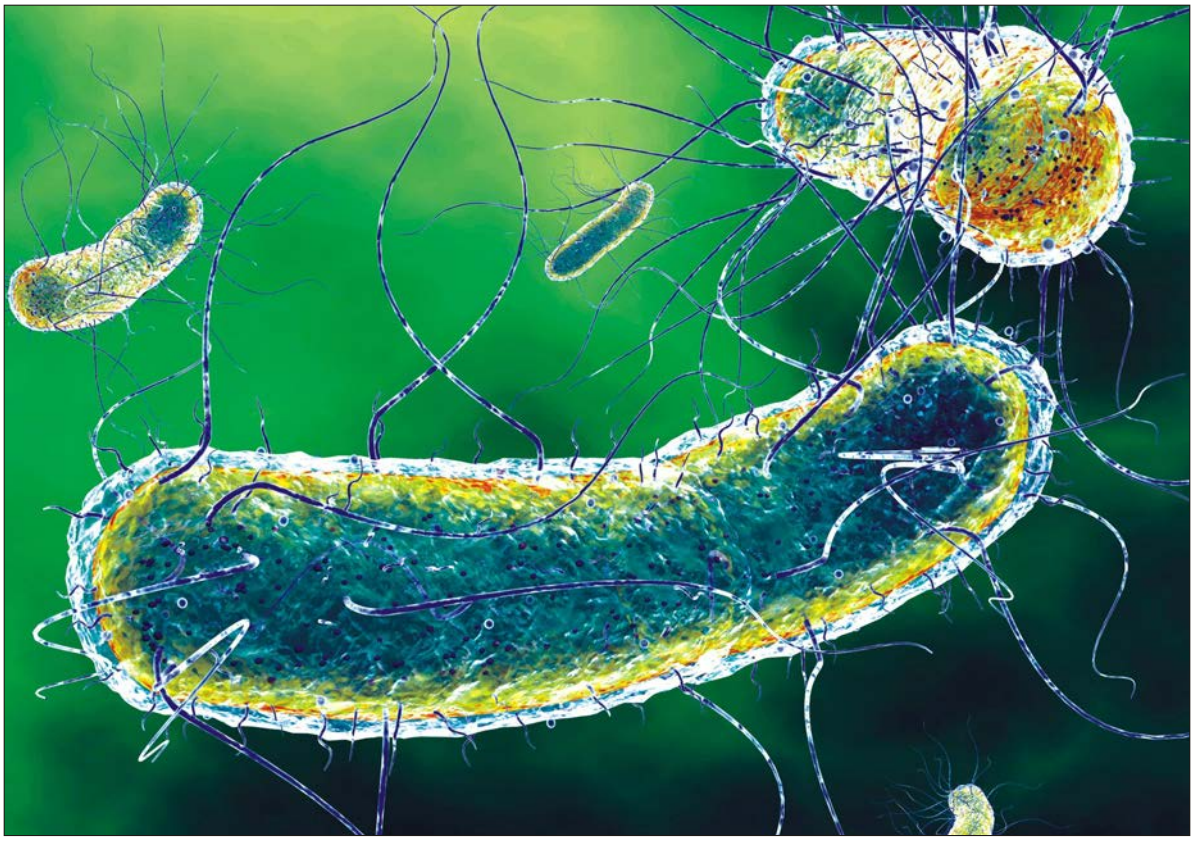

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Antioxidant Activity of a Novel *Streptomyces* Strain Eri12 Isolated from the Rhizosphere of *Rhizoma curcumae* Longae

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ABSTRACT

There are rare reports about isolated streptomyces associated with the rhizosphere of Rhizoma Curcumae Longae. In the present study, the cultural characteristics, phylogenetic analysis of the *streptomyces* strain Eri12 isolated from the rhizosphere of Rhizoma Curcumae Longae and antioxidant activity of the ethyl acetate extract of mycelia of Eri12 were investigated. The Strain Eri12 was isolated from the rhizosphere of Rhizoma Curcumae Longae which was collected from Ya'an city of Sichuan province, southwest of China. The strain Eri12 was subjected to molecular identification which included DNA extraction, amplification of 16S rDNA, 16S rDNA gene sequencing, BLASTN search and construction of 16S rDNA phylogenetic tree using MEGA4.1. Simultaneously, the mycelia extract using ethyl acetate was tested for its antioxidant activity using ABTS (2,2'-Azinobis-3-ethyl benzthiazoline-6-sulfonic acid) radicals scavenging test and DPPH [1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl] radicals scavenging test. Analysis of 16S rDNA gene sequence demonstrated that the strains Eri12 was most closely related to representatives of the genera *Streptomyces*. The ethyl acetate extract showed the antioxidant activity against the ABTS (2,2'-Azinobis-3-ethyl benzthiazoline-6-sulfonic acid) free radicals and DPPH(1,1-Diphenyl-2-picrylhydrazyl radical 2,2-Diphenyl-1-(2,4,6-trinitrophenyl) hydrazyl) free radicals with IC_{50} (The half-inhibitory concentration) of 172.43±22.19 and 842.18±161.24 μg mL^{-1}, respectively. So, it was suggested that the isolated *streptomyces* strain Eri12 could be a candidate for the nature resource of the antioxidants.

Key words: *Streptomyces,* characteristics, phylogeny, ethyl acetate extract, antioxidant activity

INTRODUCTION

Actinomycetes, phylogenetically defined as a number of taxa within the high-G+C subdivision of the gram-positive phylum (Embley and Stackebrandt, 1994), are involved in important processes in a wide range of habitats (Mincer *et al*., 2002). Actinomycetes are able to produce a large number of chemically different secondary metabolites. They are considered to be the most potent source for production of antibiotics and other bioactive secondary metabolites. Each actinomycete strain has probably genetic potential ability to produce 10-20 secondary metabolites

(Bentley *et al.*, 2002; Sosio *et al.*, 2000). The actinomycetes are noteworthy as antibiotic producers, making 75% of all known products and the *Streptomyces* are especially profilic (Nolan and Cross, 1998; Thakur *et al.*, 2009). *Streptomyces* has yielded many therapeutic agents which includes antibacterials such as tetracyclines, antifungal agents such as amphotericin, anticancer drugs exemplified by adriamycin and the immunosuppressant tacrolimus (Hopwood, 2007). *Streptomyces* has been reported to contribute nearly 70% of metabolites described under actinobacteria (Zengler *et al.*, 2005).

Actinomycetes are known to form intimate associations with plants and colonize their internal tissue. *Streptomyces* spp., *Microbispora* spp. and *Streptosporangium* spp., which isolated from roots of different plant species in Italy and from maize in Brazil, showed antagonistic activities against Gram positive bacteria and fungi (Sardi *et al.*, 1992; De Araujo *et al.*, 2000). Previous studies showed that a variety of actinomycetes inhabit a wide range of plants as either symbionts or parasites (Sardi *et al.*, 1992; Yuan and Crawford, 1995). They might play an important role in plant development and health because they could affect plant growth either by nutrient assimilation or through secondary metabolite productions.

The actinomycetes are the major microbes in the soil micro-ecosystem (Thangapandian *et al.*, 2007) and an amount of actinomycetes have already been isolated and identified (Muiru *et al.*, 2008). Recently, the rate of discovery of novel secondary metabolites produced by actinomycetes has decreased. Therefore, many efforts have been made to select and isolate actinomycetes from other biotopes, such as lake water (Singh *et al.*, 2006), marine sediments (Kumar *et al.*, 2011), plant surface and plant tissues (Zin *et al.*, 2011).

Rhizoma Curcumae Longae, one kind of typical Chinese medical plants, has many pharmacological functions, such as hypolipemic, antitumor, anti-inflammatory, antibacterial, antioxidant, cholagogic, etc. (Yang, 2010). There are rare reports about isolated streptomyces associated with the rhizosphere of Rhizoma Curcumae Longae based on literature survey. In the present study, we isolated one *Streptomyces* strain Eri12 from the rhizosphere of Rhizoma Curcumae Longae in Ya'an city, Sichuan province, Southwest of China and investigated the cultural characteristics, phylogeny analysis and antioxidant activity of isolated strain Eri12.

MATERIALS AND METHODS

Chemicals: 2, 2'-Azinobis-3-ethyl benzthiazoline-6-sulfonic acid (ABTS), 6-Hydroxy-2, 5, 7, 8-tetramethylchroman-2-carboxylic Acid (Trolox), Ascorbic acid, 1, 1-Diphenyl-2-picrylhydrazyl radical 2, 2-Diphenyl-1-(2, 4, 6-trinitrophenyl) hydrazyl (DPPH) and potassium persulfate were purchased from Sigma-Aldrich (St. Louis, MO). Nalidixic acid and potassium dichromate were purchased from Zheng Chang Glass and Reagents Co., Ltd., Sichuan.

Isolation of *Streptomyces* associated with the rhizosphere of Rhizoma Curcumae Longae: In the present study, the rhizosphere of the Chinese medicinal plant Rhizoma Curcumae Longae was collected from Ya'an city of Sichuan province, southwest of China, in November, 2009.

In the research, ISP-2 medium (Shirling and Gottlieb, 1966), S medium (Tan *et al.*, 2006), Trehalose-proline media (Jiang *et al.*, 2006) and modified Gause II media (Jiang *et al.*, 2006) were chosen for the isolation of actinomycetes. The media were added with Nalidixic acid (15 μg mL^{-1}, w/v) and potassium dichromate (25 μg mL^{-1}, w/v) to suppress the growth of non-actinobacteria and then incubated at 28°C (Cao *et al.*, 2005).

Actinomycetes were isolated according to the method reported by Schulz *et al.* (1993). And the colonies were inoculated on to ISP-2 medium for purification. Stocks were prepared on ISP-2 and kept at -70°C (under 30% of glycerol) for long-term storage and at 4°C as source cultures.

Morphological identification of the strain Eri12: Morphological characteristics are the basis for identifying actinobacteria. Isolated strain was identified according to the traditional morphological criteria including characteristics of colonies on the plate, the presence of aerial mycelium, spore mass colour, distinctive reverse colony colour, diffusible pigment and sporophore and spore chain morphology (Goodfellow and Cross, 1984). Cultural characteristics of the isolated strain after incubation for 7 days were recorded in 7 different media.

Physiological and biochemical characteristics: The utilization pattern of carbon sources by the strains was carried out according to the methods of Gottlieb (1961) since it can be used as an aid for species determination (Pridham and Gottlieb, 1948). The isolated strain was tested for its ability to grow in the presence of different concentration of sodium chloride (NaCl) (Tresner *et al.*, 1968). The ability to produce enzymes and melanin pigments was also studied (Holding and Collee, 1971).

DNA extraction and 16S rDNA sequencing: Genomic DNA extraction was done using the procedure described by Xu *et al.* (2003) and PCR amplification of the 16S rDNA gene was carried out with primers A and B (Xu *et al.*, 2003). The amplified products were sent to Invitrogen Company for purification and sequencing. The quality of the sequence was verified by sequencing both strands. This sequence was submitted to the GeneBank database.

Phylogenetic analysis: Phylogenetic trees were constructed using the Neighbor-Joining method in MEGA program version 4.1 (Kumar *et al.*, 2004). The topology of the phylogenetic tree was evaluated by bootstrap resampling method of Felsenstein with 1000 replicates (Felsenstein, 1985). The 16S rDNA gene sequences of the type strains of the various genera used in this study were retrieved from the nonredundant GeneBank database and used for cladistic analysis. These sequences have been chosen as reference sequences, in which unidentified and unpublished sequences were not included.

Extraction of the secondary metabolites: The spore suspensions of the culture were inoculated on the ISP-2 medium and incubated at 28°C for 7 days. Then, the aerial mycelia were scraped and soaked in 80% methanol for 24 h. It was centrifuged (4000 rpm, 10 min) and extracted twice more. The supernatant was dried and dissolved with distilled water. And then it was extracted with three half-volumes of ethyl acetate. The ethyl acetate extract was dried in vacuum at 40°C and 200 mbar for antioxidant tests.

ABTS radicals scavenging activity: ABTS radical scavenging activity was determined according to a modified method (Re *et al.*, 1999).

DPPH radicals scavenging activity: Scavenging activity on DPPH free radicals by the ethyl acetate extract was assessed according to the method reported by Awah *et al.* (2010).

Statistical analysis: The data of all experiments were recorded as Means±SD and were analyzed with SPSS (version 17.0 for Windows, SPSS Inc.). Differences were considered significantly at $p<0.05$.

RESULTS

Morphological identification of the isolated strain Eri12: Seven different media, including ISP-2, ISP-3, ISP-4, ISP-5, ISP-7, Czapek and PDA medium, were selected for the morphological identification. The isolated strain Eri12 could grow in each medium and it grew best in the ISP-2 medium. A dark yellow pigment diffused into the surrounding medium (Table 1).

Microscopic observation indicated that the strain Eri12 was aerobic microbe with branched aerial hyphae and non-fragmented substrate mycelia. The strain Eri12 was Gram-positive.

Biochemical traits of the isolated strain Eri12: The strain Eri12 had catalase activity and could liquefy gelatin, hydrolyze starch and reduce nitrite (Table 2). The growth of strain Eri12 at 5 different concentrations of NaCl (3, 6, 7, 8 and 10%) in the medium was thoroughly studied. The strain Eri12 could grow at 7% of NaCl in the medium but couldn't grow in the media with 8% NaCl and 10% NaCl (Table 2).

The eleven carbon sources were used in our study and the strain Eri12 could utilize many carbon sources efficiently, including glucose, xylose, arabinose, rhamnose, fructose, galactose, mannitol, sucrose, dulcitol and inositol (Table 3).

Table 1: Culture characteristics of the isolated strain Eri12 on the different medium

Medium	Growth	Arial mycelium	Substrate mycelium	Pigmentation	Spore colour
ISP-2	Good	Abundant, white	Moderate	Dark yellow	White
ISP-3	Moderate	Moderate, grey	Poor	Yellow	Grey
ISP-4	Moderate	Moderate, grey	Poor	Yellow	Few
ISP-5	Moderate	Moderate, grey	Poor	No pigment	Grey
ISP-7	Moderate	Moderate, grey	Poor	Yellow	White
Czapek	Moderate	Abundant, grey	Poor	Yellow	Grey
PDA	Moderate	Moderate, grey	Poor	Yellow	Grey

ISP-2-7: International Streptomyces Project medium, Czapek: Czapek yeast extract agar, PDA: Potato dextrose agar

Table 2: Biochemical and physiological characteristics of isolated strain Eri12

Culture	Growth characteristics
Growth under anaerobic conditions	-
Gram's staining	+
Shape and growth	Filamentous; aerial growth
Motility	Non motile
Production of diffusible pigment	+
Amylase	+
Gelatinase	+
Nitrite reduction	+
Growth in the presence of NaCl	
3%	+
6%	+
7%	+
8%	-
10%	-

+: Positive, -: Negative

Table 3: Utilization of different carbon sources by isolated strain Eri12

Carbon sources	Growth
Glucose	+
Xylose	+
Arabinose	+
Rhamnose	+
Fructose	+
Galactose	+
Raffinose	-
Mannitol	+
Dulcitol	+
Sucrose	+
Inositol	+

+: Positive utilization, -: Utilization negative

CAGAGTTTGATCCTGGCTCAGGACGAACGCTGGCGGCGTGCTTAACACATGCAAGTCGAACGATGAAGC
CTTTCGGGGTGGATTAGTGGCGAACGGGTGAGTAACACGTGGGCAATCTGCCCTTCACTCTGGGACAAG
CCCTGGAAACGGGGTCTAATACCGGATAACACTCTGTCCTGCATGGGACGGGGTTAAAAGCTCCGGCGG
TGAAGGATGAGCCCGCGGCCTATCAGCTTGTTGGTGGGGTAATGGCCTACCAAGGCGACGACGGGTAGC
CGGCCTGAGAGGGCGACCGGCCACACTGGGACTGAGACACGGCCCAGACTCCTACGGGAGGCAGCAGT
GGGGAATATTGCACAATGGGCGAAAGCCTGATGCAGCGACGCCGCGTGAGGGATGACGGCCTTCGGGT
TGTAAACCTCTTTCAGCAGGGAAGAAGCGAAAGTGACGGTACCTGCAGAAGAAGCGCCGGCTAACTACG
TGCCAGCAGCCGCGGTAATACGTAGGGCGCAAGCGTTGTCCGGAATTATTGGGCGTAAAGAGCTCGTAG
GCGGCTTGTCACGTCGGATGTGAAAGCCCGGGGCTTAACCCCGGGTCTGCATTCGATACGGGCTAGCTA
GAGTGTGGTAGGGGAGATCGGAATTCCTGGTGTAGCGGTGAAATGCGCAGATATCAGGAGGAACACCG
GTGGCGAAGGCGGATCTCTGGGCCATTACTGACGCTGAGGAGCGAAAGCGTGGGGAGCGAACAGGATT
AGATACCCTGGTAGTCCACGCCGTAAACGTTGGGAACTAGGTGTTGGCGACATTCCACGTCGTCGGTGCC
GCAGCTAACGCATTAAGTTCCCCGCCTGGGGAGTACGGCCGCAAGGCTAAAACTCAAAGGAATTGACGG
GGGCCCGCACAAGCAGCGGAGCATGTGGCTTAATTCGACGCAACGCGAAGAACCTTACCAAGGCTTGAC
ATATACCGGAAAGCATCAGAGATGGTGCCCCCCTTGTGGTCGGTATACAGGTGGTGCATGGCTGTCGTCA
GCTCGTGTCGTGAGATGTTGGGTTAAGTCCCGCAACGAGCGCAACCCTTGTTCTGTGTTGCCAGCATGCC
CTTCGGGGTGATGGGGACTCACAGGAGACTGCCGGGGTCAACTCGGAGGGAGGTGGGGACGACGTCAA
GTCATCATGCCCCTTATGTCTTGGACTGTACACGTGCTACAATGGCCGGTACAACGAGCTGCGATGCCGC
GAGGCGGAGCGAATCTCAAAAAGCCGGTCTCAGTTCGGATTGGGGTCTGCAACTCGACCCCATGAAGTC
GGAGTTGCTAGTAATCGCAGATCAGCATTGCTGCGGTGAATACGTTCCCGGGCCTTGTACACACCGCCCG
TCACGTCACGAAAGTCGGTAACACCCGAAGCCGGTGGCCCAACCCCTTGTGGGAGGGAGCTGTCGAAGG
TGGGACTGGCGATTGGGACGAAGTCGTAACAAGGTAGCCGTACCGGAAGGTGCGGCTGGATCACCTCCT

Fig. 1: 16 S ribosomal RNA gene sequence of the isolated strain Eri12

Molecular identification of the isolated strain Eri12: To elucidate the taxonomic positions of the isolated strain, we sequenced the full length of 16S rDNA gene of the strain Eri12 (Fig. 1). A continuous stretch of 1518 bp (including gaps) was used for constructing the phylogenetic Neighbor-Joining tree (Fig. 2). On the basis of phylogenetic analysis of the strain Eri12, the strain Eri12 was classified into *Streptomyces*. The 16S rDNA gene sequence of Eri12 showed high similarity of 99.59% to that of *Streptomyces griseus* whose accession number in NCBI GENEBANK is AB184347.1.

Antioxidant activity tests: The ethyl acetate extract of mycelia was tested for antioxidant activity and it showed a positive antioxidant activity.

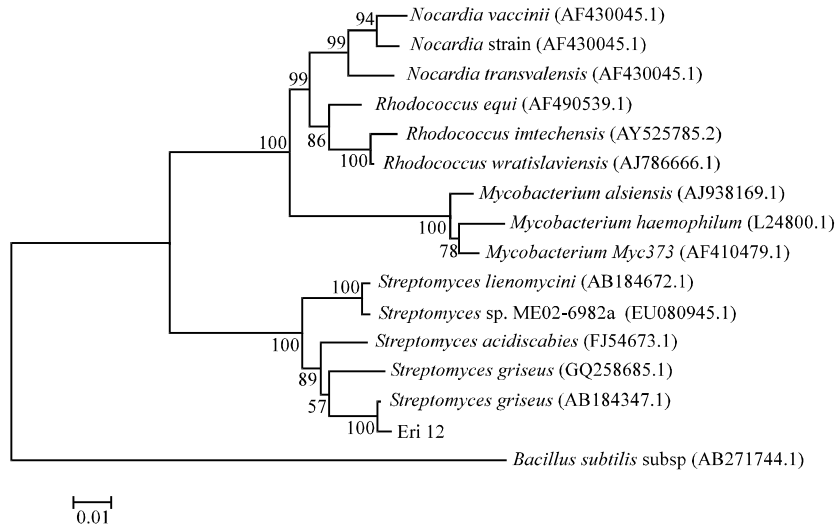

Fig. 2: Phylogenetic tree based on the 16S rDNA sequences of the showing affiliation of Eri12 strain with closely related members in GenBank. Bacillus subtilis (AB271744.1) was used as an outgroup. Phylogenetic trees were generated using MEGA version 4.1 with default parameters, K2P distance model and the Neighbor-Joining algorithm. The numbers at the branching prints are the percentages of occurrence in 1000 bootstrapped tree

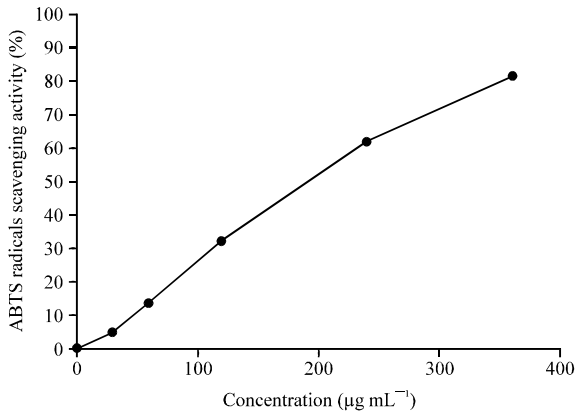

Fig. 3: ABTS radical scavenging activity of the ethyl acetate extract from *Streptomyces* strain Eri12

ABTS radical scavenging activity: After reacting for 30 min, the ethyl acetate extract showed ABTS radical scavenging activities of 4.54% at 30 μg mL^{-1}, 13.33% at 60 μg mL^{-1}, 31.91% at 120 μg mL^{-1}, 61.70% at 240 μg mL^{-1} and 81.26% at 360 μg mL^{-1}, respectively (Fig. 3). And the half-inhibitory concentration (IC_{50}) was estimated at 172.43±22.19 μg mL^{-1}.

DPPH radical scavenging activity: After reacting for 25 min, the ethyl acetate extract showed DPPH radical scavenging activities of 11.01% at 125 μg mL^{-1}, 20.66% at 250 μg mL^{-1}, 28.89% at 500 μg mL^{-1}, 51.87% at 1000 μg mL^{-1} and 78.15% at 2000 μg mL^{-1}, respectively (Fig. 4). And the half-inhibitory concentration (IC_{50}) was estimated at 842.18±161.24 μg mL^{-1}.

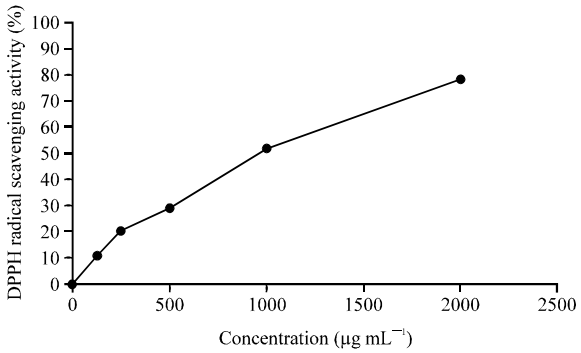

Fig. 4: DPPH radical scavenging activity of the ethyl acetate extract from *Streptomyces* strain Eri12

DISCUSSION

In the present study, the actinomycete strain Eri12 was isolated from the rhizosphere of Rhizoma Curcumae Longae using method reported by Schulz *et al.* (1993). Morphological and biochemical characteristics are two important aspects for the classification in the *Streptomycetaceae* family (Waksman and Henrici, 1943). So, the morphological and biochemical traits were investigated. According to morphological and biochemical results, the isolated strain Eri12 was found to be *Streptomyces* spp.

Phylogeny of the strain based on the sequencing of the 16S rDNA gene, had become the method of choice for tracing bacterial phylogenies and defining taxonomy (Yin *et al.*, 2008). In order to classify the isolated strain based on its morphological and biochemical traits, the phylogeny analysis of the strain Eri12 was investigated. The full length of 16S rDNA gene of the strain Eri12 was used for building the phylogenetic Neighbor-Joining tree by using MEGA version 4.1 (Kumar *et al.*, 2004). Phylogenetic tree revealed that Eri12 is strongly related to *Streptomyces* forming a distinct cluster (Fig. 2). The 16S rDNA gene sequence of Eri12 showed high similarity of 99.59% to that of *Streptomyces griseus* (AB184347.1).

The isolated strain Eri12 showed antioxidant activity in ISP-2 medium but not in broth culture medium. Similar results had been reported by Maruyama *et al.* (1975). Their study indicated that fumaradimycine, an antibiotic, isolated from *Streptomyces* was active only in agar medium but not in fermentation broth. Present result indicated that the antibiotic compounds were extracellular. Therefore, the ethyl acetate extract of mycelia was selected for antibiotic tests.

The ABTS radical cation decolorization assay is one of the methods for the screening of the antioxidant activity (Re *et al.*, 1999). Therefore, the ABTS radical scavenging activity of the ethyl acetate extract was determined. The results indicated that the ethyl acetate extract showed a lesser tendency to decay ABTS radicals at low concentrations of reaction than at high concentrations (Fig. 3). The extract scavenged ABTS radicals in a concentration-dependent manner. The IC_{50} value was estimated at 172.43±22.19 µg mL^{-1}. The antioxidant activity of the extract was about 220 times lower than that of the positive control (Trolox, IC_{50} = 0.76 µg mL^{-1}). This different half-inhibitory concentration between the ethyl acetate extract and the standard antioxidant could be explained in terms of the fact that the active components in the ethyl acetate extract comprise only a fraction of the extract.

DPPH radical scavenging activity test is another typical method to determine antioxidant activity of the extract (Awah *et al.*, 2010). The addition of the ethyl acetate extract to the DPPH

solution caused a decrease in absorbance at 517 nm indicating the scavenging activity of the extract. The extract possessed substantial dose-dependent antioxidant activity (Fig. 4). The half-inhibitory concentration (IC_{50}) was estimated at 842.18±161.24 μg mL^{-1}. The antioxidant activity of the extract was about 200 times lower than that of the positive control (Vitamin C, IC_{50} = 4.81 μg mL^{-1}). This different half-inhibitory concentration between the ethyl acetate extract and the standard antioxidant could be explained in terms of the fact that the active components in the ethyl acetate extract comprise only a fraction of the extract.

CONCLUSION

The present study indicated that the strain Eri12 isolated from the rhizosphere of Rhizoma Curcumae Longae was a novel *Streptomyces* strain based on the morphological characteristics, biochemical traits and phylogeny analysis. And the antibiotic compounds metabolized by the strain Eri12 were extracellular. According to the results of the antioxidant activity tests, it suggested that the strain Eri12 could metabolize antioxidants in the ISP-2 agar medium. The present study is still at its early stages of discovery of the potential active compounds with antioxidant activity. Further work is to elucidate the identity of compounds responsible for the antioxidant activity and it is in progress in our laboratory. Although it needs further study, the discovery of a novel *Streptomyces* strain Eri12 metabolizing antioxidant compounds in the study, undoubtedly, remains an interesting finding which would contribute to the antioxidant products of industry.

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