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In vitro antioxidant activities of endophytic fungi isolated from the liverwort *Scapania verrucosa*

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ABSTRACT. We investigated *in vitro* antioxidant activities of 49 endophytic fungi isolated from the liverwort *Scapania verrucosa*. Based on morphological and molecular identification, the endophytic fungi isolated were classified into seven genera (*Hypocrea*, *Penicillium*, *Tolypocladium*, *Chaetomium*, *Xylaria*, *Nemania*, and *Creosphaeria*), all belonging to one family (Xylariaceae). By screening with the 2,2'-azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) decolorization assay, the ethyl acetate extracts of five endophytic fungi (T7, T21, T24, T32, and T38 strains), which exhibited remarkable Trolox equivalent (TE) antioxidant capacity (ranging from 997.06 to 1248.10 μmol TE/g extract), were selected and their antioxidant capacity was further evaluated by assays for 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical

scavenging, hydroxyl radical scavenging, reducing power, and ferrous ion chelating. The ethyl acetate extracts of two endophytic fungi (T24 and T38) were found to have comparable scavenging abilities on both DPPH-free radicals (93.9 and 88.7%, respectively, at 50 μg/mL) and hydroxyl radicals (97.1 and 89.4%, respectively, at 2 mg/mL) when compared with those of the positive controls (ascorbic acid and butylated hydroxytoluene, respectively). Although their reducing powers were similar to that of butylated hydroxytoluene, as indicated by absorbance (0.35 and 0.30 at 50 μg/mL, respectively), only the T38 strain's ethyl acetate extract showed ferrous ion chelating ability (92.9% at 1 mg/mL) comparable to that of the EDTA-2Na control. These endophytic fungi in *S. verrucosa* are a potential novel source of natural antioxidants.

Key words: Endophytic fungi; Antioxidant activity; ABTS; DPPH; Scavenging ability; Ferrous ion chelating ability

INTRODUCTION

Reactive oxygen species (ROS), such as superoxide anion, hydroxyl radical and nitric oxide, may cause disruption of membrane fluidity, protein denaturation and lipid peroxidation by creating oxidative stress, which could lead to cell injury and death. They also cause alteration of platelet functions (Fridovich, 1978; Kinsella et al., 1993; Anderson et al., 1996). Under normal conditions, naturally occurring antioxidant enzymes in the body can counteract the cellular effects of ROS. However, the protective ability of these scavengers is overwhelmed by rapid generation of ROS during intense exercise training of both aerobic and anaerobic athletes, which causes muscle fatigue (Medved et al., 2004) and some pathological conditions, such as reperfusion of ischemic tissue, which results in cell death by apoptosis (Castaneda et al., 2003). To reduce the harm of ROS to the human body, sufficient amounts of exogenous antioxidants are required. In response to the growing consumer demand for food supplements that are free of synthetic antioxidants with carcinogenic potential, such as butylated hydroxytoluene (BHT) (Baardseth, 1989), there is an overwhelming trend to search for naturally occurring antioxidants in the past decades (Gould, 1995; Reische et al., 1998).

Endophytic fungi are microbes that reside in living plant tissues without causing any immediate harm to their host (Petrini, 1991). They are present in almost all plant species and have been recognized as a potential source of novel medicinal compounds (Tan and Zou, 2001). As reviewed by Schulz et al. (2002), 51% of the biologically active substances isolated from endophytes were previously unknown. Although a number of bio-pharmacological compounds with antimicrobial, antitumor, antiinflammatory, and antiviral activities have been previously isolated from endophytes (Aly et al., 2008; Lin et al., 2008; Souza et al., 2008; Liu et al., 2008), information related to their antioxidant activities is very scanty (Strobel et al., 2002).

Scapania verrucosa is a liverwort that commonly grows on forest ground, rocks and decaying wood and is mainly distributed in China, Nepal and the Himalayan region of Jammu and Kashmir (Gao and Cao, 2000; Söderström et al., 2007). We have found that the ether extracts of these fungi have strong antifungal and antitumor activity (Guo et al., 2008, 2009). In the present study, their antioxidant activity was evaluated by a variety of methods.

MATERIAL AND METHODS

Isolation of endophytic fungi

All endophytic fungi were isolated from the *S. verrucosa* specimens collected in November 2006 on Mountain Yandang, Zhejiang Province of China. A total of 35 healthy samples (whole plant, 10-15 mm long) were selected from 125 *S. verrucosa* plants. The isolation of endophytic fungi was performed as we previously described (Guo et al., 2008). Briefly, after rinsing with distilled water, all samples were surface-disinfected and then sequentially washed with 75% ethanol (1 min), 2.5% sodium hypochlorite (15 min) and sterilized water (three times). The leaves were torn off the samples, then placed on potato dextrose agar (PDA) medium supplemented with antibiotics (200 µg/mL ampicillin and 200 µg/mL streptomycin) and incubated at 28° ± 1°C for about one week. Aliquots of 1.0 mL of the last wash were also inoculated in PDA to evaluate the effectiveness of the disinfection process. The mycelium originating from the tear of the sample was purified and cultured under the same conditions. In total, 120 fragments were used in this study. The control, which was the whole plant, did not grow any mycelia after culture under the conditions used for the samples.

The purified endophytic fungi were numbered and separately transferred to fresh PDA slants prior to culturing at 28° ± 1°C for 7 days. All isolated endophytic fungi were identified based on their ribosomal DNA (rRNA gene) sequences, morphology of the fungal culture, and characteristics of their spores (Ainsworth et al., 1973; Hawksworth et al., 1983; Barnett and Hunter, 1997). Total genomic DNA was extracted from fungal mycelia grown on PDA using the cetyltrimetylammonium bromide (CTAB) method (O'Donnell et al., 1997). Primers ITS5 (5'-TCCTCCGCTTATTGATATGC-3') and ITS4 (5'-GGAAGTAAAAGTCGTAAGG-3') were used to amplify the 5.8S and ITS regions. The DNA fragment was amplified and sequenced using the method described by Guo et al. (2000). Sequences were compared against those in the GenBank database using the Basic Local Alignment Search Tool (BLAST) algorithm.

Fermentation and extraction

All strains were cultured in PDB medium (200 g dehydrated mashed potatoes, 20 g glucose in 1 L water, pH 5.5) for 7-14 days at 28° ± 1°C with gentle shaking at 1.5 *g*. The fermentation broth of individual strains was centrifuged at 2280 *g* for 10 min, and the supernatant obtained was extracted three times with ethyl acetate (v/v, 1:1). The ethyl acetate was concentrated under reduced pressure to yield the final extract. In this study, the ethyl acetate extracts of all isolated endophytic fungi were used to determine their corresponding *in vitro* antioxidant activities.

2,2'-Azino-di(3-ethylbenzthiazoline-6-sulfonic acid) (ABTS) decolorization assay

The Trolox equivalent (TE) antioxidant capacity (TEAC) was determined by the ABTS decolorization assay according to the method of Re et al. (1999). In brief, the ABTS radical cation ($ABTS^+$) was first produced by reacting ABTS stock solution (7 mM) with 2.45 mM potassium persulfate. The mixture was then placed in the dark at room temperature for 12-16 h before use. Under this condition, $ABTS^+$ can be stable in this form for more than 2 days. For the

determination of TEAC, the $ABTS^+$ solution was diluted with double-distilled water to obtain an absorbance of 0.70 ± 0.02 at 734 nm. Aliquots of 30 μL of the sample extract were then added to 3 mL diluted $ABTS^+$ solution, and the absorbance was read exactly 6 min after initial mixing. All determinations were performed in triplicate and the results are reported as μmol TE/g extract.

2,2'-Diphenyl-1-picrylhydrazyl (DPPH) radical scavenging assay

The DPPH radical scavenging assay was performed as described by Miliauskas et al. (2004) with slight modifications. In brief, individual sample extract (1 mL) at different concentrations (6.25, 12.5, 25, 50, and 100 μg/mL) was added to 2 mL DPPH (5.9 mg/100 mL methanol). After a 30-min reaction, absorbance was read at 517 nm. The scavenging ability on DPPH radicals was calculated using the following equation:

$$\text{Scavenging ability on DPPH radicals (\%)} = [(A1 - A2) / A1] \times 100$$

where *A1* is the absorbance of the control (containing all reagents except the sample extract), and *A2* is the absorbance of the sample extract. BHT and ascorbic acid were used as positive controls.

Hydroxyl radical scavenging assay

The scavenging ability of the five sample extracts on hydroxyl radicals was determined according to the method described by Smirnoff and Cumbes (1989) with some modifications. Briefly, individual sample extract (1 mL) at different concentrations (0.25, 0.5, 1, 1.5, and 2 mg/mL) was added to the reagent containing 1 mL 1.5 mM $FeSO_4$, 0.7 mL 6 mM H_2O_2 and 0.3 mL 20 mM sodium salicylate. After incubation for 1 h at 37°C, absorbance of the reaction mixture was read at 562 nm. The scavenging ability on hydroxyl radicals was calculated using the following equation:

$$\text{Scavenging ability on hydroxyl radicals (\%)} = [(A1 - A2) / A1] \times 100$$

where *A1* is the absorbance of the control reaction (containing all reagents except the sample extract), and *A2* is the absorbance of the sample extract. Again, BHT and ascorbic acid were used as positive controls.

Reducing power assay

The reducing power assay was conducted as previously described by Oyaizu (1986). In brief, each sample extract (1 mL) at different concentrations (6.25, 12.5, 25, 50, and 100 μg/mL) was first mixed with 2.5 mL 0.2 M phosphate buffer, pH 6.6, and 2.5 mL 1% potassium ferricyanide. After incubation at 50°C for 20 min, 2.5 mL 10% trichloroacetic acid was added to the mixture followed by centrifugation at 3000 rpm for 10 min. Subsequently, 2.5 mL of the upper layer of the mixture was added to 2.5 mL distilled water and 0.5 mL 0.1% ferric chloride, and the absorbance of the resulting solution was read at 700 nm against a blank. BHT and ascorbic acid were used as positive controls.

Ferrous ion chelating assay

The chelating ability of the five selected sample extracts was determined by the method previously reported by Singh and Rajini (2004). In brief, individual extracts (1 mL) at different concentrations (0.25, 0.5, 1, 1.5, and 2 mg/mL) were mixed with an equal volume of 0.1 mM $FeSO_4$ and 0.25 mM ferrozine. After incubation for 10 min, absorbance of the mixture was measured at 562 nm. The chelating ability on ferrous ion was calculated using the following equation:

$$\text{Chelating ability on ferrous ion (\%)} = [(A1 - A2) / A1] \times 100$$

where *A1* is the absorbance of the control reaction (containing all reagents except the sample extract), and *A2* is the absorbance of the sample extract. EDTA-2Na was used as the positive control.

Statistical analysis

All results are reported as means ± standard deviation. Analysis of variance was performed by ANOVA. The Duncan new multiple-range test was used to determine the differences between means. P values <0.05 were considered to be significant and P values <0.01 to be very significant.

RESULTS

Isolation, identification and classification of endophytic fungi

A total of 49 endophytic fungi were isolated from the liverwort *S. verrucosa*. Based on their morphological and molecular characteristics, isolated endophytic fungi were classified into seven genera (*Hypocrea*, *Penicillium*, *Tolypocladium*, *Chaetomium*, *Xylaria*, *Nemania*, and *Creosphaeria*) and 1 family (Xylariaceae). The majority of these isolated endophytic fungi belonged to the *Chaetomium* (18.37%), *Creosphaeria* (18.37%), *Xylaria* (16.33%), and *Xylariaceae* (16.33%).

TEAC

As shown in Table 1, TEAC of the 49 sample extracts varied considerably (ranging from 16.58 to 1248.10 µmol TE/g extract). The ethyl acetate extracts of seven endophytic fungi (T7, T21, T24, T32, T38, T44, and T45) were found to exhibit remarkable TEAC, ranging from 997.06 to 1248.10 µmol TE/g extract. Since the T24, T44 and T45 strains originated from the same species of endophytic fungus (*Chaetomium globosum*), T24 and others, T7, T21, T32, and T38, were selected for further study. Thus, a total of five of the "top seven" fungal ethyl acetate extracts were selected and their antioxidant capacity was further evaluated by the DPPH radical scavenging, hydroxyl radical scavenging assay, reducing power and ferrous ion chelating assays.

Table 1. Trolox equivalent (TE) antioxidant capacity (TEAC) of ethyl acetate extracts of the 49 endophytic fungi.

Strain	Taxon	TEAC (μmol TE/g extract)	Strain	Taxon	TEAC (μmol TE/g extract)
T1	*Hypocrea viridescens*	44.16 ± 1.44	T26	*Xylaria* sp 1	81.37 ± 2.31
T2	*Hypocrea rufa*	121.79 ± 9.93	T27	*Xylaria* sp 1	82.56 ± 1.26
T3	*Xylaria* sp 1	547.37 ± 1.03	T28	*Xylariaceae* sp 2	49.27 ± 0.62
T4	*Penicillum* sp 1	69.48 ± 2.93	T29	*Nemania diffusa*	282.28 ± 8.35
T5	*Hypocrea viridescens*	190.74 ± 15.37	T30	*Chaetomium* sp 1	38. 44 ± 0.58
T6	*Xylariaceae* sp 1	92.07 ± 9.15	T31	*Creosphaeria* sp	188.96 ± 6.26
T7	*Xylariaceae* sp 1	977.06 ± 10.30	T32	*Chaetomium* sp 1	1106.63 ± 9.54
T8	*Xylariaceae* sp 2	84.94 ± 5.48	T33	*Xylaria* sp 1	62.95 ± 4.34
T9	*Xylariaceae* sp 1	96.83 ± 7.84	T34	*Penicillum* sp 2	19.56 ± 0.82
T10	*Xylaria* sp 1	531.92 ± 12.46	T35	*Xylariaceae* sp 1	78.50 ± 2.55
T11	*Tolypoclaidium* sp	16.58 ± 0.91	T36	*Xylariaceae* sp 1	24.90 ± 0.62
T12	*Xylariaceae* sp 1	61.16 ± 2.48	T37	*Chaetomiun fusiforme*	382.73 ± 12.52
T13	*Xylaria* sp 3	46.30 ± 1.38	T38	*Creosphaeria* sp	1214.81 ± 5.45
T14	*Nemania diffusa*	531.32 ± 15.35	T39	*Creosphaeria* sp	96.83 ± 4.86
T15	*Nemania diffusa*	103.36 ± 11.60	T40	*Creosphaeria* sp	453.46 ± 9.93
T16	*Nemania diffusa*	144.97 ± 4.72	T41	*Chaetomium* sp 2	55.22 ± 5.12
T17	*Xylaria* sp 1	31.44 ± 1.29	T42	*Chaetomium* sp 2	395.80 ± 7.21
T18	*Creosphaeria sassafras*	72.46 ± 2.16	T43	*Creosphaeria sassafras*	73.05 ± 3.95
T19	*Tolypoclaidium* sp	311.40 ± 8.04	T44	*Chaetomium globosum*	1177.96 ± 13.57
T20	*Nemania* sp	564.02 ± 1.78	T45	*Chaetomium globosum*	1181.53 ± 15.85
T21	*Tolypoclaidium* sp	1086.42 ± 4.12	T46	*Creosphaeria sassafras*	335.12 ± 8.26
T22	*Xylaria* sp 1	37.39 ± 0.71	T47	*Nemania* sp	116.38 ± 7.53
T23	*Chaetomium* sp 1	106.34 ± 1.49	T48	*Penicillum* sp 3	106.87 ± 6.28
T24	*Chaetomium globosum*	1248.10 ± 15.27	T49	*Creosphaeria sassafras*	172.25 ± 5.72
T25	*Creosphaeria sassafras*	120.60 ± 7. 24			

DPPH and hydroxyl radical scavenging assays

DPPH is a relatively stable free radical and has been widely used to evaluate the antioxidant activities of various biological samples. This method is based on the reduction of DPPH in the presence of a radical scavenger or hydrogen donors due to the formation of non-radical form of DPPH-H (Jao and Ko, 2002). The hydroxyl radical is one of the most reactive free radicals, which can induce severe damage to biomolecules (Wu et al., 2007). As shown in Figures 1 and 2, all five selected ethyl acetate extracts scavenged both the DPPH radicals and hydroxyl radicals in a dose-dependent manner. It is worth noting that the DPPH and hydroxyl radical scavenging abilities of the ethyl acetate extracts of T24 and T38 were not only comparable to those of the ascorbic acid, but also significantly higher than those of the others (including BHT) at different concentrations ($P < 0.05$). The results indicate that ethyl acetate extracts of T24 and T38 strains may serve as an effective radical scavenger to react with both DPPH and hydroxyl-free radicals, converting them into stable products.

Reducing power assay

Antioxidant activities of a compound are closely associated with its reducing power, which can be used as an effective indicator (Singh and Rajini, 2004). As shown in Figure 3, similar to the DPPH and hydroxyl radical scavenging assays, the reducing power of the five selected ethyl acetate extracts was dose-dependent. Although ascorbic acid exhibited the strongest reducing power ($P < 0.05$), the reducing power of T24 ethyl acetate extract was significantly higher ($P < 0.05$) than that of the fungi T7, T21 and T32, and the values were also comparable to that of BHT. Additionally, T38 exhibited excellent reducing power of 0.07-0.53

at concentrations of 6.25-100 µg/mL. As suggested by Xing et al. (2005), their remarkable reducing power may be associated with the presence of a large amount of reductones. Reductones play an important role in reducing power, since they can break the free radical chain by donating a hydrogen atom (Xing et al., 2005).

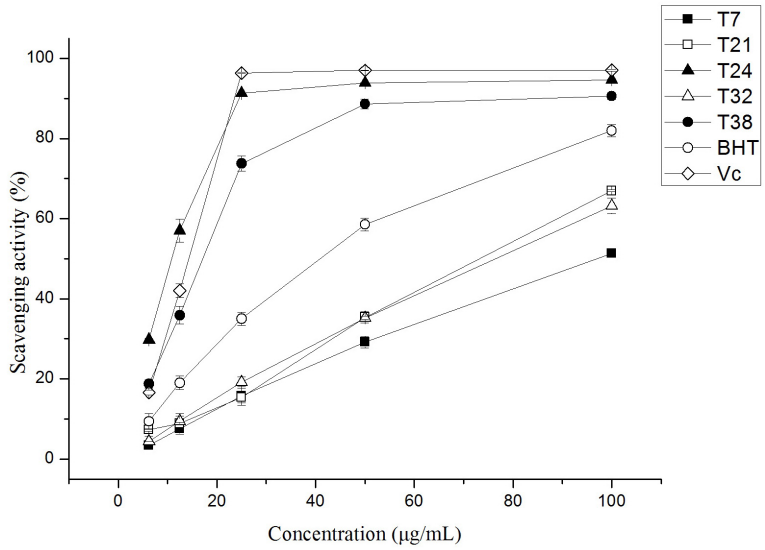

Figure 1. DPPH radical scavenging activity of the ethyl acetate extracts of five selected endophytic fungi and the positive controls [BHT and ascorbic acid (Vc)]. All values are reported as means ± SD (N = 3).

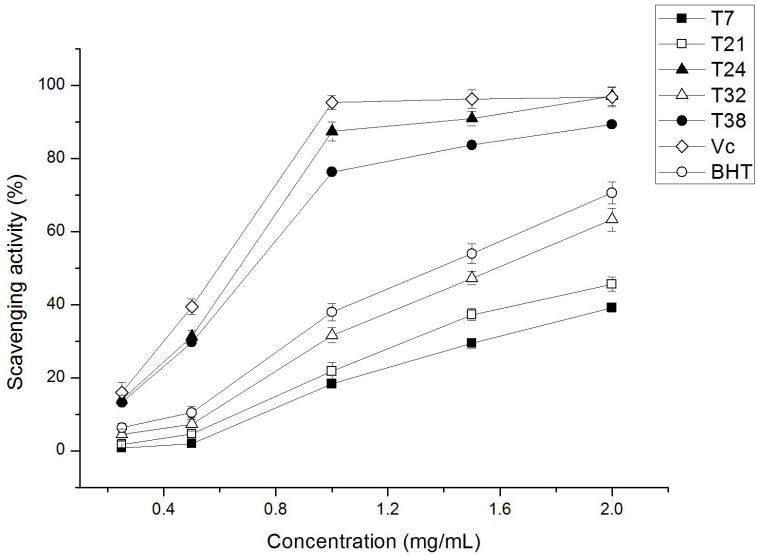

Figure 2. Hydroxyl radical scavenging activity of the ethyl acetate extracts of five selected endophytic fungi and the positive controls [BHT and ascorbic acid (Vc)]. All values are reported as means ± SD (N = 3).

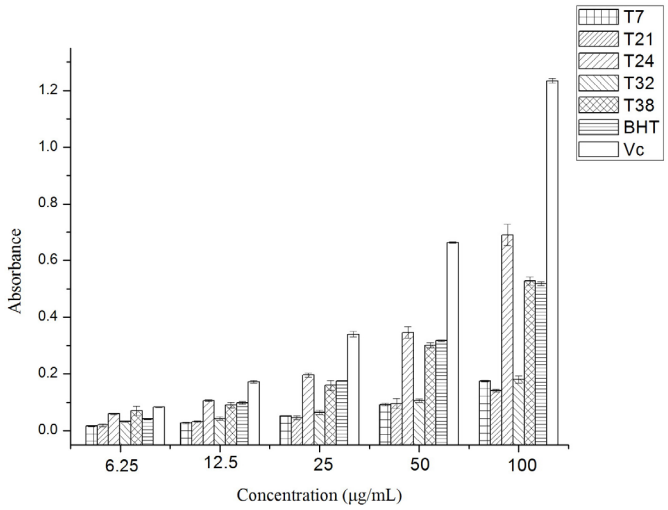

Figure 3. Reducing power of the ethyl acetate extracts of five selected endophytic fungi and the positive controls [BHT and ascorbic acid (Vc)]. All values are reported as means ± SD (N = 3).

Ferrous ion chelating assay

Ferrous ion chelating activity of an antioxidant could prevent free radical generation and consequent oxidative damage. Chelating agents, which form σ-bonds with a metal, could reduce redox potential and stabilize the oxidized form of metal ions (Srivastava et al., 2006). As shown in Figure 4, only ferrous ion chelating activity of the T38 ethyl acetate extract (>90%) was comparable to that of the positive control, EDTA-2Na. This finding may imply that certain effective chelating agent(s) may be present in this particular extract.

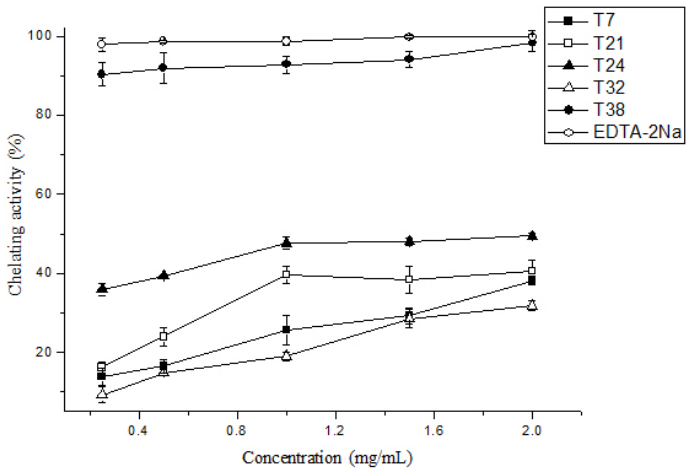

Figure 4. Ferrous ion chelating activity of the ethyl acetate extracts of the five selected endophytic fungi and the positive controls [EDTA-2Na and ascorbic acid (Vc)]. All values are reported as means ± SD (N = 3).

DISCUSSION AND CONCLUSION

Endophytic fungi, a potential source of medicinal compounds, have attracted more and more attention in the last years. It is reported that special eco-environmental microorganisms may produce special activated metabolites (Stierle et al. 1993). According to this notion, we isolated endophytic fungi from *S. verrucosa*, which grow in specific habitats such as rocks and decaying wood, to search for novel natural antioxidants.

The antioxidant activity of a certain compound can be evaluated by a variety of methods (Yu et al., 2002). In the present study, we evaluated the antioxidant activities of endophytic fungi by 5 different assays. Among the 49 strains tested, the major portion of fungi showed antioxidant activity to some extent in the $ABTS^+$ assay. The antioxidant activities of selected fungi (T7, T21, T24, T32, and T38) were further confirmed by other assays. The strain T24 exhibited the highest antioxidant capacity in the DPPH and hydroxyl radical scavenging assays and reducing power assay. The antioxidant activities of T24 and T38 were higher than those of the positive control BHT, especially in the DPPH assay. T24 showed antioxidant activity with an IC_{50} of the 8.49 μg/mL, which was lower than that of ascorbic acid (12.41 μg/mL). However, in the ferrous ion chelating assay, T38 exhibited the strongest metal ion chelating activity while T24 only showed moderate antioxidant activity. From these results, it is speculated that direct quenching of the radicals and hydrogen-donating ability, rather than iron chelation, contribute to the antioxidant activities of T24.

There is some previous research on the antioxidant activity of endophytic fungi from other medicinal plants. For example, Strobel et al. (2002) and Harper et al. (2003) obtained two antioxidants, pestacin and isopestacin, from the endophytic fungi *Pestalotiopsis microspora*. Phongpaichit et al. (2007) reported that 22.5% of the extracts from endophytic fungi and garcinia plants exhibited remarkable antioxidant activities. Recently, the endophytic fungi residing in *Nerium oleander* L. were shown to have excellent antioxidant capacity (with a TEAC value ranging from 9.59 to 150.79 μmol TE/100 mL culture in the ABTS assay) (Huang et al., 2007). The results of our study are similar to those in previous reports and indicate that endophytic fungi may serve as a potential source of antioxidants.

This is the first report on the antioxidant activity of endophytic fungi isolated from the liverwort *S. verrucosa*. Among all the strains tested, T24 (*Chaetomium globosum*) and T38 (*Creosphaeria* sp) possessed the most remarkable *in vitro* antioxidant activities as determined by five different assays mentioned above. In order to facilitate their development into a novel source of natural antioxidants, further isolation, purification and characterization of the active antioxidant constituents in ethyl acetate extracts of both T24 and T38 are underway.

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