Anti-microbial screening of endophytic fungi from *Hypericum perforatum* Linn.

Huawei Zhang*, **Chen Ying and Yifei Tang**
School of Pharmaceutical Sciences, Zhejiang University of Technology, Hangzhou, China

Abstract: Anti-microbial properties of 21 endophytic fungal strains from *Hypericum perforatum* Linn. were evaluated against three human pathogens, *Staphyloccocus aureus*, *Escherichia coli* and *Rhodotorula glutinis*, and two phytopathogens, *Rhizoctonia cerealis* and *Pyricularia grisea*. The results indicated that the ethyl acetate extracts of endophytic fermentation broth had stronger anti-microbial activities than their fermentation broth. And the inhibitory effect of the endophytic extracts on human pathogens was better than those on phytopathogens. Among these endophytic fungi, strains GYLQ-10, GYLQ-24 and GYLQ-22 respectively showed the strongest activities against *S. aureu*, *E. coli*, *R. glutinis*. GYLQ-14 and GYLQ-22 exhibited the most pronounced effect on *P. Grisea* while both GYLQ-06 and GYLQ-08 had the strongest anti-microbial activities against *R. cerealis*. Till now, this study is the first report on the isolation of endophytic fungi from *H. Perforatum* Linn. and their anti-microbial evaluation.

Keywords: *Hypericum perforatum* Linn., endophytic fungus, anti-microbial activity, extract, fermentation broth.

INTRODUCTION

Globally, almost 50 thousand people are being killed by infectious diseases every day. During the last three decades, a large number of antibiotics produced as secondary metabolites by microbes play an important role in treating these diseases (Jamil *et al*., 2007). However, these antibiotics are losing their functions because of emergence of new pathogens and multi-drug resistance. All these have necessitated the need for searching more novel molecules with better anti-microbial properties from other sources (Bhagat *et al*., 2012).

Endophytic fungi are a common and diverse group of microorganism within plant tissues or organs without causing any apparent disease (Saikkonen *et al*., 1998; Azewedo *et al*., 2000; Shen *et al*., 2012; Zhang *et al*., 2012). Since Strobel and his co-workers isolated a taxol or taxane producing endophytic fungi from *Taxus brevifolia* in 1993 (Stirele *et al*., 1993), endophytes become a research hot spot gradually. A large number of evidence have indicated that endophytic fungi could produce abundant bioactive compounds with a broad spectrum of bioactivities, such as antibacterial substances (Arivudainambi *et al*., 2011; Cui *et al*., 2011; Yu *et al*., 2010), fungicides (Strobel *et al*., 2001; Zhang *et al*., 2008), anticancer agents (Puri *et al*., 2006; Hsieh *et al*., 2009), antioxidant chemicals (Sadananda *et al*., 2011) and so on (Zhang *et al*., 2006).

Hypericum perforatum Linn., a medicinal plant known as St John's wort, widely distributed in China as well as North American and Europe. Many documents reported that *H. perforatum* Linn. has diverse clinical usages in the treatment of depressant (Jang *et al*., 2002) and kidney and lung ailment (Kumar *et al*., 2002) and has potent nootropic effect (Kumar *et al*., 1999) and anti-diarrheal activities (Khan *et al*., 2009). However, no endophytic microbe associated with this plant had been documented so far. In order to discover more bioactive metabolite-producing endophytes, investigation on the isolation and anti-microbial screening of endophytic fungi from *H. perforatum* Linn. was carried out.

**Corresponding author:* e-mail: hwzhang@zjut.edu.cn

MATERIALS AND METHODS

Plant material

The whole living plant of *H. perforatum* Linn. was collected from Qinling Mountain, Northwest China, and cultivated at campus of Zhejiang University of Technology, China. Some plant materials were used for endophyte isolation within 48 hours after harvest.

Isolation and identification of endophytic strains

A total of 21 endophytes were isolated from the healthy *H. perforatum* Linn. according to the procedure of previously reported method about endophytes isolated from *Artemisia annua* Linn. in our lab (Zhang *et al*., 2012). The purified strains were respectively numbered as GYLQ-1-21, followed by transferring into potato dextrose agar (PDA) slants separately. Then those isolates were kept at 4^{o}C after being cultured at 28^{o}C for 7 days. By observing colonial morphology, mycelium and spore characteristics (Wei, 1979), these endophytic microbes were primarily identified (table 1).

Preparation of fermentation broth and ethyl acetate extract

Each endophytic fungal strain was cultured on PDA at 28^{o}C for 7 days. Then a balanced amount of fungal colony was transferred to the culture broth in 500mL Erlenmeyer

flasks each containing sterilized 300mL of potato dextrose broth (PDB) respectively, followed by shaking at 150 rpm for 15 days at 28°C. Furthermore, the mycelium and the culture broth were separated by centrifugation at 8000 rpm for 10 minutes at 4°C. 2mL of culture broth was absorbed and filtered with a 0.22μm bacterial filter to obtain the tested fermentation broth. The residual fermentation broth was extracted three times with 300mL of ethyl acetate (Merck) and the upper solvent was dried by a rotary evaporator in vacuum to yield the each organic phase extract. Each afforded extract was dissolved in dimethyl sulfoxide (DMSO, Merck) and its final concentration reached 10mg/mL followed by preserving at 4°C.

Anti-microbial assay

Anti-bacterial test

Three human pathogens, *S. aureus*, *E. coli* and *R. glutinis*, and two plant pathogens, *R. cerealis* and *P. grisea*, were purchased from China Center for Type Culture Collection. Transferred *E. coli* and *S. aureus* were kept on nutrient agar (NA) slants at 4°C, while two fungal pathogenic microbes maintained on PDA slants at 4°C. *E. coli* and *S. aureus* were transferred into 250mL Erlenmeyer flasks each containing sterilized 150mL of NA and incubated at 28°C on a rotary shaker at 120 rpm for 8 hours. 200μL of 8h bacterial broth culture were spread evenly on NA plates. Penicillin (10 μg/disk, Sigma-Aldrich) was respectively used as the positive control. The anti-bacterial test was performed by the disk diffusion method (DDM) (Zaika, 1998; Ahmads and Farrukh, 2012). 100μL of prepared endophytic extract and the positive control were carefully dropped on a standard sterilized paper disk (Φ=6 mm) using sterilized dropping pipette and subsequently placed on NA plate. Then the plates were incubated at 37°C for 2 days. The diameter of inhibition zone (in mm) was measured to evaluate the anti-microbial effect. All tests were carried out in triplicate.

Anti-fungal test

Three fungal blocks of *R. glutinis*, *R. cerealis* and *P. grisea* were scraped from PDA slants and subsequently placed on the central of new PDA plates respectively for cultivating at 28°C. Till the colony diameter reached 3 cm, an oxford plate (6×7.8×10 mm), 1 cm away from the edge of colony, was added with 100μL of culture broth or endophytic extract followed by cultivating at 28°C for 4 days. Amphotericin B (10μg/disk, Sigma-Aldrich) was used as the positive control. Inhibition zone in diameter (mm) was measured to assess anti-fungal activity. All tests were also carried out in triplicate.

RESULTS

A total of 21 endophytic strains were isolated from *H. perforatum* Linn. and preliminary classified into 5 genus (table 1), including *Fusarium* sp., *Mucor* sp., *Aspergillus* sp., *Xylaria* sp. and *Hypocrea* sp., which *Aspergillus* sp. were the dominant strains. Anti-microbial tests revealed that the fermentation broth and ethyl acetate extracts of endophytic fungi had different inhibitory effects on *E. coli*, *S. aureus*, *R. cerealis*, *P. grisea* and *R. glutinis*. The effects of ethyl acetate extracts were stronger than those of fermentation broth at 10mg/mL. However, their anti-microbial activities were weaker than those of the positive controls.

As shown in table 1, five endophytic fungi (GYLQ-01, GYLQ-06, GYLQ-10, GYLQ-19, GYLQ-20) exhibited inhibitory effect on at least two human pathogens. Especially, the ethyl acetate extracts of GYLQ-20, GYLQ-10 and GYLQ-18 had the strongest activities against *E. coli*, *S. aureus*, *R. glutinis* with the inhibition zones of 24 mm, 15 mm, 31 mm, respectively. Among these fungal endophytes, only GYLQ-02 showed a broad spectrum of anti-fungal activity against phytopathogens. The ethyl acetate extracts of GYLQ-14 and GYLQ-18 were found to have the strongest effect on *P. grisea* with inhibition zones of 12 mm while the extracts of GYLQ-06 and GYLQ-08 exhibited the highest inhibitory activity against *R. cerealis* with an inhibition zone of 4.2 mm.

DISCUSSION

Although antibiotics play a key role in the treatment of human and plant diseases, some of them are encountering multi-drug resistance or cause severe adverse drug reactions (Rouveix, 2003; Gill *et al*., 2013). This situation leaves us with expensive and limited choices. So, it is an urgent need to search for new and more potent antibiotics. As we know, microbe is one of important sources of bioactive substances. A large number of evidence indicates that endophytic microbe is a treasure trove of natural products with new structures and/or strong bioactivities (Zhang et al., 2006). Moreover, fungal endophytes seem to be excellent producers of anti-microbial substances, such as munumbicin A-D (Castillo *et al*., 2002). cephalosol (Zhang *et al*., 2008), cercosporamide, *β*-sitosterol and trichodermin (Wang *et al*., 2012).

The present study firstly focused on investigation of the isolation and anti-microbial screening of endophytic fungi isolated from *H. perforatum* Linn. The results indicated that 21 isolated fungal endophytes had some anti-microbial activities against three human microbial pathogens *E. coli*, *S. aureus*, *R. Glutinis*, and two phytopathogenic fungi *R. Cerealis* and *P. Grisea*. The inhibitory effects of their ethyl acetate extracts were stronger than those of the fermentation broth. As far as anti-bacterial activity concerned, strains GYLQ-10 and GYLQ-20 were the best bio-control candidates. While stains GYLQ-18 and GYLQ-19 were the best endophytic strains for inhibiting phytopathogens *P. grisea* and *R. glutinis.* These selected endophytic strains would have potential application in new antibiotics production.

Table 1: Anti-microbial effects of endophytic fungal strains from *Hypericum perforatum* Linn

Strain	Genus	Antimicrobial effect[b]									
		Ec		*Sa*		*Rc*		*Pg*		*Rg*	
		E[c]	F[c]	E[c]	F[c]	E[c]	F[c]	E[c]	F[c]	E[c]	F[c]
GYLQ-01	*Fusarium* sp.	–	–	–	–	+	–	–	–	+	–
GYLQ-02	*Mucor* sp.	–	–	–	–	+	–	+	–	+	–
GYLQ-03	*Aspergillus* sp.	+++	++	–	–	–	–	–	–	–	–
GYLQ-04	*Hypocrea* sp.	++	–	+	–	–	–	–	–	–	–
GYLQ-05	*Hypocrea* sp.	++	–	–	–	–	–	–	–	–	–
GYLQ-06	*Aspergillus* sp.	+	–	+	–	+	–	–	–	+	–
GYLQ-07	*Xylaria* sp.	–	–	+	+	+	–	–	–	–	–
GYLQ-08	*Aspergillus* sp.	–	–	–	–	+	–	–	–	+	–
GYLQ-09	*Fusarium* sp.	–	–	–	–	–	–	–	–	+	–
GYLQ-10	*Aspergillus* sp.	+++	+	++	+	–	–	+	–	–	–
GYLQ-11	*Mucor* sp.	–	–	–	–	–	–	+	–	+	–
GYLQ-12	*Xylaria* sp.	–	–	–	–	–	–	–	–	–	–
GYLQ-13	*Aspergillus* sp.	+	–	–	–	+	–	–	–	–	–
GYLQ-14	*Mucor* sp.	–	–	–	–	–	–	++	–	+	–
GYLQ-15	*Hypocrea* sp.	+	–	–	–	–	–	+	–	–	–
GYLQ-16	*Aspergillus* sp.	+++	–	–	–	–	–	+	–	–	–
GYLQ-17	*Mucor* sp.	–	–	–	–	–	–	+	–	–	–
GYLQ-18	*Mucor* sp.	–	–	–	–	–	–	++	–	+++	–
GYLQ-19	*Aspergillus* sp.	+	–	–	–	–	–	++	–	+	–
GYLQ-20	*Aspergillus* sp.	+++	–	++	–	–	–	–	–	–	–
GYLQ-21	*Aspergillus* sp.	–	–	–	–	–	–	–	–	–	–
Penicillin		++++		++++							
Amphotericin B						++++		++++		++++	

A Expressed by the diameter of inhibition zones: -, no inhibition; +, <10 mm; ++, 10-15 mm; +++, 16–20 mm; ++++, >20 mm. b *Ec-Escherichia coli*, *Sa-Staphylococcus aureus*, *Rc-Rhizoctonia cerealis*, *Pg-Pyricularia grisea, Rg-Rhodotorula glutinis*. c E-ethyl acetate extract; F-fermentation broth.

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