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Isolation an antimicrobial action of endophytic fungi from *sophora flavescens* and effects on microorganism circumstances in soil

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Abstract:

The aim of this study was to isolate the endophytic fungi from Sophora flavescens and define its antimicrobial action and structure. The effects of this active substance on soil microorganism circumstances were also conducted. Column chromatography and freeze drying were used to isolate and purify the antimicrobial substance. TLC biological autoradiography was applied to trace. HPLC method was employed to measure the purity. Analysis of the structure used ^{1}H-NMR, ^{13}C-NMR and LC-MS methods. Plate method was applied to measure the effect on microorganism circumstances. The results showed that after isolating and purifying the fermentation liquor of BS001, the structure of bacteriostatic active composition was 6,7-(2′E)dibutenyl-5,8-dihydroxy-(Z)-cyclooct-2-ene-1,4-dione, which was identified by spectroscopy. The substance could increase the number of bacteria and fungi while decreased the number of antinomies in soil. A new antimicrobial substance 6,7-(2′E)dibutenyl-5,8 -dihydroxy- (Z)- cyclooct - 2-ene-1,4-dione was extracted from fermenting liquor of BS001 which was an endophytic fungi of Sophora flavescens. It could promote the beneficial flora but detrimental flora. This conclusion provides exploiture foreground for biopharmaceuticals and biopesticide.

Key words: endophytic fungi; biological activity; soil microorganism circumstances; Sophora flavescens

1. Introduction

An endophyte is an endosymbiont, often a bacterium or fungus, which lives within a plant for at least part of its life without causing apparent disease[1]. Endophyte have many characteristics, such as varied, widely distributed, variant, and diversity, novel and a variety of biological activities for secondary

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metabolites. Sophora flavescens (Sophora flavescens Ait), a medicinal plant, has medicinal function such as sterilization, pesticides, antitumor and antiviral[2]. Thus, it has great scientific research value and development potentiality for researching the endophyte of S. flavescens. Over the past decade, a new group of antibiotics, that has antitumor, antibacterial, resistance to insects function, have been separated from endophytic fungi[3, 4]. Ge[5] have reported that Cytonic acid A and B were anticancer active substance released by plant endophytes, and separated the CPT and other anticancer substances from fermenting liquor of endophytic fungi. M. Caruso had screened 150 kinds of endophytic fungi from Taxus mairei, and these substances have good resistance antitumor effect[6]. But there is almost no report about studying on isolation and identification of the endophytic fungi of S. flavescens and researching on its secondary metabolite. In this paper, the effective antibacterial component in fermenting liquor of BS001, an endophytic fungus isolated from S. flavescens, was isolated and identified，and defined its structure, and studied the effect on soil microorganism circumstances. It could provide a basis for further researching this antibacterial component.

2. Materials and Methods

2.1. Preparation

Endophytic fungi (*Aspergillu terreus*), BS001, was inoculated in PD medium (potato 200 g, sugar 10 g, glucose 10 g, sodium acetate 1.66 g, peptone 1.02 g, water1000 mL) at 25 ℃, 150 r • min^{-1} training for 3 d, then filtrated. The filtrate was evaporated and concentrated to 200 mL at 50 ℃. The pH value of concentrate was adjusted to 5, preparing for use.

2.2. Separation and purification

The concentrate was extracted by ethyl acetate, and then mixed with HPD-722 macroporous resin, static adsorbed for 40 min, filtrated. Put 0.3 L wet resin into Φ4 cm × 55 cm glass column, eluted with the concentration gradient of 10%, 20%, 40%, 60%, 80%.

The separated production was tracking by the method of biological TLC enhancement[7]. The developing agent was chloroform : methanol = 5.5 : 1.5. The plats were cultured under 25 ℃ for observing the antibacterial effect[8].

Then the fractions with antibacterial activity was collected, and reduced pressure concentrated to 1-2 mL, vacuum freeze-drying.

2.3. Structural analysis

The purity and molecular weight of the antibacterial activity substance was analyzed by Liquid chromatography-mass spectrometry (LC-MS) (1100 LC-MS APCI mass spectrometer, provided by Agilent). The Sim-pack was C_{18} Agilent; the mobile phase was methanol: water (80:20); the added quantity is 20 μL; the column temperature was 35 ℃; the detective wavelength was 254 nm. Then the peak was identified using Mass Spectrometry, determined the molecular weight. Using ^{1}H-NMR (500 MHz), ^{13}C-NMR (125 MHz), H^{1}-H^{1}-COSY, DEPT-135 and HSQC spectrum (AV 500 nuclear magnetic resonance instrument, Bruker Company) to analyze the structure of the unknown substance with solvent of MeOD. And then, the structure was validated by mass spectrometry.

2.4. Soil microbial quantity measurement

Soil was sterilized for twice, every time 2h, and 1d apart. The antibacterial activity substance after pre-

processing was diluted to 20, 40, 80, 160, 320 μg/mL, and named A_1, A_2, the A_3, A_4 and A_5, separated. There were 7 treatments. Each time was inoculated with 20 mL every 3 d. The samples were incubated at 25 ℃ with the relative humidity of above 70% for 10 d. Each treatment was replicated 3 times with distilled water as control.

Fungi, bacteria and actinomyces were then diluted and separated on Martin's medium, beef extract-peptone medium and ament Gao-surname 1 mark medium, respectively. After diluting, the microorganisms were incubated at 25 ℃ and the growth of the colonies was observed and calculated after 48 h, 72 h and 144 h.

3. Results

3.1. Antibacterial activity component BS001 structure analysis

This compound was white powder (methanol), mp183℃, $[\alpha]_D$ +3.6°. The ion peak was m/z 339.1072$[M+H]^+$, (calcd. 339.1080), given by HR-TOF-MS that suggested the molecular formula, $C_{16}H_{18}O_8$. The infrared spectrum gave ν_{OH} strong wide peak in 3383 cm^{-1}, a conjugate strong absorption peak $\nu_{C=O}$ in 1695 cm^{-1}, and a absorption peak $\nu_{C=C}$ in 1633 cm^{-1}. Ultraviolet spectrum λ_{Max} 309 nm (ε 3.20), combined with its NMR, it could be speculated that there was a conjugated system containing unsaturated carbonyl in molecule structure.

^{1}H-NMR (500MHz, MeOD) showed three hydrogen signals, 6.81 (1H, dq, J=15.9，6.6Hz), 6.43 (1H, d, J=15.9Hz), 6.09 (1H, s); two hydrogen signals in carbon linked oxygen, 4.23 (1H, br s), 4.82 (1H, br s); 1 hydrogen signal 1.90 (3H, d, J=6.6Hz). There was a propenyl fragment consist by 6.81 (1H, dq, J=15.9, 6.6Hz), 6.43 (1H, d, J=15.9Hz), 1.90 (3H, d, J=6.6Hz) according to coupled crack points of hydrogen. ^{13}C-NMR (125MHz, MeOD) showed eight carbon signals, included two carbonyl signals 205.9, 170.9; three carbon signals, 143.5, 124.6, 124.5; two oxygen carbon signals, 80.5, 76.1; and a methyl signal 18.9. It could speculate that the compound is symmetric structure combined with the data of NMR and mass spectrum.

The plane structure of this compound was deduced by 2 D nuclear magnetic resonance (NMR) technology, such as H^1-H^1-COSY, DEPT, HSQC and HMBC (Fig. 1). One of the important HMBC remote related signal was: H-3 (δ_H 6.09) and C-4 (δ_C 205.9), C-5(δ_C 80.5); H-5 (δ_H 4.23) and C-3 (δ_C 124.6), C-6 (δ_C 76.1); H-6 (δ_H 4.82) and C-4 (δ_C 205.9), C-5 (δ_C 80.5), C-1' (δ_C 170.9); H-2' (δ_H 6.43) and C-1' (δ_C 170.9); H-3' (δ_H 6.81) and C-1' (δ_C 170.9), C-2' (δ_C 124.5); H-4' (δ_H 1.90) and C-2' (δ_C 124.5), C-3' (δ_C 143.5) (Table 1) . According to the wide singlet type of H-5 and H-6, it could be judged that H-5 and H-6 in the same plane. This compound belonged to the derivative of cyclooct-2-ene- ketone, and named 6,7-(2′E)dibutenyl-5,8-dihydroxy-(Z)-cyclooct-2-ene-1,4-dione, "Anshanmycin".

3.2. Effect of Anshanmycin on soil microorganism circumstances

3.2.1. Effect of Anshanmycin on the number of soil bacteria in soil

Different concentration of Anshanmycin affected the number of bacteria in soil in different extent (Table 2). The number of soil bacteria was increased in different levels compared with control group after different concentration treatment. And the numbers were up as the training time prolonging. It could be concluded that the beneficial population in soil was improved by Anshanmycin for protecting the soil microorganism circumstances.

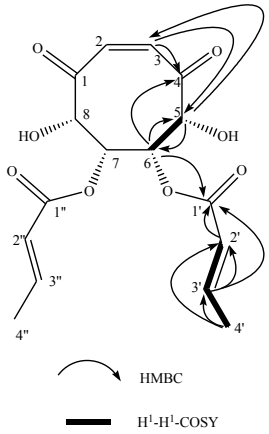

Fig. 1 the H^1-H^1-COSY and the key HMBC of the new compound.

Table 1. Data of the 13C-NMR (125MHz) and 1H-NMR (500MHz) (MeOD, δ in ppm) .

Position	C signal (δ_C)	H signal (δ_H)
1	205.9	
2	124.6	6.09(1H,s)
3	124.6	6.09(1H,s)
4	205.9	
5	80.5	4.23(1H, br s)
6	76.1	4.82(1H, br s)
7	76.1	4.82(1H, br s)
8	80.5	4.23(1H, br s)
1’	170.9	
2’	124.5	6.43(1H, d)
3’	143.5	6.81(1H, dq，J=15.9，6.6Hz)
4’	18.9	1.90(3H, d, *J*=6.6Hz)
1”	170.9	
2”	124.5	6.81(1H, dq，J=15.9，6.6Hz)
3”	143.5	6.81(1H, m)
4”	18.9	1.90(3H, d, *J*=6.6Hz)

Notes: All spectra Recorded on AV500, in MeOD, δ in ppm.

3.2.2. Effect of Anshanmycin on the number of actinomycete in soil

After treating with different concentration of Anshanmycin, the number of soil actinomycete had no significant change (Table 3). But as the time prolong, the number of soil actinomycete decreased at the high concentrations of 160 μg/mL and 320 μg/mL, while no effect at the low concentrations of 20μg/mL and 40μg/mL.

3.2.3. Effect of Anshanmycin on the number of fungi in soil

Anshanmycin had killing effect on fungi in soil in all treatments (Table 4). As the time increase, the killing effect at high concentrations was significant and the number of soil fungi was much lower than that of control.

Table 2. Effect of different concentrations of Anshanmycin on the number of bacterial in soil.

Treatment	Bacterial number（$\times10^7$）		
	48 h	72 h	144 h
CK	4.9 fF	16.1 dD	22.2 dD
A_1	4.9 fF	19.1 fF	25.6 cC
A_2	10.8 dD	22.6 cC	23.3 fF
A_3	16.9 bB	24.2 bB	24.1 eE
A_4	17.0 aA	25.9 aA	26.3 bB
A_5	12.3 cC	20.3 eE	21.3 gG

Table 3. Effect of different concentrations of Anshanmycin on the number of actinomycete in soil.

Treatment	Actinomycete number（$\times10^6$）		
	48 h	72 h	144 h
CK	12.7cC	18.3aA	8.1cC
A_1	17.2aA	18.3aA	10.7aA
A_2	12.1dD	12.3bB	9.8bB
A_3	10.1gG	10.0dD	7.8dD
A_4	14.7bB	10.7cC	10.7aA
A_5	10.7eE	5.5eE	7.8dD

Table 4. Effect of different concentrations of Anshanmycin on the number of fungi in soil .

Treatment	Fungi number($\times\mathbf{10^2}$)		
	48 h	72 h	144 h
CK	5.2aA	19.1cC	7.0cC
A_1	5.0cC	14.4aA	8.0bB
A_2	4.0dD	12.1eE	9.0aA
A_3	4.0dD	8.0 eE	3.0dD
A_4	2.0eE	5.0f	3.0dD
A_5	5.0cC	5.5dD	3.0dD

4. Discussion

Sophora flavescens is one of the traditional medicines with a long history. It used as a medicine has more than two thousand years of history according to written records in China. Alkaloids and flavonoids are main components in *S. flavescens* those has been testified have medicinal effects such as bactericidal, insecticidal, anti-tumor, anti-viral, etc[9-11]. It has been reported that some new matrine and oxymatrine compounds were isolated from *S. flavescen*s[12]. Liu proved that matrine could destroy endotoxin molecule to achieve anti-endotoxin[13]. Ji-Sang Hwang proved formononetin(1) isolated from *S. flavescens* inhibit the activity of monoamine oxidase[14]. At present, the study on *S. flavescens* focuses on separating the active components and its medicinal research. So far, there are few studies on the endophytic fungi of *S. flavescens* even on their secondary metabolites. This paper expects to obtain some structures which are the same or similar with the chemical composition or active ingredients of *S. flavescens* by studying the secondary metabolites of the endophyte of *S. flavescens*. But, the substance with antimicrobial activity is not the same with the compounds that have been found. It is a cyclic symmetric olefin structure with unsaturated double bonds and carbonyl group, excellent water solubility, lively physical and chemical properties. In this study, the compound is easy to inactivation under slightly alkaline conditions, or more than 50 ℃. And the test proved that the active component could increase the number of bacteria, while decreased the fungi.

5. Conclusions

In this sduty, it was the first time to isolate endophytic fungi BS001 from S. flavescens seeds. A new antimicrobial substance 6,7-(2′E)dibutenyl-5,8-dihydroxy-(Z)- cyclooct-2- ene -1,4-dione was extracted from fermenting liquor of BS001 which was an endophytic fungi of S. flavescens. This substance has broad-spectrum antibacterial properties. This finding has great significance and application value, and provides exploiture foreground for biopharmaceuticals and biopesticide.

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