorrected XT-4 apparatus (Figure S2-8).

Antimicrobial assay of compounds 1 and 2

Antimicrobial activity was assessed by the microbroth dilution method in 96-well culture plates according to the procedure developed by our lab (Zhang et al., 2016). All test compounds were dissolved in DMSO (100 µg mL⁻¹). Ampicillin and amphotericin B (Sigma-Aldrich) were the positive controls and DMSO was used as the negative. Antibacterial activity was assessed by the microbroth dilution method in 96-well culture plates. The test compound was initially made up to 100 µM in DMSO. The testing pathogens *E. coli* and *S. aureus* were incubated in Luria-Bertani medium for 24 hours at 37°C and *C. albicans* was cultured in the potato dextrose broth for 48 hours at 28°C at 150 rpm. Spores of different microorganism concentrations were diluted to approximately 1 × 10⁶ cfu with stroke physiological

saline solution. 20 µL of sample was added to 96-well microplates. Serial dilutions were made in the 96-well round-bottom sterile plates, and then the fungal suspension (40 µL) and nutrient solution (40 µL) were added. After incubation, the minimum inhibitory concentration (MIC) was taken as the lowest concentration of the test compounds in the wells of the 96-well plate in which the lowest microbial growth could be measured at 600 nm. All tests were also carried out in triplicate.

Results

Antimicrobial effect of the crude extracts of endophytic strains

Bioassay results indicated that all crude extracts of endophytic strains had no antimicrobial activities against *E. coli* and *C. albicans* at the concentrations ranging from 0.1 to 10 mg/mL (Table I). However, they possessed various inhibitory effects on *S. aureus*. Especially, strains L1 and R1 showed stronger inhibitory effects than those of other endophytic strains and the positive control ampicillin sodium (Figure 1). Strain L9 cultured in salted PDB also exhibited moderate inhibitory effect on *S. aureus* at 10 mg/mL. Furthermore, strain L1 still exhibited potent inhibitory effect at the lowest concentration of 0.1 mg/mL, which suggested that L1 would be a potential source for novel antibiotics.

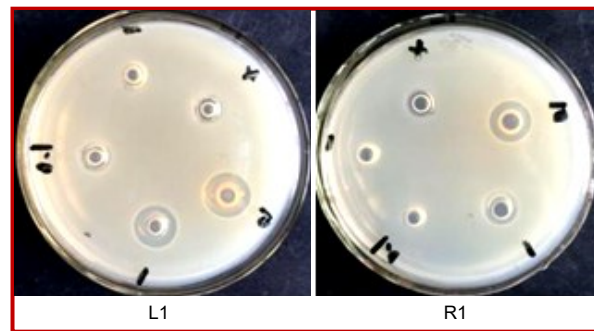


Figure 1: Inhibitory effect of crude extracts of endophytic strains L1 and R1 on *S. aureus*

Negative control (DMSO); +: positive control (ampicillin sodium); 10, 1, 0.1: concentration of crude EtOAc extract (mg/mL)

Identification of strains L1 and R1

On basis of morphological characters of mycelium as well as spores characteristics grown on five culture media including PDA medium, corn meal agar (CMA), carrot agar (CA), Winogradsky's salt-solution agar (WSA) and plate count agar (PCA), strains L1 and R1 were preliminarily identified. Strain L1 grew slowly at the beginning of cultivation. Its colony gradually changed from white into pale yellow followed by light black and its hyphae was spindly, twining, and mostly branched (Figure 2), which was very similar to

Table I				
Antimicrobial activities of ethyl acetate extracts of endophytic fungi from <i>R. madaio</i>				
Strain No.	Concentration (mg/mL)	Antimicrobial effect ^a		
		<i>E. coli</i>	<i>S. aureus</i>	<i>C. albicans</i>
L1	0.1	-	+	-
	1.0	-	++	-
	10.0	-	+++	-
L1 (with salt)	0.1	-	-	-
	1.0	-	-	-
	10.0	-	-	-
L6	0.1	-	-	-
	1.0	-	-	-
	10.0	-	++	-
L6 (with salt)	0.1	-	-	-
	1.0	-	-	-
	10.0	-	++	-
L9	0.1	-	-	-
	1.0	-	-	-
	10.0	-	-	-
L9 (with salt)	0.1	-	-	-
	1.0	-	+	-
	10.0	-	++	-
L10	0.1	-	-	-
	1.0	-	+	-
	10.0	-	++	-
L10 (with salt)	0.1	-	-	-
	1.0	-	-	-
	10.0	-	++	-
R1	0.1	-	-	-
	1.0	-	++	-
	10.0	-	+++	-
R1 (with salt)	0.1	-	-	-
	1.0	-	-	-
	10.0	-	++	-
R2	0.1	-	-	-
	1.0	-	-	-
	10.0	-	++	-
R2 (with salt)	0.1	-	-	-
	1.0	-	+	-
	10.0	-	++	-
Ampicillin sodium	30 µg/disk	++	++	-
Amphotericin B	30 µg/disk	-	-	+++
DMSO	-	-	-	-

^aExpressed by the diameter of inhibition zones: -, no inhibition; +, <10 mm; ++, 10-15 mm; +++, 16-20 mm; +++++, >20 mm

morphological characters of *Colletotrichum* genus (Wei, 1979). Amplification of its genomic DNA by forward primer NS1-GTAGTCATATGCTTGTCTC and reverse primer NS6-GCATCACAGACCTGTTATTGCCTC afforded 1679 bp fragments. Its 18S rRNA sequence was analyzed using nucleotide BLAST software of NCBI and submitted to GenBank in NCBI and an accession number MF376146 was obtained (<https://www.ncbi.nlm.nih.gov/nucore/MF376146>). The phylogenetic tree was carefully built using MEGA 6.0 adjacency method and sequence analysis results revealed 99% similarity with that of *Colletotrichum coccodes* AJ301957.1 (Figure 2).

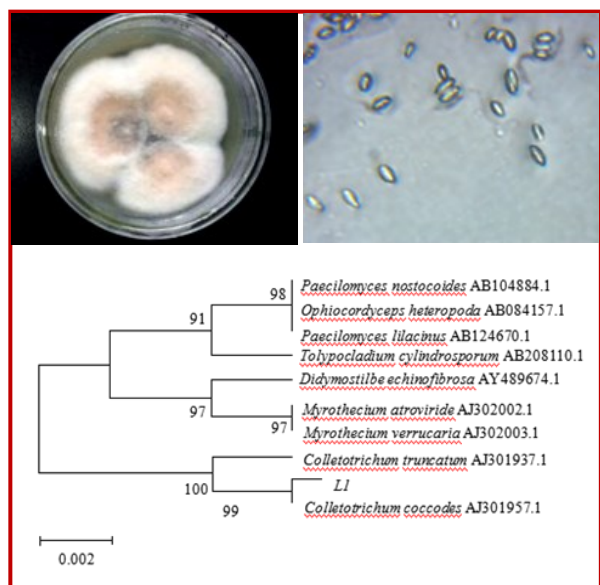


Figure 2: Morphological characteristics and phylogenetic tree of strain L1

Strain R1 grown on PDA medium quickly produced growing flesh colonies, white aerial mycelium, and fusiform conidia (Figure 3), which was very similar to morphological characters of *Fusarium* genus (Wei, 1979). Its 18S rRNA with 1613 bp was obtained through amplification using the same primers described above and submitted to GenBank in NCBI and an accession number MF376147 had been acquired (<https://www.ncbi.nlm.nih.gov/nucore/MF376147>). Sequence alignment showed that strain R1 revealed 99% identity with that of *Fusarium oxysporum* JF807402.1 (Figure 3). Therefore, on basis of their morphological properties and 18S rRNA sequence analysis, endophytic strains L1 and R1 were respectively assigned to *Colletotrichum* and *Fusarium* geni.

Structure elucidation of secondary metabolites from strain R1

Bioassay-guided fractionation of the fermentation crude of strain R1 lead to isolation of two compounds **1** and **2** using HPLC method. By a combination of their 1D NMR and ESI-MS data as well as comparison with



Figure 3: Morphological characteristics and phylogenetic tree of strain R1

literature, compounds **1** and **2** were respectively characterized as dibutyl phthalate (Wu et al., 2017), beauvericin (Zhang et al., 2016) (Figure 4). And the yield of compound **1** was about 1.17 mg/L while that of compound **2** was 2.5 mg/L.

Dibutyl phthalate (**1**): White powder; $C_{16}H_{22}O_4$; m.p. $-35^{\circ}C$; ESI-MS (positive): m/z 279 $[M + H]^+$, 301 $[M + Na]^+$;

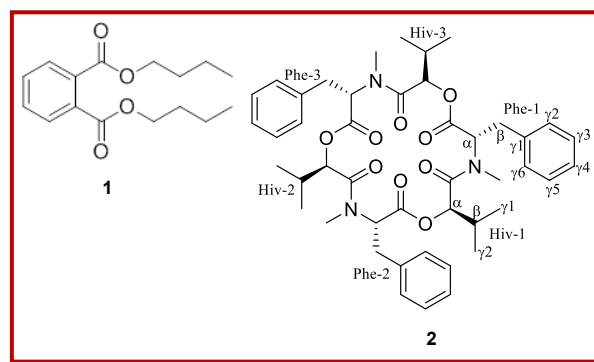


Figure 4: Chemical structures of antimicrobial compounds **1** and **2** from strain R1

1H NMR (500 MHz, $CDCl_3$, δ , ppm): 7.72 (2H, m, H-3, 6), 7.54 (2H, m, H-4, 5), 0.97 (6H, m, H-5', 5''), 1.44 (4H, m, H-4', 4''), 1.72 (4H, m, H-3', 3''), 4.31 (4H, t, H-2', 2'').

Beauvericin (**2**): White acicular crystals; m.p. $93-94^{\circ}C$; ESI-MS (positive): m/z 806 $[M + Na]^+$, 645, 545, 384; $C_{45}H_{57}N_5O$; 1H NMR (500 MHz, $CDCl_3$, δ , ppm): Hiv: 0.39 (3H, d, H- γ_1), 0.80 (3H, d, H- γ_2), 1.97 (1H, m, H- β), 5.57 (1H, d, H- α); Phe: 3.02 (3H, s, H-N- CH_3), 4.87 (1H, d, H- α), 1.98 (2H, dd, H- β); ^{13}C NMR (500 MHz, $CDCl_3$, δ , ppm): Hiv: 18.3 (C- γ_2), 17.3 (C- γ_1), 29.7 (C- β), 57.1 (C-

-α), 169.8 (C-CO); Phe: 32.13 (C-N-CH₃), 128.54 (C-γ6), 128.78 (C-γ5), 136.53 (C-γ4), 128.78 (C-γ3), 128.54 (C-γ2), 126.79 (C-γ1), 34.69 (C-β), 75.6 (C-α), 170.0 (C-CO).

A number of endophytic strains had been reported to produce antimicrobial substances, such as beauvericin from *F. oxysporum* 5-19 colonizing in *E. chrysantha* L. (Zhang et al., 2016), colletonoic acid from *Colletotrichum* sp. endophytic on Umbelliferae (Tianpanich et al., 2011; Hussain et al., 2014). Dibutyl phthalate possessed a broad spectrum of antimicrobial activity (Roy et al., 2006), including inhibitory effect on *E. coli* and *S. aureus* with the same MIC value of 37.5 µg/mL (Rajamanikyam et al., 2017). Compound **2** had remarkable effect on two Gram-negative strains (*Bacillus cereus* and *Salmonella typhimurium*) with respective MIC values of 3.12 and 6.25 µg/mL (Dzoyem et al., 2017). In the present work, bioassay results indicated that dibutyl phthalate (**1**) had no inhibitory effect on three testing pathogenic strains, while beauvericin (**2**) exhibited the strongest antimicrobial activity against *E. coli* and *S. aureus* with MIC values of 62.5, 3.9 µM, respectively.

Discussion

A number of endophytic strains had been reported to produce antimicrobial substances, such as beauvericin from *F. oxysporum* 5-19 colonizing in *E. chrysantha* L. (Zhang et al., 2016), colletonoic acid from *Colletotrichum* sp. Associated with Umbelliferae (Tianpanich et al., 2011; Hussain et al., 2014). Dibutyl phthalate (DBP) possessed a broad spectrum of antimicrobial activity against *E. coli* and *S. aureus* with the same MIC value of 37.5 µg/mL (Roy et al., 2006; Rajamanikyam et al., 2017). Beauvericin **2** was found to have remarkable inhibitory effect on two Gram-negative strains (*Bacillus cereus* and *Salmonella typhimurium*) with respective MIC values of 3.12 and 6.25 µg/ml (Dzoyem et al., 2017). In the present work, bioassay results indicated that DBP (**1**) had no inhibitory effect on three testing pathogenic strains, while beauvericin (**2**) exhibited the strongest antimicrobial activity against *E. coli* and *S. aureus* with MIC values of 62.5, 3.91 µM, respectively. Fungal strains (L1, L6, L9, L10, R1, R2) from *R. madaio* possessed selectively inhibitory effects on *S. aureus* but no activity against *E. coli* or *C. albicans*. It may be ascribes to inappropriate fermentation factors for producing bioactive secondary metabolites. Chemo-diversity of endophytic microbes is associated with their cultivation conditions, such as incubation temperature, medium composition, degree of aeration, etc (Hewage et al., 2013; Yadav et al., 2016). Therefore, more effort should be made on isolation and identification of more antimicrobial chemicals from *R. madaio*-derived endophytes using the one strain-many compounds (OSMAC) approach (Gubiani et al., 2016; Hemphill et al., 2017).

Conclusion

Isolation of endophytic fungi from *R. madaio* and antimicrobial evaluation of their culture extracts for the first time led to the discovery of two bioactive fungal strains L1 and R1, which were respectively classified as *Colletotrichum* and *Fusarium* by analysis of their morphological characteristics and 18S rRNA sequence. Chemical investigation of strain R1 resulted in isolation of two known antimicrobial agents, dibutyl phthalate (**1**) and beauvericin (**2**), which have potential application in pharmaceutical industry.

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Supplementary Materials

¹H- and ¹³C NMR spectra, and positive ESI-MS spectra for compounds **1** and **2**.

Conflict of Interest

Authors declare no conflict of interest

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