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Antimicrobial activity and cytotoxicity of endophytes from *Scapania verrucosa* Heeg.

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ABSTRACT. We evaluated the antibacterial activity and cytotoxicity of endophytes isolated from *Scapania verrucosa* Heeg., which belongs to the liverwort class. A total of 49 endophytic fungi were isolated from *S. verrucosa* and classified into seven genera and one family in our previous study. In this study, the cytotoxic activity of the endophytes was assessed using the brine shrimp lethality bioassay, seven of which showed potent toxicity against the brine shrimp with 50% lethal concentration values less than 20 µg/mL. T-30 was the most toxic, with a 50% lethal concentration value of 7.15 µg/mL. Moreover, T-27 exhibited the strongest antibacterial activity against *Staphylococcus aureus*, with minimal inhibitory concentrations below 0.25 and 4 mg/mL, which can inhibit the growth of two standard strains - ATCC 25923 (methicillin-sensitive *S. aureus*) and ATCC 43300 (methicillin-

resistant *S. aureus*) - in a time-dependent manner, respectively. These results suggest that endophytes in *S. verrucosa* are the sources for the production of natural bioactive products and thus warrant further investigation.

Key words: *Scapania verrucosa* Heeg.; Endophytic fungi; Cytotoxicity; Brine shrimp lethality bioassay; Antibacterial activity; *Staphylococcus aureus*

INTRODUCTION

Endophytic fungi are ubiquitous in plant species, in which they inhabit the tissues beneath the epidermal cell layers without causing any discernible manifestation of disease to the host (Strobel, 2002). Since a paclitaxel (Taxol)-producing endophytic fungal strain was isolated from *Taxomyces andreanae* (Stierle et al., 1993), a great deal of attention has been focused on endophytic fungi, which can produce active constituents similar to those produced by the host (Yin and Sun, 2011) or other functional metabolites with potential antitumor, anti-inflammation, antioxidation, antimicrobial, antiviral, and antipesticide properties (Aly et al., 2011; Wang et al., 2011). Notably, the endophytes from unique habitats in special environments have attracted the most attention (Tan and Zou, 2001).

Scapania verrucosa, which belongs to the liverwort class, grows on forest ground, rocks, and decaying wood and is mainly distributed in south central China, Nepal, and the Himalayan region of Jammu and Kashmir (Gao and Cao, 2000; Söderström et al., 2007). Various rare and novel natural products contained in liverworts display many interesting biological activities such as antimicrobial, cytotoxic, insect antifeedant, muscle relaxing, enzyme inhibitory, apoptosis-inducing, lipoxygenase, calmodulin, hyaluronidase, cyclooxygenase, and thrombin inhibitory properties, and neuritic sprouting. In a previous study, we examined the constituents and antitumor and antifungal activities of the ether extracts of *S. verrucosa* and its endophytic fungus *Chaetomium fusiforme*. Although the ether extracts of *S. verrucosa* and *C. fusiforme* showed little correlation in chemical composition, both exhibited potent bioactivity, and the antitumor activity of the fungi was even better than that of its host plant (Guo et al., 2008). Furthermore, the ethyl acetate extracts (EEs) of some endophytes dramatically exhibit antioxidant activity (Zeng et al., 2011). To further explore the biological effects of the endophytes from *S. verrucosa*, we evaluated their antibacterial activity against methicillin resistant/sensitive *Staphylococcus aureus* (MRSA and MSSA, respectively) as well as their cytotoxicity using the brine shrimp lethality bioassay.

MATERIAL AND METHODS

Fermentation and extraction

In our previous study, we isolated endophytic fungi from S. *verrucosa* and identified it morphologically and molecularly (Zeng et al., 2011). The strains were cultured in potato dextrose broth for 7-14 days at 28° ± 1°C with gentle shaking at 1.5 *g*. The fermentation broth of each strain was centrifuged at 2280 *g* for 10 min, and the supernatant obtained was extracted

three times with ethyl acetate (v/v, 1:1) followed by concentration under reduced pressure to yield the final extract. In this study, the EEs of all isolated endophytic fungi were evaluated for their cytotoxicity by using the brine shrimp lethality bioassay and measurements of antibacterial activity against *S. aureus*.

Brine shrimp lethality bioassay

Brine shrimp (*Artemia salina*) eggs were obtained from Qingdao Haidabaichuan Biological Engineering Co., Ltd., Qingdao, Shandong, China. A modified brine shrimp bioassay was performed (Solis et al., 1993). Briefly, EEs were dissolved in dimethylsulfoxide (DMSO; Sigma, USA) and diluted serially (10, 50, 100, 200, and 400 µg/mL) in artificial seawater. Ten to 15 newly hatched (48 h old) brine shrimp larvae were added to each well and treated with 100 µL EE of various concentrations for 24 h at room temperature. Control brine shrimp were exposed only to 1% DMSO/seawater. Under an inverted microscope, the number of inanimate larvae was recorded 24 h after exposure to the fungal extracts. One hundred microliters of methanol was then added to each well, and after 15 min, the total number of shrimp in each well was counted.

Minimal inhibitory concentration (MIC) test

The antibacterial activities of the samples were determined using a number of staphylococcal strains, including two standard (ATCC 25923, ATCC 43300) and 10 isolated clinical (SA1-10) strains provided by the Municipal Center for Disease Control and Prevention (Fuzhou, China). MIC determination was carried out using a broth dilution method described previously (Peng et al., 2011). EE dilutions were prepared as follows: 4, 2, 1, 0.5, 0.25, 0.125, and 0.0625 mg/mL. Oxacillin (OXA) was prepared as follows: 1024, 512, 256, 128, 64, 32, 16, 8, 4, 2, 1, and 0.5 µg/mL. The sterile broth used for sample dilution was supplemented with DMSO to enhance solubility. MIC values were recorded as the lowest concentration that prevented visible bacterial growth after 24 h of incubation at 37°C.

Dynamic bacterial growth assay

According to the MIC results, the EEs of T-27 and positive control OXA (concentrations of T-27 and OXA used in assays were lower than their MICs) were added to the bacterial suspension (approximately 1.0 x 10^6 CFU/mL) and cultivated aerobically at 37°C in a slowly shaking rocking bed for 24 h. Growth rate was determined by measuring optical density at 600 nm (OD_{600}) at regular intervals (Jiang et al., 2011).

Data analysis

The 50% lethal concentration (LC_{50}) value at 95% confidence interval was calculated for each fungi extract with a probit analysis. All experiments were repeated three times.

RESULTS

Endophytes reportedly have various biological activities. This study was con-

ducted to evaluate the cytotoxicity and antibacterial activities of the endophytes isolated from *S. verrucosa*. As shown in Table 1, the cytotoxicity was determined using the brine shrimp lethality bioassay. Seven strains - i.e., T-2, T-5, T-21, T-22, T-26, T-30, and T-38 - belonging to *Hypocrea rufa*, *H. viridescens*, *Tolypocladium* sp, *Xylaria* sp (T-22 and T-26), *Chaetomium* sp, and *Creosphaeria* sp were more cytotoxic than the other tested strains, with LC_{50} values less than 20 µg/mL. T-30, belonging to *Chaetomium* sp, was the most cytotoxic, with an LC_{50} value of 7.15 µg/mL, suggesting that T-30 could produce potent cytotoxic components.

Table1. Brine shrimp lethality bioassay of endophytic fungi from *Scapania verrucosa.*

Strain	Taxa	LC_{50} (µg/mL)	95%CI	Strain	Taxa	LC_{50} (µg/mL)	95%CI
T-1	*Hypocrea viridescens*	308.98	259.48-387.69	T-26	*Xylaria* sp 1	16.67	12.83-20.93
T-2	*Hypocrea rufa*	12.65	10.55-32.52	T-27	*Xylaria* sp 1	24.20	19.19-30.81
T-3	*Xylaria* sp 1	26.82	21.61-32.76	T-28	Xylariaceae sp 2	20.06	16.28-24.96
T-4	*Penicillum* sp 1	97.14	34.11-440.41	T-29	*Nemania diffusa*	65.38	56.85-75.06
T-5	*Hypocrea viridescens*	16.35	12.72-27.30	T-30	*Chaetomium* sp 1	7.15	3.58-15.02
T-6	Xylariaceae sp 1	23.18	18.38-28.83	T-31	*Creosphaeria* sp	217.76	95.45-383.12
T-7	Xylariaceae sp 1	41.49	38.23-50.78	T-32	*Chaetomium* sp 1	41.43	38.73-46.58
T-8	Xylariaceae sp 2	53.56	48.30-61.30	T-33	*Xylaria* sp 1	22.15	16.12-29.08
T-9	Xylariaceae sp 1	21.67	16.65-27.63	T-34	*Penicillum* sp 2	42.78	36.58-49.52
T-10	*Xylaria* sp 1	69.21	21.63-147.52	T-35	Xylariaceae sp 1	581.39	219.21-988.19
T-11	*Chaetomium* sp	270.91	224.29-356.59	T-36	Xylariaceae sp 1	33.38	27.51-39.81
T-12	Xylariaceae sp 1	150.59	127.79-180.41	T-37	*Chaetomiun fusiforme*	237.32	128.27-328.34
T-13	*Xylaria* sp 3	95.06	69.43-135.50	T-38	*Creosphaeria* sp	12.03	10.75-15.44
T-14	*Nemania diffusa*	213.06	176.52-268.53	T-39	*Creosphaeria* sp	189.60	156.83-233.15
T-15	*Nemania diffusa*	51.11	43.91-59.62	T-40	*Creosphaeria* sp	36.55	33.12-40.96
T-16	*Nemania diffusa*	71.87	63.13-83.68	T-41	*Chaetomium* sp 2	242.56	107.11-407.67
T-17	*Xylaria* sp 1	33.46	30.43-36.76	T-42	*Chaetomium* sp 2	72.45	58.72-104.84
T-18	*Creosphaeria sassafras*	40.10	23.16-45.47	T-43	*Creosphaeria sassafras*	69.46	59.63-80.42
T-19	*Tolypoclaidium* sp	21.11	18.00-24.48	T-44	*Chaetomium globosum*	120.67	105.44-142.03
T-20	*Nemania* sp	58.83	50.98-65.74	T-45	*Chaetomium globosum*	104.86	90.86-121.56
T-21	*Tolypoclaidium* sp	16.09	10.78-21.68	T-46	*Creosphaeria sassafras*	51.39	44.14-59.76
T-22	*Xylaria* sp 1	10.48	7.09-15.47	T-47	*Nemania* sp	359.34	301.45-463.98
T-23	*Chaetomium* sp 1	163.70	80.53-350.97	T-48	*Penicillum* sp 3	622.69	287.39-806.52
T-24	*Chaetomium globosum*	49.41	40.85-55.65	T-49	*Creosphaeria sassafras*	57.34	50.55-64.60
T-25	*Creosphaeria sassafras*	35.41	5.39-102.95				

LC_{50} = 50% lethal concentration; 95%CI = 95% confidence interval.

Antibacterial activity was tested against some *S. aureus* strains. According to the results in Table 2, the endophytes displayed moderate antibacterial activity against *S. aureus*, including two standard strains (ATCC 25923 and ATCC 43300) and 10 clinical isolated strains (SA1-10) that were divided into two types (MSSA and MRSA). T-27, belonging to *Xylaria* sp, displayed the strongest antibacterial activity among these endophytes, inhibiting 8 MSSA and 4 MRSA strains with MICs less than 0.25 and 4 mg/mL, respectively. Moreover, the bacterial dynamic growth curves of ATCC 25923 and ATCC 43300 were observed. The doses of T-27 were 31.25 µg/mL and 1 mg/mL, and the doses of OXA were 0.125 and 128 µg/mL for ATCC 25923 and ATCC 43300, respectively, which were both less than their MICs. As shown in Figures 1 and 2, T-27 and OXA dose-dependently decreased the growth rate in ATCC 25923 and ATCC 43300, ATCC 25923 was much more sensitive to T-27 and OXA than ATCC 43300 was. These results indicated that T-27 might be a source for antibacterial drugs.

Table 2. Results of the minimal inhibitory concentration (MIC).

Strains	MIC (mg/mL)											
	ATCC* 25923	ATCC** 43300	SA-1*	SA-2**	SA-3*	SA-4*	SA-5*	SA-6*	SA-7*	SA-8**	SA-9*	SA-10**
T-1	1	>4	0.5	>4	2	4	1	>4	2	>4	>4	>4
T-2	1	>4	2	>4	0.5	>4	1	>4	1	>4	>4	>4
T-3	2	>4	>4	>4	>4	1	2	>4	>4	>4	>4	>4
T-4	0.5	>4	2	>4	4	1	0.5	4	2	>4	0.25	>4
T-5	0.25	>4	0.25	>4	>4	0.5	2	>4	1	>4	>4	>4
T-6	0.5	>4	0.25	>4	0.5	1	1	>4	0.5	>4	2	>4
T-7	1	>4	0.25	>4	2	2	2	>4	2	>4	>4	>4
T-8	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-9	0.5	>4	1	>4	0.5	2	2	2	2	>4	1	>4
T-10	0.125	>4	0.125	>4	0.125	0.5	0.5	0.25	0.25	>4	0.5	>4
T-11	1	>4	1	>4	2	1	2	>4	2	>4	2	>4
T-12	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-13	1	>4	0.5	>4	2	1	2	4	1	>4	1	>4
T-14	1	>4	0.5	>4	1	1	1	2	1	>4	1	>4
T-15	0.125	>4	0.125	>4	0.125	0.5	0.25	0.5	0.5	>4	0.25	>4
T-16	0.5	>4	0.25	>4	1	0.5	1	2	0.25	>4	0.5	>4
T-17	0.25	>4	0.125	>4	0.5	0.5	0.5	1	0.5	>4	1	>4
T-18	1	>4	1	>4	2	0.5	2	4	4	>4	4	>4
T-19	2	>4	2	>4	4	4	4	>4	4	>4	2	>4
T-20	2	>4	2	>4	2	4	4	>4	4	>4	4	>4
T-21	4	>4	4	>4	2	4	1	>4	4	>4	>4	>4
T-22	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-23	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-24	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-25	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-26	1	>4	1	>4	1	2	1	4	0.5	>4	1	>4
T-27	0.125	4	0.125	2	0.125	0.125	0.125	0.25	0.125	4	0.125	2
T-28	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-29	0.25	>4	0.5	>4	1	1	1	2	0.5	>4	0.5	>4
T-30	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-31	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-32	0.5	>4	1	>4	2	0.5	2	>4	1	>4	4	>4
T-33	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-34	1	>4	1	>4	4	2	2	1	2	>4	2	>4
T-35	2	>4	1	>4	4	1	2	2	2	>4	4	>4
T-36	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-37	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-38	1	>4	4	>4	4	2	4	>4	>4	>4	>4	>4
T-39	1	>4	1	>4	2	1	>4	>4	0.5	>4	4	>4
T-40	1	>4	1	>4	2	1	4	>4	2	>4	2	>4
T-41	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4	>4
T-42	2	>4	1	>4	0.5	1	2	0.5	0.5	>4	1	>4
T-43	2	>4	2	>4	>4	1	>4	2	>4	>4	2	>4
T-44	4	>4	2	>4	>4	>4	4	>4	0.5	>4	0.5	>4
T-45	0.5	>4	0.5	>4	1	0.5	1	2	0.5	>4	1	>4
T-46	1	>4	0.5	>4	1	1	2	4	2	>4	1	>4
T-47	1	>4	1	>4	0.5	1	1	4	1	>4	1	>4
T-48	2	>4	1	>4	1	1	2	4	1	>4	1	>4
T-49	2	>4	4	>4	4	2	4	4	2	>4	2	>4
OXA	0.5	512	<0.5	64	1	<0.5	1	2	<0.5	128	1	256

*MSSA = methicillin-sensitive *Staphylococcus aureus*; **MRSA = methicillin-resistant *S. aureus*; OXA = oxacillin (μg/mL) was used as the positive drug. The concentration of the bacterial suspension was 1.0 x 10^5 CFU/mL.

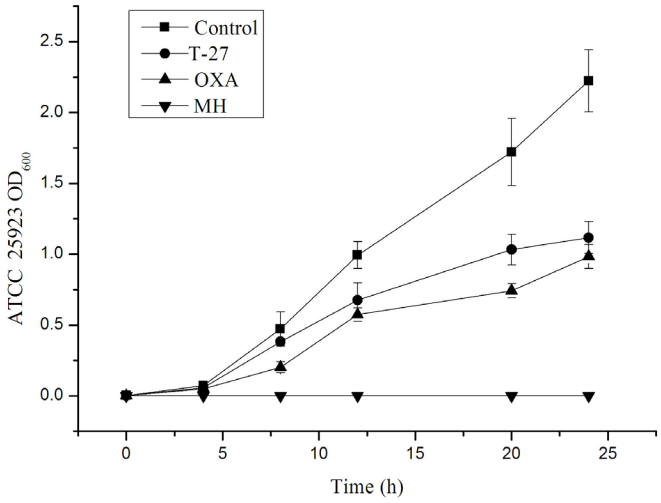

Figure 1. Inhibitory effect of T-27 on the growth of ATCC 25923.

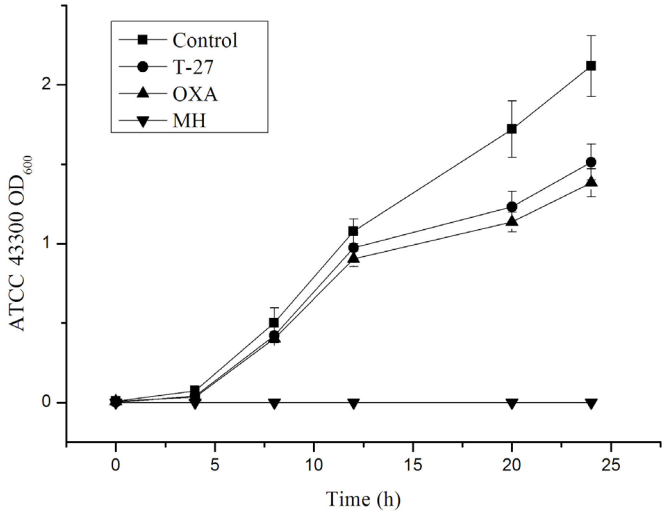

Figure 2. Inhibitory effect of T-27 on the growth of ATCC 43300.

DISCUSSION

S. verrucosa lives in a special environment, spreading over wild areas and occurring in small populations. Owing to the difficulty of obtaining large amounts of sample, the investigation of chemical composition was halted (Guo et al., 2008). Seeking a way out of the stalemate, researchers have recently recognized endophytic fungi from medical plants as alternative producers of bioactive components (Weber et al., 2007). These fungi can be cultured quickly from

biomass that can be accumulated through large-scale fermentation (Xu et al., 2009).

Our previous report detailed the separation of 49 endophytic fungi from fresh *S. verrucosa* plants, which were classified into 7 genera (namely, *Hypocrea*, *Penicillium*, *Tolypocladium*, *Chaetomium*, *Xylaria*, *Nemania*, and *Creosphaeria*) and 1 family (Xylariaceae) using morphological and molecular identification. The majority of these isolated endophytic fungi belonged to the Chaetomium (18.37%), Creosphaeria (18.37%), Xylaria (16.33%), and Xylariaceae (16.33%) (Zeng et al., 2011). As a part of our ongoing search for biologically active metabolites, we have reported the antioxidant, antitumor, and antifungal activities of these endophytes. This study was conducted to elucidate the bioactivity more comprehensively. First, for cytotoxicity assessment, the brine shrimp lethality bioassay was selected because it is rapid, simple, easily mastered, inexpensive, and requires only a small amounts of test material. In addition, the bioassay has a robust correlation with cytotoxic activity in some human solid tumors (Ghisalberti, 1993; McLaughlin et al., 1998). Since its introduction, this *in vivo* lethality test has been successively used to provide a frontline screen that can be supported by more specific and sophisticated bioassays once active compounds have been isolated. As shown in Table 1, 7 strains displayed remarkable cytotoxicity against brine shrimp - particularly, T-30, with an LC_{50} value of 7.15 μg/mL. In our previous study, the *Pyricularia oryzae* P-2b model and 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide method were used for *in vitro* screening of antitumor endophyte strains from *S. verrucosa* [such as T-11, T-12, T-24, T-28, T-31, T-32, T-35, and T-36 (Guo et al., 2009)], revealing the active strains from the brine shrimp lethality bioassay. T-11 was the most active strain and was identified as *Chaetomium* sp, the same fungal taxon as that of T30, thus suggesting that *Chaetomium* sp isolated from *S. verrucosa* produces toxic substances that accounted for the significant cytotoxicity of their EEs. Endophytes belonging to *Chaetomium* sp, isolated from *Ginkgo biloba*, can produce active constituents with significant cytotoxic activity against brine shrimp (Qin et al., 2009). Accordingly, various bioassays should be performed to understand the biological activities of these compounds more thoroughly. Both T-11 and T-30, belonging to *Chaetomium* sp, presumably produced the active compounds responsible for the potent cytotoxicity.

Owing to drug resistance and severe adverse drug reactions to many antibiotics, the search for new and effective antibiotic agents is essential (de León et al., 2010). Mounting evidence suggests that metabolites of endophytes can inhibit or kill a wide variety of harmful microorganisms, including phytopathogens, bacteria, fungi, viruses, and protozoans (Strobel et al., 2004). In this study, we observed the antibacterial activity of endophytes against some strains of *S. aureus* including MSSA and MRSA. We found that T-27, belonging to *Xylaria* sp, was the most effective against *S. aureus* strains with MICs less than 0.25 and 4 mg/mL (see Table 2). T-27 exhibited time-dependent inhibition against ATCC 25923 (MSSA) and ATCC 43300 (MRSA) with EE concentrations of 31.25 μg/mL and 1 mg/mL, respectively, according to the bacterial dynamic growth curves shown in Figures 1 and 2. Recently, an endophytic *Xylaria* sp isolated from *G. biloba* L. has been shown to have broad antimicrobial activity (Liu et al., 2008); its metabolites contained the antimicrobial compound 7-amino-4-methylcoumarin. Furthermore, crude EEs of *Xylaria* sp strains (Ascomycetes) showed dramatic antibacterial activity against MRSA strains (Ramesh et al., 2012), suggesting that T-27 consumes and transfers the composition of the culture medium to produce antibacterial metabolites.

In conclusion, our results demonstrated that the endophytes in *S. verrucosa* exhibited moderate antibacterial activity and potent cytotoxicity, suggesting that they are a source for the production of natural bioactive products and thus warrant further investigation.

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