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Isolation, Identification and Antioxidant Properties of *Xylaria escharoidea* Associated with Termite Nest

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Abstract: The strain of Xylaria Y-4 was isolated from an active termite nest and identified as Xylaria escharoidea based on ITS1-5.8S-ITS2 sequences. Intracellular Polysaccharide (IPS) and Extracellular Polysaccharide (EPS) were extracted from *Xylaria escharoidea* (strain Y-4) in submerged culture. Antioxidant potency was investigated by assays of various established antioxidant *in vitro* systems such as hydroxyl (·OH) and DPPH radical scavenging ability, reducing power and ferrous ion chelating ability. Results revealed that the antioxidant activities of IPS extracts were generally more potent than EPS extracts in *in vitro* assays. IPS exhibited stronger scavenging activity towards DPPH radicals with EC_{50} of 0.75 mg mL^{-1}.

Key words: Free radicals, polysaccharide, reducing power, IPS, *in vitro*

INTRODUCTION

Reactive Oxygen Species (ROS) including hydrogen, peroxidesuperoxide anion, hydroxyl radicals and singlet oxygen is generated by aerobic metabolism of the body (Lee *et al*., 2004). They can cause protein and lipid oxidation, DNA strand break and base modification, resulting in various diseases and disorders such as cancer, cardiovascular diseases, atherosclerosis and aging (Wu and Hansen, 2008; Xu *et al*., 2009). Almost all organisms are well protected against free radical damage by oxidative enzymes such as superoxide dismutase, catalase and chemical compounds (Niki *et al*., 1994). However, these systems are frequently insufficient to totally prevent the damage, resulting in diseases and accelerated ageing (Ames *et al*., 1993). Some synthetic antioxidants such as Butylated Hydroxy-Anisole (BHA) and Butylated Hydroxytoluene (BHT) have been used in preservation of foods but several studies suggest that they could promote tumour formation (Cheung *et al*., 2003). Therefore, considerable interest has arisen in looking for natural antioxidants.

Fungus combs are the fecal-based fungus gardens built by macrotermitine termites in their nests where they cultivate mycelia of the genus *Termitomyces*. Some species of Xylaria are often found in the fungus combs abandoned by termites and *Xylaria nigripes* (Koltz.) Sacc., a chinese traditional medicine was studied widely in China. *Xylaria nigripes*, also known as Wu Ling Shen, is known to enhance immunity and hematopoiesis (Xu, 1997). It is also used for treating insomnia, trauma and as a diuretic and nerve tonic (Dai and Yang, 2008; Ma *et al*., 1999; Xu, 1997). Moreover, other studies have shown that it possesses good antioxidant and hepatoprotective activities (Ko *et al*., 2009; Song *et al*., 2011; Wu, 2001). Another species *Xylaria gracillima* (Fr.) Fr., also found in abandoned nest of termites was reported to have anti-tumor activities (Dong, 1998) and antioxidant activities (Li and Wen, 2008).

Xylaria escharoidea (Berk.) Fr. is a species undoubtedly associated with termite nest. So far, there has been no official report on the antioxidant activities of *X. escharoidea*. The aim of this research was to study the antioxidant properties of extracts from *X. escharoidea* (Y-4) in submerged culture.

MATERIALS AND METHODS

Chemicals and reagents: Ferrozine, potassium ferricyanide, ferric trichloride, absolute ethyl alcohol, 1,1-Diphenyl-2-Picryl-Hydrazyl (DPPH), soldium salicylate, ferrous sulfate, Hydrogen peroxide (H_2O_2), ascorbic acid (VC), monosodium phosphate, disodium hydrogen phosphate, ethanoic acid, sodium acetate. All chemicals and solvents used in this study were of analytical grade available commercially.

Fungal isolation and identification: The fungus comb sample was collected from an active termite nest in Changsha, Hunan Province, China, Sep. 2011. When back to the laboratory, fungus comb was incubated at room temperature in a moist chamber where filter paper soaked

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in sterile demineralized water was added to keep moist environment. After 5-7 days, Xylaria appeared and was inoculate on Potato Dextrose Agar (PDA) media to obtain pure cultures. The strain of Xylaria Y-4 was isolated, purified and preserved on PDA slant at 4°C. Cultures and anamorph of Xylaria Y-4 researchers obtained in this study had colony and anamorphic features much like those *X. escharoidea* strains described by Ju and Rogers (1999).

Phylogenetic analyses: Total genomic DNA was extracted from fungal mycelia using the Cetyl Trimethyl Ammonium Bromide (CTAB) Method (O'Donnell *et al.* 1997). Primers ITS1 (5'-TCCGTAGGTGAACCTGCGG-3') and ITS4 (5'-TCCTCCGCTTATTGATATGC-3') (White *et al.*, 1990) were used in amplifying the regions of ITS1-5.8S-ITS2. DNA fragment was amplified and sequenced using the method described by Qian *et al.* (2011). The other sequence data of ITS rDNA included in this study were downloaded from GenBank.

DNA sequences used in this study were aligned using the alignment program ClustalX Version 1.81 (Thompson *et al.*, 1997). The alignment was manually adjusted with Se-Al v.2.03a (Rambaut, 2000). The aligned dataset was analyzed with Maximum Parsimony (MP) using PAUP×4.0b10 (Swofford, 2002). Maximum parsimony analysis was conducted using heuristic searches with 1000 replicates of random-addition sequence, Tree Bisection Reconnection (TBR) branch swapping algorithm. All characters were equally weighted and unordered. Gaps were treated as missing data to minimize homology assumptions. A Bootstrap (BS) analysis was performed with 1000 replicates, each with 10 random taxon addition sequences. TBR branch swapping was employed.

Fungal cultivation: Strain Y-4 was cultured on a PDA agar plate for 5-6 days at 25°C. Four active mycelial agar discs (diameter 0.4 cm) were inoculated into a 250 mL erlenmeyer flask containing 100 mL submerged medium of basic medium (pH 6.0) including 2% glucose, 1% peptone, 0.1% KH_2PO_4, 0.1% $MgSO_4$ and then incubated at 25°C, 150 r min^{-1} for 1 week on a rotary shaker.

Extraction of water soluble polysaccharides: Mycelia of the cultured fungus were filtered through a filter paper and then washed with distilled water. After lyophilization, the dried mycelia were boiled with distilled water at 100°C, 2 h for 3 times and the supernatant was condensed and was mixed with ethanol (95%, v/v) at 4°C overnight for 3 times, to obtained Intracellular Polysaccharide (IPS). The yield of IPS from dried mycelia was 13.68 mg g^{-1}. To extract Extracellular Polysaccharide (EPS), the residual fermentation broth was condensed by evaporator and mixed with four volumes of ethanol (95%, v/v) at 4°C, 18 h for 3 times. Then, supernatant was discarded after centrifugation at 3000 rpm for 10 min. The yield of EPS from fermentation broth was 6.51 g L^{-1}. IPS and EPS were lyophilized to powder. The two kinds of crude polysaccharide were redissolved in distilled water for determination of antioxidant activity.

Hydroxyl radical scavenging assay: Hydroxyl radical scavenging activity was measured by a modified Smirnoff and Cumbes (1989)'s Method. The reaction mixture, containing 0.3 mL soldium salicylate (20 mmol L^{-1}), 1 mL ferrous sulfate (1.5 mmol L^{-1}), 0.1 mL different samples (10-90 mg mL^{-1}) and 0.7 mL H_2O_2 (6 mmol L^{-1}) was incubated in a water bath at 37°C for 1 h. The absorbance was measured at 510 nm using Microplate Reader and VC was used for comparison. The capability of scavenging hydroxyl radical was calculated using the equation:

$$\text{Scavenging effect (\%)} = \frac{A_0 - A_1}{A_0} \times 100$$

Where:
A_0 = Absorbance of the control (blank, without samples)
A_1 = Absorbance of the samples

Reducing power assay: The reducing power of the extracts was determined according to the method described earlier by Yen and Chen (1995) with slight modifications. About 1 mL of each extract (10-90 mg m L^{-1}) in deionised water was mixed with 2.5 mL of sodium phosphate buffer (200 mmol L^{-1}, pH 6.6) and 2.5 mL of 1% potassium ferricyanide. The mixture was incubated at 50°C for 20 min and then rapidly cooled. After 2.5 mL of 10% trichloroacetic acid were added, the mixture was centrifuged at 4000 r min^{-1} for 10 min, 2.5 mL of the supernatant was mixed with 5 mL of distilled water and 0.5 mL of ferric chloride (0.1%) for 10 min. The absorbance was measured at 700 nm and VC was used for positive control. A higher absorbance indicates a higher reducing power.

DPPH radical scavenging assay: The effect of extracts on DPPH radical was estimated according to the method of Blois (1958). The 0.1 mL sample was mixed with 2.0 mL of acetic acid buffer solution (0.05 mol L^{-1}, pH 5.5), 1.9 mL of anhydrous ethanol and 1 mL DPPH solution (0.5 mmol L^{-1}) and the mixture was left to stand in dark at room temperature for 30 min. The absorbance was measured at 517 nm. VC and distilled water were used as positive control and blank control, respectively. The scavenging ability was calculated by using the equation:

$$\text{Scavenging ability (\%)} = \frac{[A_0-(A_1-A_2)]}{A_0}\times 100\%$$

Where:

A_0 = Absorbance of the control (blank, without samples)
A_1 = Absorbance of the samples
A_2 = Absorbance of the samples and the other solvent (without DPPH)

Ferrous ion chelating activity assay: The chelating activity of ferrous ions by the extracts was estimated by the methods of Decker and Welch (1990). Briefly, 1 mL sample (10-90 mg mL^{-1}) was added to 3.7 mL distilled water and 0.1 mL $FeCl_2$ (2.0 mmol L^{-1}). The reaction was initiated by the addition of 0.2 mL ferrozine (5 mmol L^{-1}) and the mixture was shaken vigorously and left standing at room temperature for 10 min. The absorbance of the resulting solution was then measured spectrophotometrically at 562 nm. Distilled water was used as blank control and VC was used as positive control. The chelating activity of the samples for ferrous ion was calculated as:

$$\text{Chelating rate (\%)} = \frac{A_0-A_1}{A_0}\times 100\%$$

Where:

A_0 = Absorbance of the control (blank, without samples)
A_1 = Absorbance of the presence of samples

Statistical analysis: All data were expressed as Mean±(SD) for at least triplicate analyses on the same sample. Analysis of variance was performed by ANOVA procedures using SPSS 11.5 (SPSS Inc., Chicago, IL, USA). Significant differences between means were determined by Duncan's multiple range tests ($p<0.05$).

RESULTS

Phylogenetic analysis: Strain Y-4 was isolated from the fungus comb with the method of *in situ* cultivation and exhibited 99% similarity with *Xylaria escharoidea* (EU179864) (Ju and Hsieh, 2007) in GenBank database based on ITS1-5.8S-ITS2 sequences. Phylogenetic analysis (Fig. 1) based on ITS1-5.8S-ITS2 regions shows

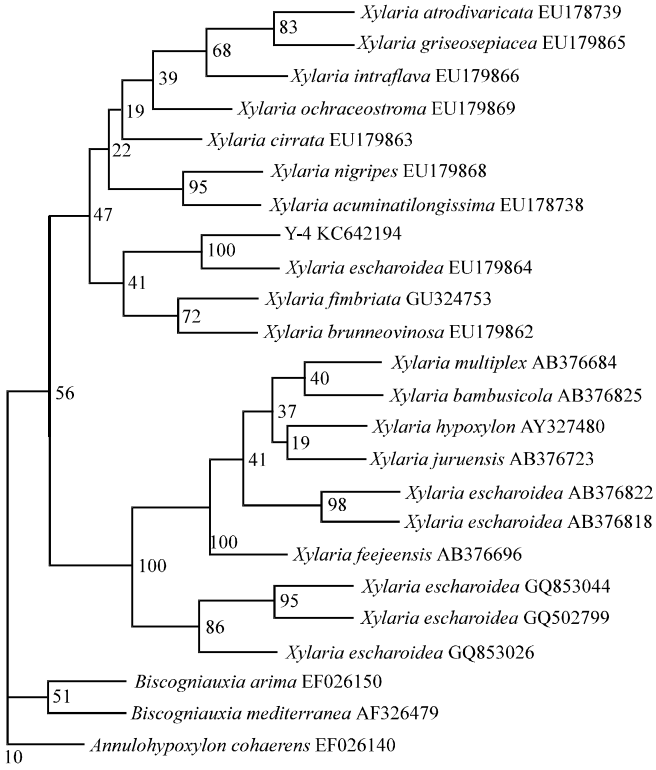

Fig. 1: Phylogenetic tree constructed with the maximum parsimony method according to *rDNA-ITS* (ITS1-5.8S-ITS2) gene evolutionary distance among the strain of Y-4 and other termite-associated *Xylaria* species. *Annulohypoxylon cohaerens* (EF026140), *Biscogniauxia arima* (EF026150) and *Biscogniauxia mediterranea* (AF326479) were used as out-groups. GenBank accession numbers are given in parentheses. The confidence values from 1,000 replicate bootstrap samplings were shown at each node

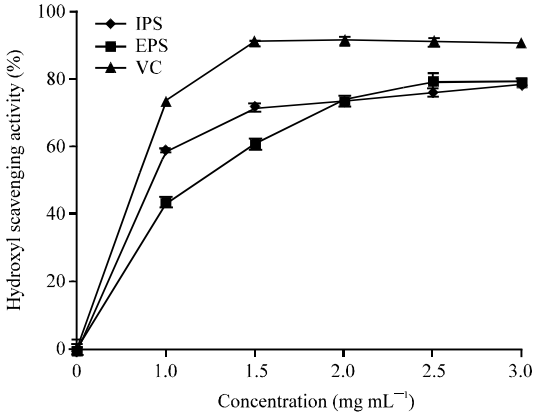

Fig. 2: Hydroxyl radical scavenging activity of IPS and EPS from *X. escharoidea* (strain Y-4) in submerged culture. Each value is the Mean±SD of triplicate measurements

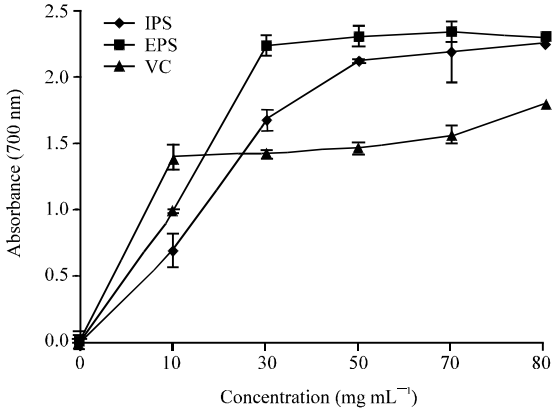

Fig. 3: Reducing power of IPS and EPS from *X. escharoidea* (strain Y-4) in submerged culture. Each value is the Mean±SD of triplicate measurements

the sequence of Y-4 also grouped in one clade with the epitype sequence of *X. escharoidea*c (EU179864) with a strong support (BP = 100). So, strain Y-4 was identified as *X. escharoidea*.

Hydroxyl radical scavenging activity: Hydroxy radical is known as the strongest reactive oxygen radical and can induce severely damage to the adjacent biomolecules like DNA, lipids and proteins (Spencer *et al*., 1994). Figure 2 shows that IPS and EPS from *X. escharoidea* (strain Y-4) exhibited excellent and concentration dependent scavenging abilities toward hydroxyl radicals. At a concentration of 3 mg mL^{-1}, the scavenging ability of IPS and EPS against hydroxyl radical reached 78.54 and 79.42%, respectively. In general, IPS had almost the same power with EPS at all tested concentrations. However, based on EC_{50} values, IPS (0.70 mg mL^{-1}) was more effective than EPS (1.13 mg mL^{-1}) on scavenging hydroxyl radical. The antioxidant properties are inversely correlated with their EC_{50} values and <10 mg mL^{-1} are indicative of the effective antioxidant activity (Lee *et al*., 2007).

Reducing power: The reducing capacities are generally associated with the presence of reductants (antioxidants). The presence of reductants in the samples would result in the reducing of Fe^{3+} to Fe $^{2+}$by donating an electron. Amount of Fe^{2+} complex can be then be monitored by measuring the formation of Perl's Prussian blue at 700 nm. Increasing absorbance at 700 nm indicates an increase in reductive ability. The reducing power of ISP and ESP extracted from *X. escharoidea* (strain Y-4) were shown in Fig. 3. Higher absorbance value means stronger reducing power of samples. Apparently, the reducing power of all samples was moderate. However, it is worthwhile to note

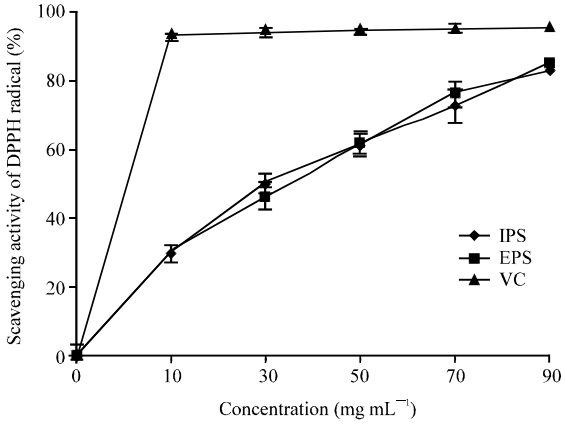

Fig. 4: DPPH radical scavenging activity of IPS and EPS from the *X. escharoidea* (strain Y-4) in submerged culture. Each value is the Mean±SD of triplicate measurements

that IPS and EPS were much stronger than that of ascorbic acid when concentration above 30 mg mL^{-1}. The EC_{50} values of IPS and EPS were 1.36 and 1.23 mg mL^{-1}, respectively which reveals that the reducing power of EPS are higher than that of IPS (Table 1).

DPPH radical scavenging activity: The model of scavenging DPPH radical is based on the reduction of the absorbance of methanolic DPPH solution at 517 nm in the presence of a proton-donating substance. The method is widely used to evaluate the free radical-scavenging capacities of samples (Soare *et al*., 1997; Naik *et al*., 2003). The scavenging abilities of water-soluble polysaccharides from the *X. escharoidea* on DPPH correlated well with the increasing of concentrations (Fig. 4). IPS and EPS had almost the same activities on scavenging DPPH radical.

Table 1: EC_{50} values obtained in the different antioxidant activity assays of IPS and EPS from *X. escharoidea* (strain Y-4) (mg/mL)

Samples	Hydroxyl radicals	Reducing power	DPPH radicals	Ferrous ions
IPS	0.75±0.021^{b}	1.36±0.027^{a}	25.88±1.034^{c}	1.29±0.042^{a}
EPS	1.13±0.036^{c}	1.23±0.045^{b}	27.01±1.22^{b}	2.01±0.108^{b}
VC	0.70±0.005^{a}	<0.2	1.21±0.087^{a}	ND
EDTA	ND	ND	ND	<0.10

EC_{50} value = The effective concentration at which hydroxyl radicals were scavenged by 50% DPPH radicals were scavenged by 50%, the absorbance was 0.5 and ferrous ions were chelated by 50%, respectively; IPS = Intracellular Polysaccharide; EPS = Extracellular Polysaccharide. Each value is expressed as Mean±Standard Deviation (n = 3). Means with different letters within a column are significantly different ($p<0.05$); ND = Not Done

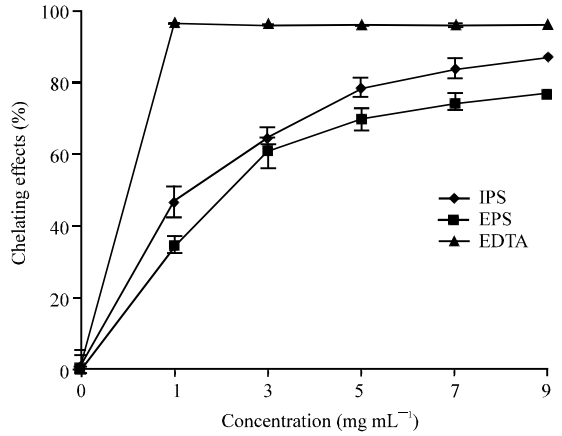

Fig. 5: Ferrous ion chelating activity of IPS and EPS from the *X. escharoidea* (strain Y-4) in submerged culture. Each value is the mean±SD of triplicate measurements

EC_{50} values of IPS and EPS were 25.88 and 27.01 mg mL^{-1}, respectively. At concentration of 90 mg mL^{-1}, the highest inhibition rate of IPS and EPS reached 82.68 and 84.90%, respectively.

Ferrous ion chelating ability: Ferrozine can quantitatively form complexes with ferrous ion. However in the presence of chelating agents, the complex formation is disrupted with the result that the red colour of the complex is decreased. Therefore, measurement of colour reduction can estimate the chelating activity of the coexisting chelator. As shown in Fig. 5, IPS and EPS exhibited obvious chelating activity on ferrous ion in a concentration dependent manner. At concentration of 9 mg mL^{-1}, the chelating effects IPS and EPS were 87.16 and 77.20%. The EC_{50} values of IPS and EPS were 1.29 and 2.01 mg mL^{-1}. Obviously, the chelating activity of EPS was lower than that of IPS.

DISCUSSION

The present study provides the first analyses for the antioxidant activities of *X. Escharoidea* (strain Y-4) associated with fungus comb using *in vitro* assays. The water-soluble polysaccharide IPS and EPS of *X. escharoidea* (strain Y-4) exhibited excellent hydroxyl radical scavenging activity and ferrous ion chelating activity. The antioxidant activities of IPS were generally more potent than EPS as antioxidants. In extremely high concentrations, the reducing power of IPS and EPS even was much higher than positive control VC. According to Li and Wen (2008), IPS of *X. gracillima* founded on waste termite nest was more effective than EPS on chelating ferrous ion whereas which was weaker than EPS on scavenging hydroxyl radical.

In ferrous ion chelating assay, the potency of IPS of *X. escharoidea* (strain Y-4) was weaker than that of *X. gracillima* at the same concentration (Li and Wen, 2008). While in hydroxyl radicals scavenging assay, from 0.125-16 mg mL^{-1}, the highest activity of IPS extract from *X. gracillima* was 54.9% (Li and Wen, 2008) which was lower than IPS from *X. escharoidea* (strain Y-4) (58.91%, 1 mg mL^{-1}). Based on EC_{50} values, the mycelial extract from *Xylaria nigripes* (Koltz.) Sacc. (EC_{50} 73.49 μg mL^{-1}) (Ko *et al*., 2009) exhibited stronger scavenging activity of DPPH radical than that from *X. escharoidea* (strain Y-4) (EC_{50} 25.88 mg mL^{-1}). In addition, the antioxidant and anti-tumor activity of nine compounds from mycelium of *X. nigripes* were studied by Gong *et al.* (2008). The results showed that (2, 6-dihydroxyphenyl)-3-hydroxybutan-one had strong DPPH radical scavenging ability and reducing capacity (Gong *et al*., 2008).

CONCLUSION

There are few reports about antioxidant activities of EPS from Xylaria associated with termite nest. According to Li and Wen (2008), the components extracted from *X. gracillima* exhibited a dose-dependent scavenging activity towards hydroxyl radicals. At concentration of 2 mg mL^{-1}, the DPPH radical scavenging activity of EPS from *X. gracillima* was about 18.79% which was weaker than that of *X. escharoidea* (strain Y-4) (74.15%) at the same concentration. But the chelating ferrous ion activity of EPS from *X. gracillima* was stronger than that of *X. escharoidea* (strain Y-4) at concentration 2 mg mL^{-1}.

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