

## A Novel Source of Bioactive Compounds: Endophytic Bacteria Isolated from *Centella asiatica*

Arash Rafat<sup>1\*</sup>, Koshy Philip<sup>1</sup> and Sekaran Muniandy<sup>2</sup>

<sup>1</sup>Microbiology Division, Institute of Biological Sciences, Faculty of Science

<sup>2</sup>Department of Molecular Medicine, Faculty of Medicine,  
University of Malaya, 50603 Kuala Lumpur, Malaysia.

(Received: 14 June 2011; accepted: 18 July 2011)

Endophytic bacteria were isolated from aerial parts of *Centella asiatica* (L.) Urban and identified using 16S ribosomal DNA sequences. Erythrocyte haemolysis protection, 2,2-diphenyl-1-picrylhydrazil scavenging activity, superoxide dismutase activity and antioxidant reductive capacity assays were carried out to evaluate the antioxidant potential of the cell free supernatant from the bacterial cultured broth of the isolated endophytic bacteria. The antibacterial potential of the endophytic bacteria was investigated using a disc diffusion method. *Pantoea agglomerans* AR\_PSBH2 showed the highest antioxidant properties in all tests except the superoxide dismutase activity assay in which *Erwinia soli* AR\_PINLTS1 showed the highest activity among all endophytic bacteria. Antioxidant activity of none of the isolated bacteria was significantly compatible with ascorbic acid (1mg/ml) as the positive control in all four employed assays. Meanwhile the 2,2-diphenyl-1-picrylhydrazil free radical scavenging activity of tert-butylated hydroxytoluene (1 mg/ml) as the positive control was significantly lower than all of the isolated bacteria. All isolated endophytic bacteria except *Bacillus gibsonii* AR\_PBSTSB inhibited the growth of *Pseudomonas aeruginosa* a species pathogenic bacteria while none of them showed antibacterial activity against *Escherichia coli*, *Staphylococcus aureus* and *Bacillus cereus*.

**Key words:** *Centella asiatica*, Endophytic bacteria, Antioxidant activity, Antibacterial activity.

Although for long time the existence of bacteria inside plants tissues without causing diseases was patented (Tervet and Hollis, 1948), the attention to the benefits and potential applications of these bacteria is quite recent. While in many cases endophytic bacteria neither show any damage to the host plant nor any beneficial effects (Sturz, 1995), there are also several reports

on beneficial effects of these plant associated bacteria such as growth promoting effects (Joseph *et al.*, 2011) and plant disease protection (Ziedan, 2005). Termination of growth of the plant host in shoot cultures of *Echinacea* caused by the endophytic bacterial growth is one of the reported negative effects of endophytic bacteria on plants growth (Lata *et al.*, 2006). Hence, the three neutral, beneficial, and deleterious effects on plant host by endophytic bacteria shows the importance of this group of bacteria in plant biological studies.

Apart from the contribution of endophytic bacteria to plant health and growth, some of the isolated endophytic bacteria from different plants have shown potent bioactivities such as

\* To whom all correspondence should be addressed.

Tel.: +60379675839; Fax: +60379674178

E-mail: rafat\_arash@siswa.um.edu.my

antibacterial (Ravikumar *et al.*, 2010), antifungal (Cho *et al.*, 2007) and antitumor (Taechowisan *et al.*, 2007) properties. Although the number of valuable phytochemicals isolated from endophytic bacteria is not as high as pharmaceutical compounds extracted from endophytic fungi, a few reports shows this group of bacteria has a tremendous potential for producing bioactive compounds. For example, the novel antimicrobial lipopeptides, namely ecomycins, are produced by *Pseudomonas viridiflava*, the bacterium which is normally associated with the leaves of various grass species (Miller *et al.*, 1998). Another example is the production of 4-Arylcoumarins, an antitumor active compound, by the *Streptomyces aureofaciens* bacteria associated with root tissues of *Zingiber officinale* (Taechowisan *et al.*, 2007).

*Centella asiatica* (L.) Urban from Apiaceae family is an important Asian medicinal plant which is consumed traditionally in several countries such as India, Malaysia, China and Sri Lanka (Somchit *et al.*, 2004; Panthi and Chaudhary 2006). Medicinal value of this herb in traditional medicine for treatment of several diseases such as asthma, lupus, leprosy, cellulitis and epilepsy is reported (Samy, *et al.*, 2005). Although there are several studies to show the pharmaceutical values of this herb such as cytotoxic potential (Ullah *et al.*, 2009), antioxidant capacity (Rafat *et al.*, 2011) and anti-inflammatory activity, but endophytic microbes associated with this plant are hardly studied. Rakotoniriana *et al.* (2008) isolated some of the endophytic fungi from leaves of *C. asiatica* and showed they have a potent antifungal activity.

In this study, some of the endophytic bacteria associated with different parts of *C. asiatica* are isolated and identified using 16S rRNA sequencing method. The antibacterial and antioxidant properties of these bacterial products were also evaluated to understand the potential of the bacterial world associated with this important medicinal plant.

## MATERIALS AND METHODS

### Bacteria Isolation and Identification

Aerial parts of healthy *Centella asiatica* plants of 2-3 months age were cut using a sterile surgical blade (Wuxi Xinda Medical Device, China). A voucher specimen has been deposited

in the herbarium of University of Malaya (Voucher Specimen: KLU047364). The stems and leaves were excised and surface sterilized separately by first placing under running tap water for 30 minutes, immersed in 70% ethanol for 3 minutes and rinsed once with sterile distilled water. The stems and leaves were then immersed in 20% Clorox® added with 1-2 drop(s) of Tween 20 for 20 minutes, rinsed with sterile distilled water thrice. The last used sterile distilled water (50 µl) was spread on nutrient broth plate to confirm the success of surface sterilization protocol.

Surface sterilized stems and petioles were separated from leaf into two groups. Both groups of tissue were macerated with a sterile mortar and pestle in a sterile condition and macerated tissue (0.1 g) was diluted in 500 µl sterile phosphate buffered saline (PBS). Each diluted sample was plated on nutrient broth agar and incubated at 25 °C for 5 days. The number of colony-forming units (cfu) per gram was measured using a serial dilution method described by Nautiyal (1997) to obtain the total population of bacteria.

Based on the morphological appearance of the bacterial colonies, colonies were randomly picked and subcultured separately. A few subculture cycles were performed to purify the bacteria. Twenty-four distinct colony morphotypes were finally selected and glycerol stocks from cultured single colonies were prepared and stored at -80 °C.

The antagonistic strains were subjected to Gram-stain for primary identification. Their genomic DNA were extracted using *i-genomic DNA Extraction Mini Kit* (iNtRON Biotechnology, Korea). The 16S ribosomal RNA gene was amplified with universal forward primer F29 (AGAGTTTGATCATGGCTCAG) and universal reverse primer R1492 (TAC GGC TAC CTT GTT ACG ACTT). PCR reaction mixtures contained 2 µl of 10X PCR buffer, 2 µl of dNTP mixture (2.5 mM each), 1µl of each primer (10 pmoles), 5 µl of the extracted genomic DNA, 0.5 µl of *Taq* DNA polymerase (5U/µl) from *iNtRON Biotechnology* (Korea) and finally were made up to 20 µl with double distilled water (ddH<sub>2</sub>O). PCR condition comprised an initial denaturation at 94 °C, continued by 35 cycles of denaturation at 94 °C for 1 min, annealing at 52 °C for 1 min, extension at 72 °C for 1.5 min and ended with a final

extension at 72 °C for 10 min. The PCR products sizes were confirmed to be 16S rDNA by electrophoresis on a 1.5% agarose gel and then were purified using *PCR Quickspin Kit* (iNtRON Biotechnology, Korea). The result of DNA sequencing by Macrogen Inc. (Korea) were matched with NCBI nucleotide database using alignment search tool of BlastN and the bacteria were identified on the basis of at least 98% similarity to the 16S rRNA gene sequences.

#### **Bacterial Products Solution Preparation**

The identified bacteria were cultured in nutrient broth at 25°C for 96 h and then were centrifuged at 10000Xg for 10 min. The supernatant of each bacterial culture was collected and filter sterilized using a 0.20 µm *Minisart®* sterile syringe filter (Sartorius Stedim Biotech, Germany) and refrigerated at 4 °C.

#### **Antioxidant Potential Evaluation**

##### **Erythrocyte hemolysis inhibition assay**

This assay was carried out based on the method described before (Rafat *et al.*, 2010). Briefly, the cells of rabbit blood were harvested by centrifugation at 1000Xg at 4°C for 20 min. The cells were washed thrice with PBS (pH 7.4) and finally were added to an equal volume of PBS to prepare the erythrocyte suspension. The bacterial product solution (1 ml) was added to 500 µl of erythrocyte suspension and incubated at 37°C for 40 min as the pretreatment stage. Then, the volume of solutions was adjusted to 9 ml using PBS and 1 ml of 10mM hydrogen peroxide (H<sub>2</sub>O<sub>2</sub>) was added to induce the oxidative stress. After incubating the mixtures at 37°C for 150 min, the amount of released hemoglobins into the supernatant was measured at 540 nm. The results were expressed as a percentage based on a complete hemolysis (100%) acquired by pure water. Ascorbic acid (1 mg/ml) and non-pretreated erythrocyte were used as the positive and negative controls respectively.

##### **DPPH\* scavenging capacity assay**

DPPH free radical scavenging protocol was obtained from Rafat *et al.* (2010). Bacterial product solution (50 µl) was mixed with 950 µl of DPPH\* (90 µM) and incubated for 120 min at room temperature after adjusting the final volume to 4 ml using 95% ethanol. The reduction of mixture colour was measured at 515 nm and compared with mixture without sample (blank). Both ascorbic acid

(1 mg/ml) and 1 mg/ml of tert-butylated hydroxytoluene (BHT) (1 mg/ml) were used as the positive controls in this assay. The DPPH radical scavenging activity was expressed as a percentage value using the following equation:

$$\text{Free Radical Scavenging Activity (\%)} = \frac{[(A_{\text{blank}} - A_{\text{sample}}) / A_{\text{blank}}] \times 100}{}$$

##### **SOD activity assay**

SOD Assay Kit (19160) from Sigma-Aldrich (Japan) was employed and the assay was carried out based on the Kit protocol as we have described before (Rafat *et al.* 2010). Bacterial product solution (20 µl) was mixed with reaction mixtures of the Kit and incubated at 37 °C for 20 min before measuring the absorbance of mixtures at 450 nm. Ascorbic acid (1 mg/ml) and BHT (1 mg/ml) were applied as the positive controls in this experiment. The inhibition rates were expressed as percentage values based on the mixture with our sample.

##### **Reductive capacity assay**

*NWLSST<sup>TM</sup>* Antioxidant Reductive Capacity Assay Kit from Northwest Life Science Specialties, LLC (USA) was used for determining the antioxidant capacity of bacterial products solutions in terms of their reductive capacity. The ability of samples to reduce Cu<sup>++</sup> to Cu<sup>+</sup> was measured based on the manual description of the Kit. The bacterial product solutions were diluted 1:40 with the Assay Dilution Buffer and the absorbance of 200 µl of the mixture was measured at 490 nm before adding 50 µl of Cu<sup>++</sup> Solution to each sample. The Stop Solution (50 µl) was added to the mixture 3 minutes after adding Cu<sup>++</sup> Solution and the absorbance was measured for the second time at 490 nm. The net difference between absorbance readings was compared to the standard curve which was prepared using uric acid and the reduction capacity was expressed as an equivalent uric acid concentration (method adapted from Shvedova *et al.*, 2008).

##### **Antibacterial activity assay**

The antibacterial potential of isolated endophytic bacteria products was evaluated using a disc diffusion method described by Lee *et al.* (2008) with some modifications. Two Gram-Negative pathogenic bacteria (*Escherichia coli* and *Pseudomonas aeruginosa*) and two Gram-Positive

pathogenic bacteria (*Staphylococcus aureus* and *Bacillus cereus*) were obtained from Microbiology Division of Institute of Biological Sciences, University of Malaya and then grown in Nutrient Broth medium to yield a final concentration of  $10^7$  CFU/ml. The test bacteria (0.1 ml) were streaked on whole Mueller Hinton medium plates using sterile swab cotton. Sterilized filter paper discs were soaked in endophytic bacteria products solutions and were then placed in the center of the test bacteria plates. The plates were incubated at 37 °C for 24 h and diameters of the produced inhibition zones were measured to evaluate the antibacterial activity of samples. Tetracycline disc (30 µg) and sterile distilled water were used as the positive and negative controls respectively.

#### Statistical analysis

All experiments were carried out in triplicate. One-way analysis of variance (ANOVA) was used to analyze the data using SPSS version

17. The means were compared with Duncan's Multiple Comparison Test (DMCT) and  $p = 0.05$  was considered to indicate statistical significance.

## RESULTS

### Isolation and Identification of Endophytic Bacteria

There were 6 endophytic bacteria isolated from *C. asiatica* stems and leaf petioles as well as 6 bacteria from leaf (Table 1) while three strains are common between them. The total endophytic bacteria population in the stem and petiole parts ( $10^4$  to  $10^5$  cfu/g) was more than the leaves ( $10^3$  to  $10^4$  cfu/g).

### Antioxidant Activity Assays

#### Erythrocyte hemolysis inhibition assay

Based on erythrocytes hemolysis inhibition assay result (Fig. 1), all of the endophytic bacteria products reduced the percentage of

**Table 1.** Isolated endophytic bacteria associated with different parts of *Centella asiatica*

Bacteria Name	Bacteria Strain	Accession No. (NCBI)	Location
<i>Bacillus gibsonii</i>	AR_PBSTSB	HM582875	Petiole-Stem
<i>Methylobacterium radiotolerans</i>	AR_PSLBHI	HM582876	Leaf & Petiole-Stem
<i>Pantoea agglomerans</i>	AR_PSBH2	HM582877	Leaf & Petiole-Stem
<i>Pantoea agglomerans</i>	AR_PINLBH4	HM582878	Leaf & Petiole-Stem
<i>Erwinia tasmaniensis</i>	AR_PINLTS5	HM582879	Leaf
<i>Erwinia soli</i>	AR_PINLTS1	HM582880	Leaf
<i>Providencia vermicola</i>	AR_PSBH1	HM582881	Petiole-Stem
<i>Pseudomonas fulva</i>	AR_PSTS1	HM582882	Petiole-Stem
<i>Xanthomonas axonopodis</i>	AR_PINLBH3	HM582883	Leaf

**Table 2.** Antioxidant reductive capacity of isolated endophytic bacteria expressed as an equivalent uric acid concentration based on a uric acid standard curve

Sample	Accession No. (NCBI)	Uric Acid Equivalent (mM)
<i>Bacillus gibsonii</i>	(HM582875)	0.059 ± 0.003 <sup>b</sup>
<i>Methylobacterium radiotolerans</i>	(HM582876)	0.043 ± 0.003 <sup>b</sup>
<i>Pantoea agglomerans</i>	(HM582877)	0.120 ± 0.013 <sup>b</sup>
<i>Pantoea agglomerans</i>	(HM582878)	0.003 ± 0.001 <sup>b</sup>
<i>Erwinia tasmaniensis</i>	(HM582879)	0.065 ± 0.006 <sup>b</sup>
<i>Erwinia soli</i>	(HM582880)	0.071 ± 0.007 <sup>b</sup>
<i>Providencia vermicola</i>	(HM582881)	0.102 ± 0.01 <sup>b</sup>
<i>Pseudomonas fulva</i>	(HM582882)	0.071 ± 0.017 <sup>b</sup>
<i>Xanthomonas axonopodis</i>	(HM582883)	0.077 ± 0.005 <sup>b</sup>
Vitamin C (1mg/ml)	-	3.018 ± 0.188 <sup>a</sup>

Means followed by the same letter (s) in the same column are not significantly different based on Duncan's Multiple Comparison Test at  $p = 0.05$ .

erythrocytes hemolysis and all compared to the non-pretreated sample were significant except *P. agglomerans* AR\_PINLBH4 (HM582878). Among all the endophytic bacteria, *P. agglomerans* AR\_PSBH2 (HM582877) sample showed the lowest hemolysis percentage. None of the endophytic bacteria significantly inhibited the hemolysis percentage compared to the positive control (1 mg/ml ascorbic acid).

**DPPH radical scavenging assay**

The result of DPPH radical scavenging assay is shown in Fig. 2. Among all endophytic bacteria, the highest free radical scavenging percentage was obtained with *P. agglomerans* AR\_PSBH2 (HM582877) followed by *P. vermicola* AR\_PSBH1 (HM582881) and showing significantly higher scavenging activity compared to other isolated bacteria. The lowest free radical scavenging capacity among the examined endophytic bacteria was obtained with *B. gibsonii* AR\_PBSTSB (HM582875) and *M. radiotolerans* AR\_PSLBHI (HM582876). Although the free radical scavenging activities of all endophytic bacteria was significantly lower than ascorbic acid (1 mg/ml) but all examined bacteria product neutralized DPPH free radical significantly higher than BHT (1 mg/ml) did.

**SOD activity assay**

Results of SOD activity assay (Fig. 3) shows that *E. coli* AR\_PINLTS1 (HM582880) had the highest SOD capacity among the examined

bacteria products which was not significantly different with *B. gibsonii* AR\_PBSTSB (HM582875), *M. radiotolerans* AR\_PSLBHI (HM582876), *P. agglomerans* AR\_PINLBH4 (HM582878) and *X. axonopodis* AR\_PINLBH3 (HM582883). The lowest inhibition rates among all endophytic bacteria were obtained by *P. fulva* AR\_PSTS1 (HM582882) followed by *P. agglomerans* AR\_PSBH2 (HM582877). SOD activity of all endophytic bacteria products was significantly lower than both positive controls (ascorbic acid at 1mg/ml and BHT at 1 mg/ml).

**Reductive capacity assay**

The results of the quantitative measurement of antioxidant status in endophytic bacteria product samples (Table 2) showed that although the highest reductive capacity belonged to *P. agglomerans* AR\_PSBH2 (HM582877) but is not significantly different with other endophytic bacteria samples. Meanwhile another strain of *P. agglomerans* namely AR\_PINLBH4 (HM582878) showed the lowest antioxidant reductive property among all samples. Antioxidant capacity of all endophytic bacteria products measured using this assay was significantly lower than positive control (1 mg/ml of ascorbic acid).

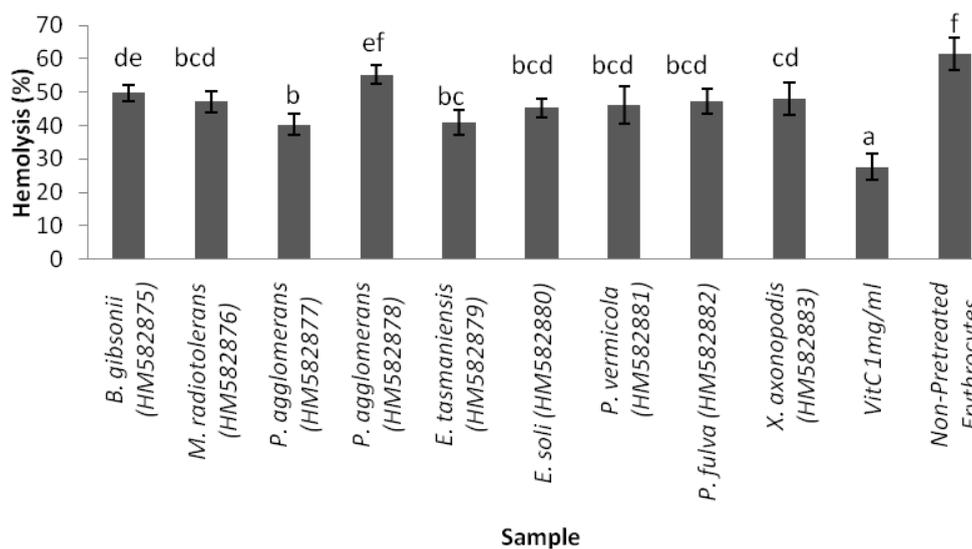
**Antibacterial Assay**

The results of disc diffusion antimicrobial assay (Table 3) showed that although none of the endophytic bacteria products showed inhibition activity against *E. coli*, *B. cereus* and *S. aureus*,

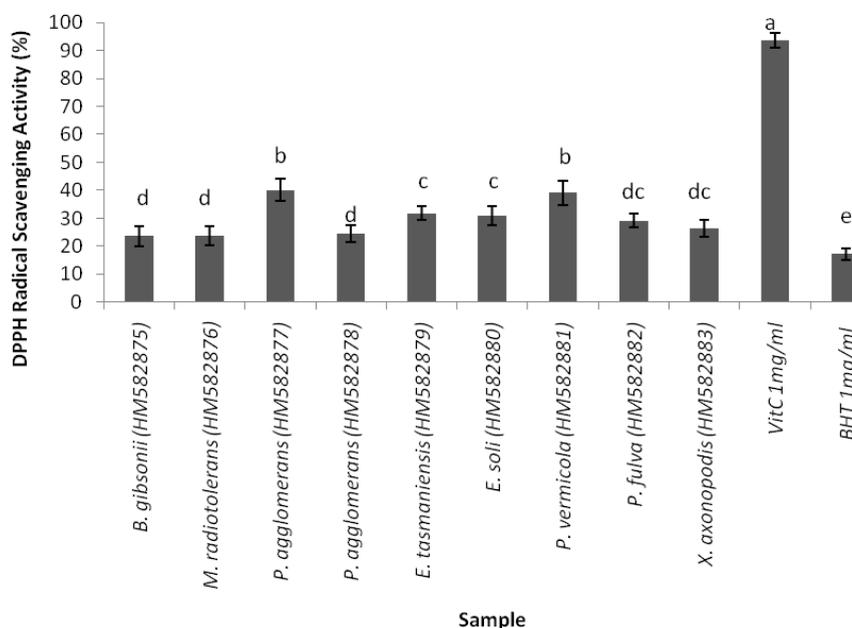
**Table 3.** Antibacterial activity of isolated endophytic bacteria products and controls against pathogenic bacterial species tested by disc diffusion assay

Bacteria	Inhibition Zone (mm)			
	<i>Escherichia coli</i>	<i>Pseudomonas aeruginosa</i>	<i>Bacillus cereus</i>	<i>Staphylococcus aureus</i>
<i>B. gibsonii</i> (HM582875)	NI	NI	NI	NI
<i>M. radiotolerans</i> (HM582876)	NI	9	NI	NI
<i>P. agglomerans</i> (HM582877)	NI	9	NI	NI
<i>P. agglomerans</i> (HM582878)	NI	9	NI	NI
<i>E. tasmaniensis</i> (HM582879)	NI	9	NI	NI
<i>E. soli</i> (HM582880)	NI	9	NI	NI
<i>P. vermicola</i> (HM582881)	NI	11	NI	NI
<i>P. fulva</i> (HM582882)	NI	8	NI	NI
<i>X. axonopodis</i> (HM582883)	NI	8	NI	NI
Tetracycline (30 µg)	26	18	22	31
Sterilized dH <sub>2</sub> O	NI	NI	NI	NI

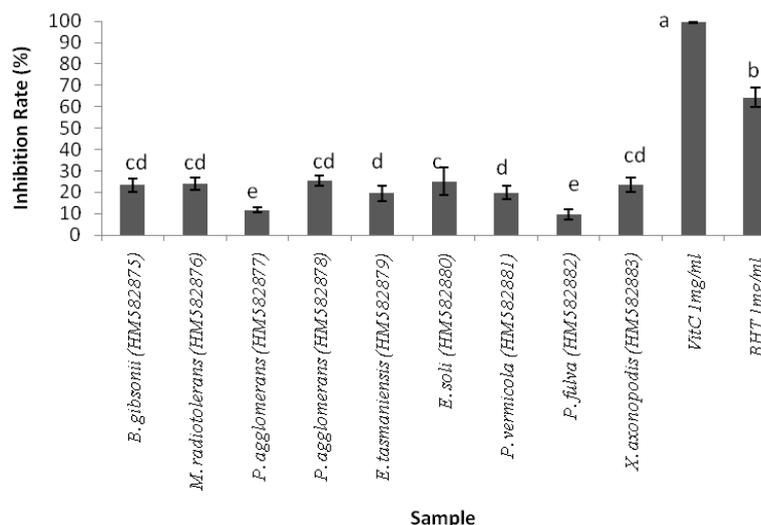
NI: No Inhibition



**Fig. 1.** Antioxidant activity of isolated endophytic bacteria: hemolysis of rabbit erythrocytes expressed as percentage values. Ascorbic acid at concentration of 1 mg/ml (VitC 1mg/ml) was used as the positive control. The mean changes between the samples were analyzed by one-way ANOVA followed by Duncan's Multiple Comparison Test. Samples represented with different letters are significantly different ( $p = 0.05$ )



**Fig. 2.** Antioxidant activity of isolated endophytic bacteria: neutralization of DPPH radical of samples in the free radical scavenging activity assay. Ascorbic acid at concentration of 1 mg/ml (VitC 1mg/ml) and tertbutylated hydroxytoluene at concentration of 1 mg/ml (BHT 1mg/ml) were used as the positive controls. The mean changes between the samples were analyzed by one-way ANOVA followed by Duncan's Multiple Comparison Test. Samples represented with different letters are significantly different ( $p = 0.05$ )



**Fig. 3.** Antioxidant activity of isolated endophytic bacteria: SOD activities of samples are presented as inhibition rate. Ascorbic acid at concentration of 1mg/ml (VitC 1mg/ml) and tertbutylated hydroxytoluene at concentration of 1 mg/ml (BHT 1mg/ml) were used as the positive controls. The mean changes between the samples were analyzed by one-way ANOVA followed by Duncan's Multiple Comparison Test. Samples represented with different letters are significantly different ( $p = 0.05$ )

all of them could inhibit the growth of *P. aeruginosa* except *B. gibsonii* (HM582875). *P. vermicola* (HM582881) products solution showed the highest inhibition activity against *P. aeruginosa* while the lowest inhibition obtained by *P. fulva* (HM582882) and *X. axonopodis* (HM582883).

## DISCUSSION

While Hallmann *et al.* (1997) showed that bacterial endophytes can be isolated from both surface disinfected plant tissue and internal plant tissue extracts, several methods for the isolation of endophytic bacteria have been reported recently. Qin *et al.* (2009) applied three different methods of isolation of endophytic actinobacteria from the dried surface sterilized plant samples after a 15 minute heat surface treatment at 100°C. While their first method, the samples were broken into small pieces and placed on medium, their second using method was established based on a method described by Otoguro *et al.* (2001) using calcium carbonate powder, phosphate buffer and centrifugation. Their third method was based on a procedure established by Jiao *et al.* (2006) which is

a combination of enzymatic methods and separation using centrifugation. Qin *et al.* (2009) state that using various methods of isolation increase the possibility of obtaining many endophytic bacteria of interest compared to the usage of a single isolation procedure. Based on several reports on isolation of endophytic bacteria from plants, fresh surface sterilized plant tissue can be cultured for several days on a normal plant growth medium (Dias *et al.* 2009) or on an enriched growth medium (Reed *et al.* 1995) or on a bacterial growth medium (Tiwari *et al.* 2010). In other methods surface sterilized plant tissues are usually macerated with a homogenizer, blender or mortar and pestle and then diluted in sterile water (Hung and Annapurna 2004), sterile potassium phosphate buffer (Vetrivelkai *et al.*, 2010) or sterile magnesium sulfate solution (Wang *et al.* 2006) and plated and incubated. The result of our study showed that the applied isolation method is able to isolate a variety of endophytic bacteria from different families.

The population and diversity of plant endophytic microorganisms is in a relationship with various factors such as environmental and soil condition, plant species, plant age and tissue type

(Zinniel *et al.* 2002; Adams and Kloepper 2002; Conn and Franco, 2004). The present study shows the population of endophytic bacteria in stem and petiole parts of *C. asiatica* is higher than the leaves which is in agreement with the study by Altalhi (2009) on endophytic bacteria associated with old leaves and stems of grapevine.

Although endophytic microorganisms have been shown to be a profuse source of bioactive compounds, they are seldom screened and evaluated for their properties (Strobel, 2007). Evaluation of antioxidant activity of endophytic *Pestalotiposis microspora* fungus showed the high antioxidant capacity of pestacin (Harper *et al.* 2003) and isopestacin (Strobel *et al.* 2002) produced by this fungus. To our knowledge there is no specific report on antioxidant potential of endophytic bacteria and our study may be the first report of its kind. Application of various antioxidant evaluation methods to achieve a more reliable result is recommended (Rafat *et al.* 2010). In the present study, a combination of enzymatic and non-enzymatic base assays to measure the antioxidant properties of endophytic bacteria products was applied. The study clearly showed that the result of each antioxidant assay may or may not be in agreement with the other assays. For example, *P. agglomerans* (HM582877) could show the highest antioxidant activity based on three erythrocytes hemolysis, free radical scavenging and reductive capacity assays while it showed one of the lowest SOD activity among all isolated endophytic bacteria. Hence, using different antioxidant activity techniques to gain complete assessment is necessary.

This study confirmed that the bioactivity properties of the bacteria species vary by strain. For example, among all isolated endophytic bacteria, *P. agglomerans* AR\_PSBH2 showed the highest erythrocytes hemolysis inhibition while the lowest inhibition was obtained from another strain of this bacterium namely *P. agglomerans* AR\_PINLBH4.

Antimicrobial activity of endophytic microbes has been studied more than other bioactivity properties. Some of the endophytic fungi isolated from different plants showed potent antimicrobial activity. Ramos *et al.* (2010) reported the antimicrobial properties of some endophytic fungi isolated from *Smallanthus sonchifolius* roots

while Maria *et al.* (2005) and Sette *et al.* (2006) showed the antimicrobial potential of some mangrove endophytic fungi of southwest coast of India and coffee plant respectively. Several of endophytic bacteria isolated from Thai medicinal plants also showed a promising antibacterial activity against variety of human pathogenic bacteria (Laorpaksa *et al.* 2008). Roy and Banerjee (2010) showed that endophytic bacteria associated with *Vinca rosea* plant have the potential of being a new source of antimicrobial compound. The activity of endophytic actinomycetes isolated from tomato plants against *Ralstonia solanacearum* (Tan *et al.* 2006) is another example to clarify the importance of endophytic bacteria as the bioactive phytochemicals producer. All isolated endophytic bacteria in this study, except *B. gibsonii* (HM582875), showed a good inhibition activity against *P. aeruginosa* but no inhibition against the other test bacteria. The reason for such selectivity is unclear and needs further investigation. The study also suggests that the secondary metabolites produced by *C. asiatica*-associated endophytic bacteria can be analyzed for isolation of antimicrobial compounds.

## CONCLUSION

A wide range of endophytic bacteria from different families were isolated from leaf and petiole-stem of *C. asiatica*. There were similar strains such as *Methylobacterium radiotolerans* AR\_PSLBHI and *Pantoea agglomerans* AR\_PSBH2 which were found in both examined morphological parts of plant. This finding can confirm that some of the endophytic bacteria migrated between different plant parts. *Bacillus gibsonii* was the only Gram-positive endophytic bacteria isolated. All isolated endophytic bacteria except *B. gibsonii* could inhibit the growth of *Pseudomonas aeruginosa* as the pathogenic test bacteria. This confirmed that endophytic bacteria associated with *C. asiatica* are a novel source for antimicrobial compounds. Although the isolated endophytic bacteria cell-free cultures showed fair antioxidant activities compared to the applied positive controls, DPPH radical scavenging activity of these bacteria free cultures were significantly higher than one of the positive controls (BHT at concentration of 1 mg/ml).

## ACKNOWLEDGMENTS

The authors would like to thank University of Malaya for providing a research grant (RG086/09AFR) and for the use of laboratory facilities to carry out this study.

## REFERENCES

- Adams, P.D., Klopper, J.W. Effect of host genotype on indigenous bacterial endophytes of cotton (*Gossypium hirsutum* L.). *Plant Soil*, 2002; **240**: 181-9.
- Altalhi, A.D. Plasmids profiles, antibiotic and heavy metal resistance incidence of endophytic bacteria isolated from grapevine (*Vitis vinifera*). *Afr. J. Biotechnol.*, 2009; **8**(21): 5873-5882.
- Conn VM, Franco CMM Analysis of the endophytic actinobacterial population in the roots of wheat (*Triticum aestivum* L.) by terminal restriction fragment length polymorphism and sequencing of 16S rRNA clones. *Appl. Env. Microbiol.*, 2004; **70**(3): 1787-1794.
- Cho, K.M., Hong, S.Y., Lee, S.M., Kim, Y.H., Kahng, G.G., Lim, Y.P., Kim, H., Yun, H.D. Endophytic bacteria communities in ginseng and their antifungal activity against pathogens. *Microb. Ecol.*, 2007; **54**(2): 341-51.
- Dias, A.C.F., Costa, F.E.C., Andreote, F.D., Lacava, P.T., Teixeira, M.A., Assumpção, L.C. Isolation of micropropagated strawberry endophytic bacteria and assessment of their potential for plant growth promotion. *World J. Microbiol. Biotechnol.*, 2009; **25**: 189-195.
- Hallmann, J., QuadtHallmann, A., Mahaffee, W.F., Klopper, J.W. Bacterial endophytic in agricultural crops. *Can. J. Microbiol.*, 1997; **43**(10): 895-914.
- Harper, J.K., Arif, A.M., Ford, E.J., Strobel, G.A., Jr Porco, J.A., Tomer, D.P., Oneill, K.L., Heider, E.M., Grant, D.M. Pestacin: a 1,3-dihydro isobenzofuran from *Pestalotiopsis microspora* possessing antioxidant and antimycotic activities. *Tetrahedron*, 2003; **59**(14): 2471-6.
- Hung, P.Q., Annapurna, K. Isolation and characterization of endophytic bacteria in soybean (*Glycine* sp.). *Omonrice*, 2004; **12**: 92-101
- Jiao, J.Y., Wang, H.X., Zeng, Y., Shen, Y.M. Enrichment for microbes living in association with plant tissues. *J. Appl. Microbiol.*, 2006; **100**: 830-7.
- Joseph, B., Sankarganesh, P., Justinraj, S., Edwin, B.T. Need exploration of endophytic *Streptomyces* – Review. *J. Pure Appl. Microbiol.*, 2011; **5**(1): 153-160.
- Laorpaksa, A., Jianmongkol, S., pothiwong, W. Antimicrobial activity of endophytic bacteria isolated from Thai medicinal plant. *Thai J. Pharm. Sci.*, 2008; **32**: 21-32.
- Lata, H., Li, X.C., Silva, B., Moraes, R.M., Halda-Alija, L. Identification of IAA-producing endophytic bacteria from micropropagated *Echinacea* plants using 16S rRNA sequencing. *Plant Cell Tiss. Organ. Cult.* 2006; **85**: 353-9.
- Lee, J.H., Yang, H.Y., Lee, HS., Hong, S.K. Chemical composition and antimicrobial activity of essential oil from cones of *Pinus koraiensis*. *J. Microbiol. Biotechnol.*, 2008; **18**(3): 497-502.
- Maria, G.L., Sridhar, K.R., Raviraja, N.S. Antimicrobial and enzyme activity of mangrove endophytic fungi of southwest cost of India. *J. Agri. Tech.*, 2005; **1**: 67-80.
- Miller, C.M., Miller, R.V., Garton-Kenny, D., Redgrave, B., Sears, J., Condron, M.M., Teplow, D.B., Strobel, G.A. Ecomycins, unique antimycotics from *Pseudomonas viridiflava*. *J. Appl. Microbiol.*, 1998; **84**(6): 937-944.
- Nautiyal, C.S. A method for selection and characterization of rhizosphere-competent bacteria of chickpea. *Curr. Microbiol.*, 1997; **34**: 12-7.
- Otoguro, M., Hayakawa, M., Yamazaki, T., Iimura, Y. An integrated method for the enrichment and selective isolation of *Actinokineospora* spp. in soil and plant litter. *J. Appl. Microbiol.*, 2001; **91**: 118-130.
- Panthi, M.P., Chaudhary, R.P. Antibacterial activity of some selected folklore medicinal plants from west Nepal. *The. Sci. World*, 2006; **4**(4): 16-21.
- Qin, S., Li, J., Chen, H.H., Zhao, G.Z., Zhu, W.Y., Jiang, C.L., Xu, L.H., Li, W.J. Isolation, diversity, and antimicrobial activity of rare actinobacteria from medicinal plants of tropical rain forests in Xishuangbanna, China. *Appl. Environ. Microbiol.*, 2009; **75**(19): 6176-6186.
- Rafat, A., Philip, K., Muniandy, S. Antioxidant potential and content of phenolic compounds in ethanolic extracts of selected parts of *Andrographis paniculata*. *J. Med. Plants Res.*, 2010; **4**(3): 197-202.
- Rafat, A., Philip, K., Muniandy, S. Antioxidant properties of indigenous raw and fermented salad plants. *Int. J. Food Prop.*, 2011; **14**(3): 599-608.
- Rakotoniriana, E.F., Munaut, F., Decock, C., Randriamampionona, D., Andriamboloniaina, J. *Pure & Appl. Microbiol.*, **6**(1), March 2012.

- M., Rakotomalala, T., Rakotonirina, E.J., Rabemanantsoa, C., Cheuk, K., Ratsimamanga, S.U., Mahillon, J., El-Jaziri, M., Quetin-Leclercq, J., Corbisier, A.M. Endophytic fungi from leaves of *Centella asiatica*: occurrence and potential interactions within leaves. *Antonie van Leeuwenhoek*, 2008; **93**: 27-36.
23. Ramos, H.P., Braun, G.H., Pupo, M.T., Said, S. Antimicrobial activity from endophytic fungi *Arthrinium* state of *Apiospora montagnei* Sacc. and *Papulaspora immerse*. *Braz. Arch. F. Biol. Tech.*, 2010; **53**(3): 629-632.
24. Ravikumar, S., Thajuddin, N., Suganthi, P., Inbaneson, S.J., Vinodkumar, T. Bioactive potential of seagrass bacteria against human bacterial pathogens. *J. Environ. Biol.*, 2010; **31**: 387-9.
25. Reed, B.M., Buckley, P.M., DeWilde, T.N. Detection and eradication of endophytic bacteria from micropropagated mint plants. *In Vitro Cell Dev. Biol. Plant*, 1995; **31**: 53-7.
26. Roy, S., Banerjee, D. Isolation of antimicrobial compound by endophytic bacteria from *Vinca rosea*. *Inter. J. Curr. Res.*, 2010; **5**: 47-51.
27. Samy, J., Sugumaran, M., Lee, K.L.W., Wong, K.M. (eds): Herbs of Malaysia: an introduction to the medicinal, culinary, aromatic and cosmetic use of herbs. Kuala Lumpur: Marshall Cavendish Editions, 2005.
28. Sette, L.D., Passarini, M.R.Z., Delarmelina, C., Salati, F., Duarte, M.C.T. Molecular characterization and antimicrobial activity of endophytic fungi from coffee plants. *World J. Microbiol. Biotechnol.*, 2006; **22**: 1185-1195.
29. Shvedova, A. A., Kisin, E., Murray, A.R., Johnson, V.J., Gorelik, O., Arepalli, S., Hubbs, A.F., Mercer, R.R., Keohavong, P., Sussman, N., Jin, J., Yin, J., Stone, S., Chen, B.T., Deye, G., Maynard, A., Castranova, V., Baron, P.A., and Kagan, V.E. Inhalation vs. aspiration of single-walled carbon nanotubes in C57BL/6 mice: inflammation, fibrosis, oxidative stress, and mutagenesis. *Am. J. Physiol. Lung Cell Mol. Physiol.*, 2008; **295**(4): L552-L565.
30. Somchit, M.N., Sulaiman, M.R., Zuraini, A., Samsuddin, L., Somchit, N., Israf, D.A., Moin, S. Antinociceptive and antiinflammatory effects of *Centella asiatica*. *Ind. J. Pharmacol.*, 2004; **36**(6): 377-380.
31. Strobel, G.: Plant-associated microorganisms (Endophytes) as a new source of bioactive natural products. In: *Medicinal Plant Biotechnology, From Basic Research to Industrial Applications* (Kayser O, Quax WJ, ed). Weinheim: WILEY-VCH Verlag GmbH & Co. KGaA, 2007; pp 49-72.
32. Strobel, G., Ford, E., Worapong, J., Harper, J.K., Arif, A.M., Grant, D.M., Fung, P.C., Chau, R.M.W. Isopestacin, an isobenzofuranone from *Pestalotiopsis microspora*, possessing antifungal and antioxidant activities. *Phytochem*, 2002; **60**(2): 179-183.
33. Sturz, A.V. The role of endophytic bacteria during seed piece decay and potato tuberization. *Plant Soil*, 1995; **175**: 257-263.
34. Taechowisan, T., Lu, C., Shen, Y., Lumyong. Antitumor activity of 4-Arylcoumarins from endophytic *Streptomyces aureofaciens* CMUAc130. *J. Canc. Res. Therapeut.*, 2007; **3**(2): 86-91.
35. Tan, H.M., Cao, L.X., He, Z.F., Su, G.J., Lin, B., Zhou, S.N. Isolation of endophytic actinomycetes from different cultivars of tomato and their activities against *Ralstonia solanacearum* in vitro. *World J. Microbiol. Biotechnol.*, 2006; **22**: 1275-1280.
36. Tervet, I.W., Hollis, J.P. Bacteria in the storage organs of healthy plants. *Phytopathol.*, 1948; **38**: 960-7.
37. Tiwari, R., Kalra, A., Darokar, M.P., Chandra, M., Aggarwal, N., Singh, A.K., Khanuja, S.P.S. Endophytic bacteria from *Ocimum sanctum* and Their Yield Enhancing Capabilities. *Curr. Microbiol.*, 2010; **60**: 167-171.
38. Ullah, M.O., Sultana, S., Haque, A., Tasmin, S. Antimicrobial, cytotoxic and antioxidant activity of *Centella asiatica*. *Eur. J. Sci. Res.*, 2009; **30**(2): 206-254.
39. Vetrivelkalai, P., Sivakumar, M., Jonathan, E.I. Biocontrol potential of endophytic bacteria on *Meloidogyne incognita* and its effect on plant growth in bhendi. *J. Biopesticides*, 2010; **3**(2): 452-7.
40. Wang, E.T., Tan, Z.Y., Guo, X.W., Rodríguez-Duran, R., Boll, G., Martínez-Romero, E. Diverse endophytic bacteria isolated from a leguminous tree *Conzattia multiflora* grown in Mexico. *Arch. Microb.* 2006; **186**: 251-9.
41. Ziedan, E.H.E. Manipulating Endophytic Bacteria for Biological Control to Soil Borne Diseases of Peanut. *J. Appl. Sci. Res.*, 2006; **2**(8): 497-502.
42. Zinniel, D.K., Lambrecht, P., Harris, B.N., Feng, Z., Kuczmarski, D., Higley, P., Ishimaru, C.A., Arunakumari, A., Barletta, R.G., Vidaver, A.K. Isolation and characterization of endophytic colonizing bacteria from agronomic crops and prairie plants. *Appl. Environ. Microbiol.*, 2002; **68**: 2198-2208.