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ANTIMICROBIAL AND ANTIOXIDANT ACTIVITY OF BACTERIAL ENDOPHYTES ISOLATED FROM LEAVES OF THE MANGROVE PLANT *Rhizophora stylosa*

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ABSTRACT

Mangroves are the most productive ecosystems and contain highly diverse plants and microbial communities. Mangrove endophytes are proved to be a rich source of bioactive secondary metabolites. The biological molecules produced by endophytes play an important role in protection of mangrove plants against herbivores, insects as well as pathogens. The present study aimed to isolate the endophytic bacteria from the mangrove plant *Rhizophora stylosa* and screen antimicrobial and antioxidant activity of ethyl acetate extracts from the isolated endophytic bacteria. A total of 64 endophytic bacterial strains from *R. stylosa* leaves were isolated, of which ethyl acetate extracts of 14 isolated endophytic strains showed antimicrobial activity against at least one of reference microorganisms *Escherichia coli* ATCC 25922, *Pseudomonas aeruginosa* ATCC 25923, *Bacillus subtilis* ATCC 27212, *Staphylococcus aureus* ATCC 12222 and *Candida albicans* ATCC 7754 with MIC values from 32 to 512 µg/ml. Among them, four strains showed activity against one reference microorganism, five strains showed activity against two reference microorganisms, four strains showed activity against three reference microorganisms, and one strain showed activity against four reference microorganisms. Additionally, the ethyl acetate extracts of 12 isolated endophytic bacteria showed ATBS and DPPH radical scavenging activity with scavenging values from 36.3 ± 2.6% to 71.5 ± 6.6% and from 26.2 ± 3.3% to 57.4 ± 5.8%, respectively. The identification of the five most active endophytic bacteria by 16S rRNA sequences revealed that the endophytes belonged to four genera, including *Bacillus*, *Streptomyces*, *Pseudovibrio* and *Pseudomonas*. The obtained results suggest that the endophytic bacteria from mangrove plants are a promising reservoir of antimicrobial and antioxidant agents.

Keywords: *Rhizophora stylosa*, antimicrobial activity, antioxidant activity, endophytic bacteria, mangroves.

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INTRODUCTION

Endophytes were defined as "microbes that colonize living, internal tissues of plants without causing any immediate, overt negative effects" (Stone et al., 2000). It is reported that endophytes can be transmitted over generations through the tissue of the host, seed or plant propagules (Carroll, 1988). Endophytic bacteria are able to prevent the deleterious effects on certain pathogenic organisms. These microbes function as a biological defense for the plant against phytopathogens by producing secondary metabolites that inhibit phytopathogens or by inducing host defense mechanisms (Alvin et al., 2014). In addition, endophytes play important roles in promotion of plant growth by producing phytohormones (Tudzynski, 1997), synthesizing of siderophores (O'Sullivan & O'Gara, 1992), fixing nitrogen, solubilizing minerals (Richardson et al., 2009), suppressing ethylene (Glick et al., 1998) or assisting phytoremediation (Ryan et al., 2008).

Mangrove ecosystem is characterized by periodic tidal flooding and provides unique environmental characteristics for developing diverse groups of microorganisms (Sivaramakrishnan et al., 2006, Thatoi et al., 2013). Although the mangrove ecosystem is rich in microbial diversity, the described species have been less than 5%. Furthermore, their ecological role and their technological potential are poorly understood (Thatoi et al., 2013). Interestingly, recent studies reveal that the endophytic bacteria from mangrove plants are an important source of bioactive secondary metabolites. The secondary compounds derived from endophytes of mangrove plants are structurally diverse and pharmacologically active. These known compounds from mangrove plant endophytes include terpenes, chromones, coumarins, polyketides, alkaloids, and peptides (Kui-Wu et al., 2014; Xu, 2015; Ancheeva et al., 2018). These compounds exhibit a broad spectrum of biological properties such as antioxidant, cytotoxic, antifungal, antibacterial, α-glucosidase, acetylcholinesterase, antivirus (Kui-Wu et al., 2014; Xu, 2015; Ancheeva et al., 2018; Zhou et al., 2018; Manganyi et al., 2019).

With the length of coastline up to 3260 km, coastal mangroves are among the most productive and biologically important ecosystems in Vietnam (e.g., habitat for animal and vegetation species, medicinal sources, carbon storage and coastal protection from storm events). Regarding medicinal potential from Vietnamese mangrove microorganisms, a few studies on the antimicrobial activity of mangrove microorganisms have been reported (Hong & Phuong, 2013; Dat et al., 2019). Herein, the present study isolated and screened antimicrobial and antioxidant bacterial endophytes from the mangrove plant *R. stylosa* for discovery of potential medicinal sources from Vietnamese mangrove endophytic bacteria.

MATERIALS AND METHODS

Sample plant collection

The plant *R. stylosa* was collected in the mangrove forest Canh Duong, Phu Loc, Thua Thien Hue. The samples were contained in sterile bags and transferred immediately to the laboratory for isolation of bacterial endophytes.

Isolation of endophytic bacteria from leaves of *R. stylosa*

The fresh plant leaves were washed in running water to remove soil particles. The sample surface was then sterilized by sequential immersion in 70% ethanol for 5 min and sodium hypochlorite for 10 min. The samples then washed three times in sterile distilled water to remove surface sterilizing agents before being soaked in 10% sodium bicarbonate. The samples were cut into small pieces and placed on nutrient agar (Himedia) and ISP medium No. 4 (Himedia) (Hoai et al., 2018). The plates were incubated for 3−5 days at 37°C for the growth of endophytic bacteria. Representative bacterial isolates with different colony morphotypes were selected, pure cultured, and stored with 20% glycerol (v/v) at -80°C.

Preparation of ethyl acetate extracts

The endophytic bacterial strains were cultivated in 500 mL nutrient broth at 37°C, 150 rpm for 7 days. The fermentation broths were extracted with ethyl acetate (5 times) and then were evaporated under reduced pressure to yield ethyl acetate extracts.

Screening antimicrobial activity of the extracts

The ethyl acetate extracts of endophytic bacteria were tested against five reference microorganisms, including the Gram-positive bacteria (*S. aureus* ATCC12222, *B. subtilis* ATCC27212), the Gram-negative bacteria (*P. aeruginosa* ATCC25923, *E. coli* ATCC25922) and the yeast *C. albicans* ATCC7754. Antimicrobial activity of the ethyl acetate extracts against reference microorganisms was determined as minimum inhibition concentration (MIC) values that recorded by the microdilution method (Dat et al., 2018). MIC value is the lowest concentration of the extracts that completely inhibits the growth of microorganisms.

Screening antioxidant activity of the extracts

ABTS radical scavenging assay

The ABTS radical scavenging activity of the extracts was determined by measuring the decrease in absorbance of ABTS radical solution in the presence of the extracts. Briefly, two solutions ABTS 7 mM and potassium persulfate 2.45 mM were mixed and allowed to stand in the dark at room temperature for 16 h before use in order to produce ABTS radical solution. The ABTS radical solution was then diluted with ethanol to give an absorbance of 0.700 ± 0.02 at 734 nm. Ten microliters of each extract (500 µg/ml) were added to 190 µl of ABTS radical solution in 96 well plates. The solution incubated at room temperature for 10 min and then the absorbance of the reaction was recorded at 734 nm on a microplate reader. Ascorbic acid was used as positive control. The ABTS radical scavenging activity was calculated as follow:

$$\text{ABTS scavenging activity (\%)} = 100 \times [(Ac - As)/(Ac - Ab)]$$

Where: Ac was the absorbance of the control, As was absorbance of the extract and Ab was the absorbance of the blank.

DPPH radical scavenging assay

The DPPH radical scavenging activity of the extracts was determined by measuring the decrease in absorbance of DPPH radical solution in the presence of the extracts. Briefly, 10 µl of each extract (500 µg/ml) was added to 190 µl of DPPH (0.1 mg/ml) in 96 well plates. The solution was mixed for 1 min and incubated at room temperature for 30 min. Then the absorbance of the reaction mixture was recorded at 517 nm on a microplate reader. Ascorbic acid was used as positive control. The DPPH radical scavenging activity was calculated as follow:

$$\text{DPPH scavenging activity (\%)} = 100 \times [(Ac - As)/(Ac - Ab)]$$

Where: Ac was the absorbance of the control, As was absorbance of the extract and Ab was the absorbance of the blank.

Identification of the isolates by the 16S rRNA sequence

The most potential bioactive isolates were identified using 16S rRNA gene sequencing. The 16S rRNA gene was amplified with universal primers: 27f (5′-AGAGTTTGATCCTGGCT CAG-3′) and 1492r (5′- GGTTACCTTGTTACGACTT-3′). The PCR cycling parameters: an initial denaturation at 94°C for 5 min followed by 30 cycles of denaturation at 94°C for 1 min, annealing at 56°C for 50 s, amplification at 72°C for 1.5 min and a final extension at 72°C for 7 min. The 16S rRNA gene sequencing was carried by DNA Analyzer (ABI PRISM 3100, Applied Bioscience). The obtained DNA sequences were removed poor quality ends using BioEdit software v.2.7.5, and then were blasted to sequences in the GenBank database using the Blast search programme (http://www.ncbi.nlm.nih.gov/) to find their highest similarity sequences. The sequences were aligned using the ClustalW algorithm.

The phylogenetic tree of 16S rRNA sequences was created by the Neighbor-Joining algorithm with 1000 bootstraps using MEGA v.7.0.0.

RESULTS

Isolation of endophytic bacteria from leaves of *R. stylosa*

From leaves of the plant *R. stylosa* collected from mangrove forest Canh Duong, Phu Loc, Thua Thien Hue, 64 endophytic bacterial strains were isolated and pure cultured, of which 39 strains were isolated from NA medium and 25 strains were isolated from ISP4 medium. Among them, 30 representative strains with different colony morphotypes were selected for preparing ethyl acetate extracts and screening antimicrobial and antioxidant activity.

Antimicrobial activity of the ethyl acetate extracts of the endophytic bacteria

Ethyl acetate extracts of 30 endophytic bacteria were tested their antimicrobial activity against five pathogenic microorganisms (*E. coli* ATCC 25922, *P. aeruginosa* ATCC 25923, *B. subtilis* ATCC 27212, *S. aureus* ATCC 12222, and *C. albicans* ATCC 7754). The tested results showed that 14 out of 30 endophytic bacteria exhibited antimicrobial activity against at least one reference microorganism with MIC values ranging from 32 to 512 µg/mL (Table 1). Among them, 4 strains showed activity against one reference microorganism, 5 strains showed activity against two reference microorganisms, 4 strains showed activity against 3 reference microorganisms, and only one strain showed activity against 4 reference microorganisms. It was found that isolated strains were more active towards Gram-positive bacteria (14 strains) than Gram-negative bacteria (11 strains), whereas number of strains displaying activity against yeast *C. albicans* was lowest (5 strains). Frequencies of the antimicrobial activities against the microbial pathogens were as follows: *B. subtilis* (23.3%), *S. aureus* (23.3%), *E. coli* (20.0%), *P. aeruginosa* (16.7%), *C. albicans* (16.7%).

Table 1. Antimicrobial activity (µg/mL) of ethyl acetate extracts from isolated endophytes

Isolates	Gram-positive		Gram-negative		Yeast
	B. subtilis ATCC 27212	*S. aureus* ATCC 12222	*P. aeruginosa* ATCC 25923	*E. coli* ATCC 25922	*C. albicans* ATCC 7754
RSL-N1	64	256	-	128	-
RSL-N3	-	-	256	-	-
RSL-I4	32	-	-	64	256
RSL-I8	-	128	-	-	-
RSL-N13	256	-	128	32	64
RSL-N14	64	64	-	-	-
RSL-I16	-	-	64	64	32
RSL-N19	512	32	-	-	-
RSL-N20	-	128	-	-	-
RSL-I23	32	-	-	-	512
RSL-N24	-	64	512	32	-
RSL-N26	-	-	128	64	-
RSL-N29	128	-	-	-	256
RSL-I30	-	64	-	-	-

Antioxidant activity of the ethyl acetate extracts of the endophytic bacteria

The antioxidant activity of ethyl acetate extracts of the endophytic bacteria was determined by the ABTS and DPPH radical scavenging assays (Figure 1). The ABTS and DPPH radical scavenging assays showed the extracts of 12 endophytic bacteria exhibited ABTS and DPPH radical scavenging activity with scavenging values from 36.3 ± 2.6% to 71.5 ± 6.6% and 26.2 ± 3.3% to 57.4 ± 5.8%, respectively. Among them, the ethyl acetate extracts of five bacterial endophytes RSL-N1, RSL-I4, RSL-N13, RSL-I16, RSL-N24 showed the scavenging activity more than 50%. The positive control (ascorbic acid) showed ABTS and DPPH radical scavenging activity with scavenging values of 89.2 ± 6.5% and 83.4 ± 6.1%, respectively.

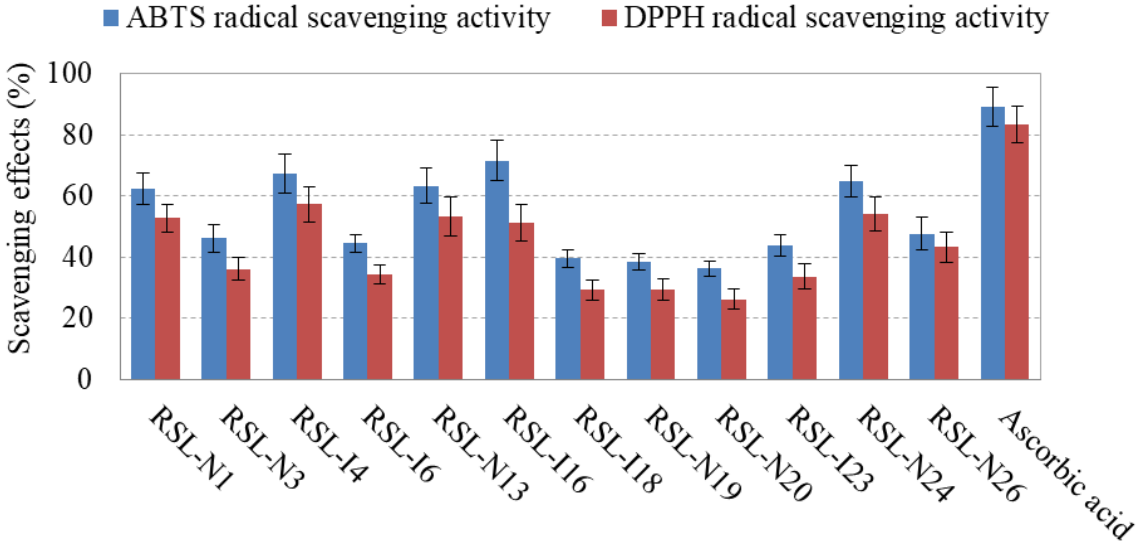

Figure 1. DPPH and ABTS radical scavenging activity of the ethyl acetate extracts

Molecular identification of active bacterial endophytes

The 16S rRNA genes of five endophytic bacteria with highly antimicrobial and antioxidant activities were amplified and sequenced to identify their taxa. The 16S rRNA sequences of strains were high similarity (99 - 100%) to that of bacteria on GenBank (Table 2). The phylogenetic analysis revealed that five endophytic bacteria belonged to 4 genera, including *Bacillus*, *Pseudovibrio*, *Pseudomonas* and *Streptomyces* (Figure 2).

Table 2. The closest sequences of 16S rRNA sequences of isolates obtained in NCBI for

Isolates	Media	Closest homologs	Similarity (%)
RSL-N1	NA	*Bacillus pumilus* CE92, MK618603	100
RSL-I4	ISP4	*Bacillus licheniformis* MSWS30, KX785167	99.9
RSL-N13	NA	*Streptomyces viridis* BK199, NR_117083	99.8
RSL-I16	ISP4	*Pseudovibrio japonicus* WSF2, NR_041391	99.6
RSL-N24	NA	*Pseudomonas synxantha* IAM 12356, NR_043425	99.7

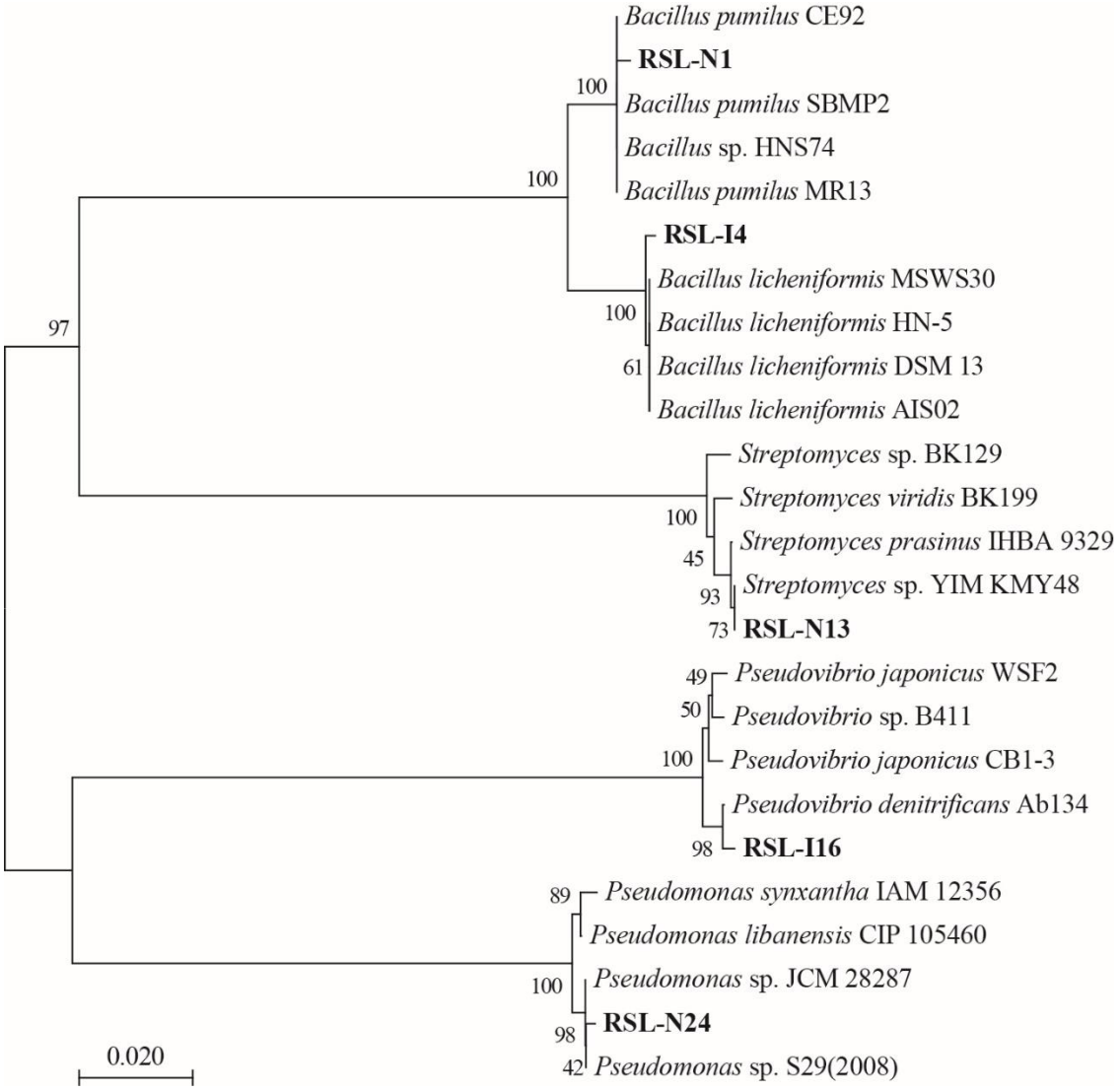

Figure 2. Phylogenetic tree based on 16S rRNA gene sequences of isolates and their closest sequences derived from NCBI

DISCUSSION

Mangrove plants are considered as an ecological niche for diverse endophytic microorganisms and are potential sources of bioactive compounds. The aim of this study was to isolate endophytic bacteria from the mangrove plant *R. stylosa* and screen antimicrobial and antioxidant activities of ethyl acetate extracts of the isolated endophytic bacteria. The antimicrobial and antioxidant assays in our study revealed endophytic bacteria of *R. stylosa* as potential producers of antimicrobial and antioxidant compounds. Among ethyl acetate extracts of 30 isolated endophytic bacteria, 14 extracts showed antimicrobial activity and 12 extracts showed antioxidant activity. Previous studies have reported that endophytic microorganisms from mangrove plants exhibit a wide range of biological activity, including antimicrobial and antioxidant activities. In respect of the antimicrobial activity, Fareza et al. (2018) isolated two endophytic fungi

Neopestalotiopsis sp. and *Peniophora lycii* from leaves of *R. mucronata*. The extracts from two isolated fungi showed antimicrobial activity against *E. coli* ATCC 25922 and *S. aureus* ATCC 25923 with MIC values from 125 to 500 µg/ml. Handayani et al. (2017) isolated 12 endophytic fungi from mangrove plant *Sonneratia griffithii*. These fungi belonged to two genera *Aspergillus* and *Candida*. Among them, the extracts of 10 endophytic fungi exhibited antibacterial activity against *E. coli* and *S. aureus* with zone inhibition from 8.0 to 15.75 mm at a concentration of 10 mg/ml extract in DMSO. Eldeen et al. (2014) also isolated 33 endophytic bacteria from five mangrove plants *Avicennia lanata*, *R. mucronata*, *R. apiculata*, *Sonneratia caseolaris*, and *Xylocarpus moluccensis*. Of these, 18 isolated bacteria exhibited antibacterial activity against *B. cereus*, *S. aureus*, *E. coli*, *Salmonella typhimurium* with MIC values of 19-250 µg/ml. The identification of isolated bacteria revealed that they belonged to genera *Bacillus* and *Staphylococcus*. In another study, Nia et al (2017) isolated 12 endophytic bacteria from two mangrove plants *R. apiculata* and *Bruguiera gymnorrizha*, of which, two fungi *Penicillin* spp. showed antibacterial activity against *Klebsiella pneumonia* (Nia et al., 2017). In regard of the antioxidant activity, Rahmawati et al. (2019) isolated six fungi and three bacteria from two mangrove plants *Avicennia marina* and *Xylocarpus granatum*. Of these, six fungi and two bacteria showed DPPH radical scavenging activity with IC_{50} values from 1 - 19 ppm. Zhoe et al. (2018) isolated 225 fungal strains from two mangrove plant *R. stylosa* and *R. mucronata*. Antioxidant assays revealed that the crude extracts of 40 isolated endophytic fungi showed the DPPH and ABTS radical scavenging activities with IC_{50} values from 0.33 ± 0.02 to 14.36 ± 0.68 mg/ml. Obtained results in our study and the previous studies show that the endophytic microorganisms from mangrove plants are promising sources of antimicrobial and antioxidant agents for pharmaceutical applications.

CONCLUSION

The present study isolated endophytic bacteria from the mangrove plant *R. stylosa* and screened antimicrobial and antioxidant activity of ethyl acetate extracts from the isolated endophytic bacteria. From leaves of *R. stylosa*, 64 endophytic bacterial strains were isolated, of which the ethyl acetate extracts of 14 isolated endophytic strains showed antimicrobial activity against at least one of reference microorganisms with MIC values from 32 to 512 µg/ml. Furthermore, the ethyl acetate extracts of 12 isolated endophytic bacteria showed ABTS and DPPH radical scavenging activity with scavenging values from 36.3 ± 2.6% to 71.5 ± 6.6% and 26.2 ± 3.3% to 57.4 ± 5.8%, respectively. The identification of five promising endophytic bacteria by 16S rRNA sequences revealed that the endophytes to 4 genera, including *Bacillus*, *Streptomyces*, *Pseudovibrio*, *Pseudomonas*. The obtained results suggest that mangrove endophytes are a potential source of antimicrobial and antioxidant agents.

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