JIDC | THE JOURNAL OF INFECTION IN DEVELOPING COUNTRIES

Original Article

Investigation on the bioactivity of culturable endophytic and epiphytic bacteria associated with ethnomedicinal plants

Fenella Mary War Nongkhlaw, Santa Ram Joshi

Microbiology Laboratory, Department of Biotechnology and Bioinformatics, North-Eastern Hill University, Shillong, India

Abstract

Introduction: The growing need for the bioactive compounds of ethnomedicinal plants for the treatment of diseases has resulted in exploitation of medicinal plants. The present investigation aimed to study the diversity of endophytic and epiphytic bacteria associated with ethnomedicinal plants and to explore their potential as source of bioactive compounds.

Methodology: Characterization of culturable endophytic and epiphytic bacteria associated with 11 ethnomedicinal plants and their potential as natural antioxidants was assessed through free radical scavenging activity, total phenolics, total flavonoids, metal ion chelation, and antagonistic activity. Genetic screening to assess the potential of endophytes and epiphytes to synthesize bioactive compounds was achieved by screening for the presence of the non-ribosomal peptide synthetase (NRPS) gene.

Result: The frequently isolated endophytic bacterium recovered was *Bacillus* sp. Antioxidative property of the bacterial extracts revealed endophytes with potent antioxidant activity and better antagonistic activity as compared to epiphytes. Genetic screening revealed the presence of the NRPS gene in seven plant-associated bacteria, indicating the production of natural products.

Conclusions: The study indicated the extracts of bacterial endophytes associated with ethnomedicinal plants as good sources of natural products with potential application in oxidative stress. The isolates could be used as new bioactive agents.

Key words: ethnomedicinal plants; bacteria; endophytes; epiphytes; antioxidant; antimicrobial

J Infect Dev Ctries 2015; 9(9):954-961. doi:10.3855/jidc.4967

(Received 06 March 2014 – Accepted 29 March 2015)

Introduction

There is an emerging need for compounds derived from natural sources for the development of drugs to treat diseases, drug-resistant bacteria, and fungal infections [1]. The present study carries significance because the tribal people of the northeastern region of India are known to use plants as folk remedies for treating a variety of ailments [2], and this has resulted in the exploitation of ethnomedicinal plants. In recent years, research on medicinal plants have attracted considerable attention globally [3-5], but very little is known about the bioactive properties of bacteria living in association with plants. Compounds derived from plants are largely products of plant metabolism. However, plant-associated microorganisms also produce bioactive compounds; taxol and camptothecin are examples of anti-cancer compounds synthesized by both plants and endophytes [6-7]. Exploring bioactive compounds from associated microbes may not only reduce the unwanted destruction of ethnomedicinal plants [8], but may also provide an alternative source for the extraction of potential metabolites on a large scale. Endophytes are a specific group of microorganisms that can be found in internal, healthy plant tissue without causing visible damage to their hosts [9]. Microorganisms that colonize plant surfaces are referred to as epiphytes.

The present study was an attempt to assess the diversity of predominant and culturable endophytic and epiphytic bacteria associated with ethnomedicinal plants and to explore their potential as natural antioxidants through assessment of free-radical scavenging activity, total phenolics, total flavonoids, metal ion chelation, and antagonistic activity. The biosynthetic potential of endophytes and epiphytes was considered via the detection of non-ribosomal peptide synthetase (NRPS) biosynthesis genes [10]. NRPSs are involved in the biosynthesis of antibiotics and anti-inflammatory and immunosuppressive compounds. NRPSs are composed of a series of modules, and the adenylation (A) domain is targeted for the detection of NRPS biosynthesis genes [11-12]. Though there are other methods for bioprospection of microbes, isolation in culture can be an important

process for the study of microbes and discovery of novel biological activity.

Methodology

Plant sample collection

Healthy plants used by different traditional medicinal practitioners (TMPs) were collected from different parts of northeast India based on their ethnomedicinal uses. The taxonomic identity of the plants was confirmed with the help of the herbarium curator of the parent university. All samples were collected in sterile polythene bags, brought to the laboratory, and used for isolation within 24 hours of collection.

Isolation and molecular characterization of endophytic and epiphytic bacteria

Endophytic and epiphytic bacteria were isolated from healthy plant samples devoid of any apparent infections per the method previously reported [13,14]. Total genomic DNA was extracted using HiPurA bacterial and yeast genomic DNA Isolation Kits (HiMedia Labs, Mumbai, India). Polymerase chain reaction (PCR) amplification and sequencing of 16S rRNA gene was carried out in a 25 μL reaction mixture using general primers 27F 5'-AGAGTTTGATCCTGGCTGAG-3' and 1541R 5'-AAGGAGGTGATCCAGCCGCA-3'(Applied Biosystems, Foster City, USA) as described previously [15]. The V6–V8 hypervariable region of bacteria-specific 16S rRNA gene was amplified with the primer pair 984GCF 5'-AACGCGAAGAACCTTAC-3' and 1378R 5'-CGGTGTGTACAAGGCCCGGGAACG-3' [16,17]. The DNA template replaced with sterile water was used as a negative control. The amplified 16S rRNA gene (approximately 1,400 base pairs) and V6–V8 region (approximately 400 base pairs) was then purified using QIAquick Gel Extraction Spin Kit (QIAGEN, Hilden, Germany). The purified PCR products were bi-directionally sequenced using both forward and reverse primers in a sequencer Genetic Analyzer (ABI 3130 Applied Biosystems) with Big Dye (3.1) terminator protocol as described previously [15].

Phylogenetic analyses

Sequence similarities were determined by the BLAST program against the database of type strains with validly published prokaryotic names in the EzTaxon 2.1 server [18]. Molecular Evolutionary Genetics Analysis software (MEGA, version 4.0) [19] was used for phylogenetic analysis as described previously [15].

Bacterial extract preparation

All selected isolates were grown in 100 mL nutrient broth incubated in a shaker incubator at 120 rpm at 32°C for three days. The bacterial culture broth was centrifuged at 10,000 rpm for 15 minutes, and the supernatant was filtered using autoclaved 0.22 μm membrane filter paper. The filtrate was concentrated using a rotary evaporator (Stuart RE300P, Stuart Science Equipment, Stone, UK) under reduced pressure at 45°C. The concentrated aqueous extract of each selected isolate was then diluted to perform various assays.

Total phenolics

Total phenolic content was determined colorimetrically using Folin-Ciocalteu reagent [20] with minor modifications. Diluted aqueous extract (3.5 mL) was mixed with 0.5 mL of Folin-Ciocalteu reagent and incubated at room temperature for 3 minutes, and 1 mL of saturated Na_2CO_3 was added to the mixture. Control was maintained using water in place of the bacterial extract. All tubes were incubated at 45°C for 5 minutes, and the absorbance was measured at 760 nm (Cecil CE7200, Cecil Instruments, Milton, UK). All determinations were performed in triplicate. Total phenolic content was quantified using a known concentration of gallic acid. The concentration was expressed as milligram of gallic acid equivalent per gram of extract. Ascorbic acid (1 mg mL^{-1}) was taken as a positive control for comparative analysis.

Total flavonoids

The total flavonoid content was determined using the aluminium chloride method having the absorbance maximum at 430 nm. One milliliter of diluted sample was mixed with 1 mL of 2% aluminium chloride methanolic solution, 0.2 mL of 1M potassium acetate, and kept at room temperature for 30 minutes. Control was maintained using water in place of the bacterial extract. The absorbance of the reaction mixture was measured at 430 nm (Cecil CE7200). The total flavonoid content was determined from the standards obtained for quercetin. The concentration of total flavonoids was expressed as milligram of quercetin equivalent per gram of extract. Rutin (1 mg mL^{-1}) was taken as a positive control for comparative analysis.

Antioxidant activity by 2,2'-diphenyl-1-picrylhydrazyl (DPPH) radical scavenging method

The antioxidant activity of bacterial aqueous extract was evaluated using the DPPH (2,2'-diphenyl-1-picrylhydrazyl free radical) modified assay [21]. This method is based on the reduction of alcoholic DPPH solution in the presence of an antioxidant. For each antioxidant, different concentrations of the extract were tested. The aqueous extract (0.1 mL) was added to 3.9 mL of a 6×10^{-5}mol L^{-1} methanol DPPH solution. The control consisted of 0.1 mL water added to 3.9 mL DPPH solution. The reaction mixture was vortexed thoroughly and left in the dark at ambient temperature for 30 minutes. DPPH solution was prepared fresh daily and kept in the dark at 4°C between measurements. All determinations were performed in triplicate. Methanol was used to set zero blank and the decrease in absorbance was read at 517 nm (Cecil CE7200). A solution of methanol DPPH without extract was used as a control. The percentage of radical scavenging was calculated using the equation

$$\%Scavenging\ effect = \left[\frac{A_0 - A_1}{A_0}\right] * 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of sample extract or standard. Ascorbic acid (1 mg mL^{-1}) was taken at various concentrations as a known antioxidant for comparative analysis. Then percentage inhibitions were plotted against respective concentrations used, and from the graph, IC_{50} was calculated.

Reducing power assay

Different concentrations of aqueous extract of bacteria were mixed with phosphate buffer (2.5 mL, 0.2M, pH 6.6) and potassium ferricyanide (2.5 mL, 1%). This mixture was kept at 50°C in a water bath for 20 minutes. After cooling, 2.5 mL of 10% trichloroacetic acid was added and centrifuged at 3,000 rpm for 10 minutes. The upper layer of solution (2.5 mL) was mixed with distilled water and 0.5 mL of freshly prepared ferric chloride solution (0.1%, w/v). The absorbance was measured at 700 nm [22]. The control was prepared in a similar manner but excluded samples. Ascorbic acid (1 mg mL^{-1}) at various concentrations was used as a positive control. An increase in absorbance of the reaction mixture indicated an increase in reducing power. The extract concentration providing 0.5 of absorbance (IC_{50}) was calculated from the absorbance graph generated at 700 nm against extract concentration.

Metal chelating activity

Determination of metal chelating activity was based on chelating of Fe^{2+} ions by ferrozine, which forms a complex with Fe^{2+} ions. Different concentrations of aqueous extract were added to 50 µL of 2 mM $FeCl_2$. The reaction was initiated by the addition of 0.2 mL of 5 mM ferrozine, and the mixture was shaken vigorously and left standing at room temperature for 10 minutes. Water was added in place of the bacterial extract in the control. Absorbance of the solution was measured spectrophotometrically at 562 nm [23]. The percentage inhibition of ferrozine-Fe^{2+} complex formation was calculated as

$$\%Inhibition = \left[\frac{A_0 - A_1}{A_0}\right] * 100$$

Where A_0 is the absorbance of control and A_1 is the absorbance of sample extract or standard. NaEDTA (1 mg mL^{-1}) was used at various concentrations as positive control. The extract concentration providing 50% inhibition (IC_{50}) was calculated from the graph.

Antagonistic activity

The concentrated aqueous extracts of different isolates were tested using the agar well diffusion method [24] for their antimicrobial activity against selected human pathogenic strains, including *Streptococcus pyogenes* MTCC 1925, *Candida albicans* MTCC183, *Escherichia coli* MTCC730, and *Staphylococcus aureus* MTCC96. The test organisms were spread on the surface of Müeller-Hinton Agar (MHA) (HiMedia) using a sterile swab stick as described previously [13]. Zones of inhibition were measured, and the mean value was obtained [25]. Control experiments were carried out under similar conditions using standard drugs: chloramphenicol (30 µg) for antibacterial activity and nystatin (50 µg) for antifungal activity.

Screening for NRPSs

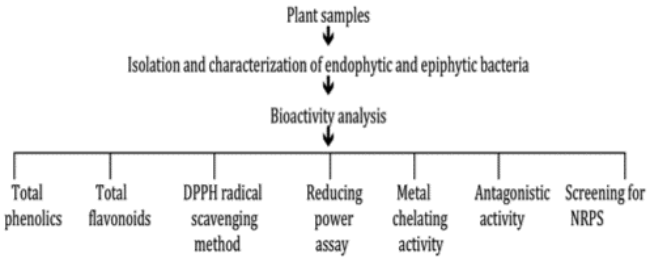
PCR amplification and sequencing of the NRPS gene was carried out in a 25 µL reaction mixture using primers MTF2 5'GCNGGYGGYGCNTAYGTNCC3' and MTR2 5'CCNCGDAYTTNACYTG3' [26] under the following conditions: template DNA denaturation at 94°C for 5 minutes followed by 30 cycles at 94°C for 10 seconds, annealing at 52°C for 30 seconds, and extension at 72°C for 30 seconds. The final step was carried out at 72°C for 5 minutes and then 4°C until infinity using PCR Gene Amp 9700 (Applied Biosystems). The DNA template was replaced with sterile water used as a negative control.

Statistical analysis

The experimental results of biological activity tests were expressed as mean ± standard deviation (SD) of three replicates. The results were processed using Microsoft Excel 2007 and Origin 6.0.

The methods are summarized in Figure 1.

Figure 1. A flowchart of the methods adopted in the study



Results

The results provided an understanding of the association of bacterial endophytes and epiphytes in the different ethnomedicinal plants (Table 1). The most frequently isolated endophytic bacteria was *Bacillus* sp., followed by *Pseudomonas* sp. Phytochemical studies revealed similarity in the amount of total phenolics in both endophytes and epiphytes, in the range of 10.5 ± 0.01 to 16.0 ± 0.005 and 8.1 ± 0.017 to 15.0 ± 0.015 milligrams of gallic acid equivalent per gram of extract, respectively

Table 1. Antioxidant activity of endophytic/epiphytic bacteria associated with various ethnomedicinal plants

Ethnomedicinal plants	Endophyte/ epiphyte	Isolate/GenBank accession no.	Antioxidant activity (IC_{50})		
			a	b	c
Rubia cordifolia (fruit bearing)	Endophyte	*Bacillus mycoides* M31 / JX298809	31.25 ± 0.01	> 20	> 60
	Epiphyte	*Citrobacter youngae* MEB5 / JX390623	> 50	> 20	> 60
		Raoultella ornithinolytica MEB11/ JX390624	50 ± 0.005	6.1 ± 0.019	> 60
Centella asiatica	Endophyte	*Serratia marcescens* cenA / JN613282	> 50	14.5 ± 0.01	2 ± 0.005
		Bacillus subtilis cenB / JN613283	27 ± 0.013	11 ± 0.005	2 ± 0.017
	Epiphyte	*Pantoea eucalypti* CEN7E / JN628292	> 50	12.5 ± 0.009	11.5 ± 0.005
Potentilla fulgens (flower bearing)	Endophyte	*Bacillus methylotrophicus* POTA / JQ236632	34 ± 0.014	7.3 ± 0.017	13.25 ± 0.015
	Epiphyte	*Stenotrophomonas maltophilia* POT5 / JQ281541	34 ± 0.004	9.9 ± 0.01	> 60
Acmella oleracea (flower bearing)	Endophyte	*Pseudomonas palleroniana* Y1 / JQ770187	> 50	> 20	3 ± 0.007
Aloe vera	Endophyte	*Herminiimonas saxobsidens* AA / JQ770186	> 50	> 20	11 ± 0.01
Zingiber montanum	Endophyte	*Pseudomonas baetica* ENIB7 / JQ236625	> 50	19.7 ± 0.01	11.5 ± 0.004
Houttuynia cordata	Endophyte	*Bacillus* sp. F21 / JX298807	20 ± 0.012	7.7 ± 0.005	> 60
	Epiphyte	*Bacillus thuringiensis* F41 / JX390622	37 ± 0.01	16.5 ± 0.014	> 60
		Curtobacterium citreum E4 / JN418873	> 50	14.2 ± 0.02	> 60
		Enterobacter sp. EP2a / JN653461	> 50	> 20	> 60
Litsea cubeba	Endophyte	*Paenibacillus uliginis* C22 / JN585959	36.5 ± 0.005	10.2 ± 0.005	1 ± 0.01
		Bacillus siamensis C53 / JN585960	32 ± 0.02	16.3 ± 0.01	2 ± 0.005
Pouzolzia hirta (flower bearing)	Epiphyte	*Comamonas* sp. POUX / JQ007727	> 50	> 20	53.75 ± 0.01
Drymarie cordata	Epiphyte	*Leclercia adecarboxylata* EPCC4 / JQ074052	> 50	12.4 ± 0.015	51.5 ± 0.009
		Exiguobacterium indicum EPCC8 / JQ074054	22 ± 0.015	4.5 ± 0.02	> 60
Standard		Ascorbic acid	0.0615 ± 0.007	0.32 ± 0.005	-
Standard		NaEDTA	-	-	0.11 ± 0.005

Antioxidant activity: [a] DPPH assay; [b] Reducing power; [c] Metal chelating

Figure 2. Comparison of total phenolic content between endophytic and epiphytic aqueous extracts at different concentration (the results are average of three readings ± standard error of mean)

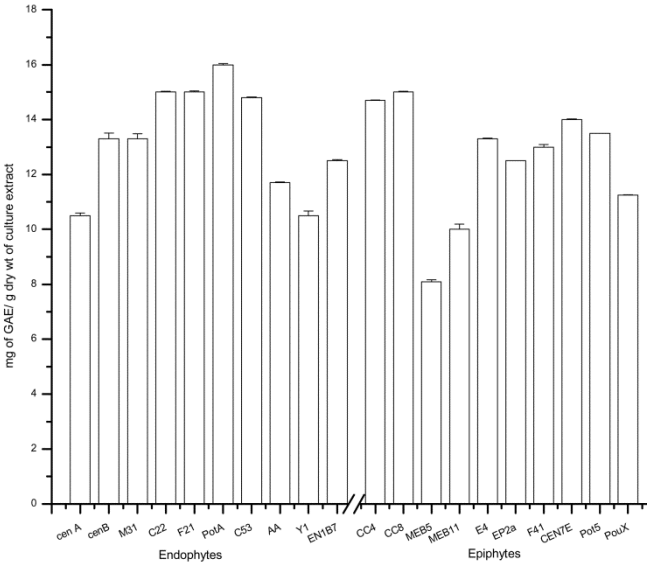
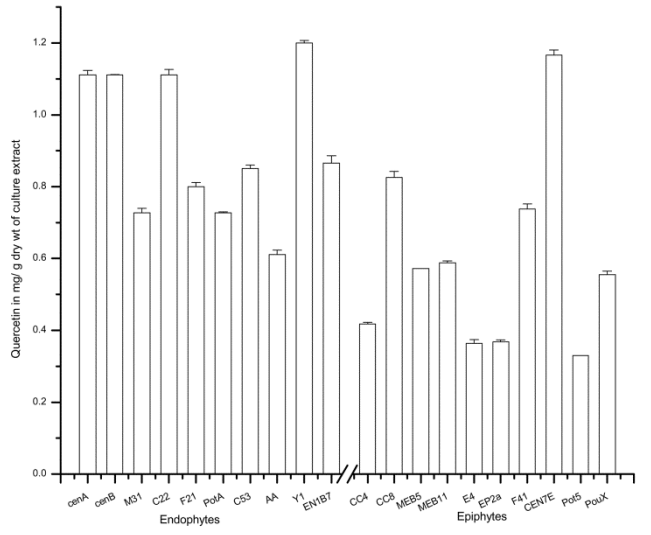


Figure 3. Comparison of total flavonoids between endophytic and epiphytic aqueous extracts at different concentration (the results are average of three readings ± standard error of mean)

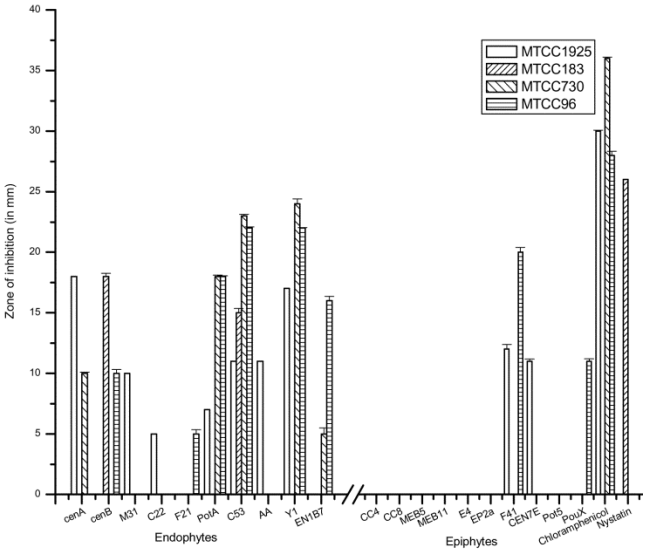


(Figure 2) ($R^2 = 0.954$, $y = 0.145$ x). A Differences in the amount of total flavonoids were observed among bacterial populations, with endophytes producing in the range of 0.611 ± 0.017 to 1.2 ± 0.004 and epiphytes in the range of 0.33 ± 0.005 to 0.825 ± 0.01 milligrams of quercetin equivalent per gram of culture extract, respectively (Figure 3) ($R^2 = 0.991$, $y = 0.065$ x).

Both endophytic and epiphytic bacterial extracts were able to reduce the stable radical DPPH to a yellow-colored diphenylpicryl hydrazine. The aqueous extract of *Bacillus* sp. F21 (associated with *Houttuynia cordata*) showed the highest radical scavenging activity, with IC_{50} of 20 ± 0.012 mg mL^{-1} (Table 1). The formation of the ferrozine-Fe^{2+} complex was found to be incomplete in the presence of endophytic and epiphytic extracts, indicating their capacity to chelate iron. Extract of *Paenibacillus uliginis* C22 showed the highest iron chelating activity, with IC_{50} of 1 ± 0.01 mg mL^{-1} (Table 1). The metal chelating capability of the isolates showed the lowest EC_{50} of 1 mg mL^{-1} compared to reducing power and DPPH radical scavenging activity (Table 1). Bacteria associated with *Centella asiatica*, *Potentilla fulgens*, and *Houttuynia cordata* showed better antioxidant and antagonistic activities compared to the isolates associated with the other ethnomedicinal plants (Table 2). The antagonistic activity of the culture extracts of endophytes was evident in the agar well diffusion assay (Figure 4). All (100%) of the endophytic isolates showed antagonistic activity, whereas only 20% of the epiphytic isolates showed antagonistic activity against the tested pathogens. Among the different endophytic isolates, *B. siamensis* C53 and *B. subtilis* cenB showed the highest antagonistic activity (Figure 4).

Amplification of seven plant-associated bacteria NRPS adenylation domain was confirmed via sequencing and BLASTX (translated) analysis. BLASTN (nucleotide) analysis of the PCR products did not reveal any significant matches to the

Figure 4. Comparison of antagonistic activity between endophytes and epiphytes against tested human pathogens (the results are average of three readings ± standard error of mean)



MTCC296: *Trichophyton rubrum*; MTCC183: *Candida albicans*; MTCC 730: *Escherichia coli*; MTCC96: *Staphylococcus aureus*

nucleotide database, indicating that the sequences were novel at this level. The GenBank accession numbers of the NRPS genes obtained are KF765939 through KF765945 for *Raoultella ornithinolytica* MEB11, *Serratia marcescens* cenA, *Comamonas testosteroni* POUX, *Bacillus methylotrophicus* POTA, *Bacillus siamensis* C53, *Bacillus subtilis* cenB, and *Bacillus mycoides* M31.

Discussion

This study presents a comprehensive assemblage of endophytic and epiphytic bacteria associated with plants of medicinal relevance and highlights a high level of diversity in the sampled niches. The study revealed the antioxidant and antagonistic activities of plant-associated endophytes and epiphytes. The antioxidant activity of the isolates is due to their redox properties, which play an important role in absorbing and neutralizing free radicals, quenching singlet and triplet oxygen, or decomposing peroxides [26-27]. Lipid peroxidation not only causes loss in food quality, but is also associated with diseases such as carcinogenesis, mutagenesis, ageing, and arteriosclerosis [28]. Tissue damage caused in such

Table 2. Activity and phytochemical parameters assessed for endophytic/epiphytic bacteria

Ethnomedicinal plants	Isolate	Activity and parameters assessed for endophytic/epiphytic bacteria								
		Antioxidant activity			Antagonistic activity				Phytochemicals	
		[a]	[b]	[c]	[d]	[e]	[f]	[g]	[h]	[i]
Rubia cordifolia	*Bacillus mycoides* M31	++	+	+	-	-	-	-	++	+
	Citrobacter youngae MEB5	+	+	+	-	-	-	-	+	+
	Raoultella ornithinolytica MEB11	+	+++	+	-	-	-	-	+	+
Centella asiatica	*Serratia marcescens* cenA	+	++	+++	-	-	+	-	+	+++
	Bacillus subtilis cenB	+++	++	+++	+	++	-	+	++	+++
	Pantoea eucalypti CEN7E	+++	++	++	-	-	-	-	++	+++
Potentilla fulgens	*Bacillus methylotrophicus* POTA	++	+++	++	-	-	++	++	+++	+
	Stenotrophomonas maltophilia POT5	++	+	+	-	-	-	++	++	+
Acmella oleracea	*Pseudomonas palleroniana* Y1	+	+	+++	-	-	+++	+++	+	+++
Aloe vera	*Herminiimonas saxobsidens* AA	+	+	+	-	-	-	-	+	+
Zingiber montanum	*Pseudomonas baetica* ENIB7	+	+	++	-	-	+	++	++	++
Houttuynia cordata	*Bacillus* sp. F21	+++	+++	+	-	-	-	+	+++	+
	Bacillus thuringiensis F41	+	++	+	-	-	-	++	++	+
	Curtobacterium citreum E4	+	++	+	-	-	-	-	++	+
	Enterobacter sp. EP2a	+	+	+	-	-	-	-	++	+
Litsea cubeba	*Paenibacillus uliginis* C22	++	++	+++	-	-	-	-	+++	+++
	Bacillus siamensis C53	++	++	+++	++	-	+++	+++	+++	++
Pouzolzia hirta	*Comamonas* sp. POUX	+	+	+	-	-	-	+	+	+
Drymarie cordata	*Leclercia adecarboxylata* EPCC4	+	++	+	++	-	-	-	+++	+
	Exiguobacterium indicum EPCC8	+++	+++	+	-	-	-	-	+++	++
Standard	Ascorbic acid	+++	+++	-	-	-	-	-	+++	-
Standard	Rutin	-	-	-	-	-	-	-	-	+++
Standard	NaEDTA	-	-	+++	-	-	-	-	-	-
Standard	Chloramphenicol (30 µg)	-	-	-	+++	-	+++	+++	-	-
Standard	Nystatin (50 µg)	-	-	-	-	+++	-	-	-	-

Antioxidant activity

[a] DPPH assay: + indicates IC_{50} > 50 mg mL^{-1}, ++ indicates IC_{50} = 32–40 mg mL^{-1}, +++ indicates IC_{50} < 32 mg mL^{-1}; [b] Reducing power: + indicates IC_{50} > 20 mg mL^{-1}, ++ indicates IC_{50} = 10–20 mg mL^{-1}, +++ indicates IC_{50} < 10 mg mL^{-1}; [c] Metal chelating: + indicates IC_{50} > 60 mg mL^{-1}, ++ indicates 5–60 mg mL^{-1}, +++ indicates IC_{50} < 5 mg mL^{-1}

Antagonistic activity

[d] MTCC1925, [e] MTCC183, [f] MTCC730, [g] MTCC96; + indicates inhibition zone < 10 mm, ++ indicates 10–20 mm and +++ indicates > 20 mm

Phytochemicals

[h] Total phenolics: + indicates < 12 mg, ++ indicates 12–14 mg, +++ indicates > 14 mg; [i] Total flavonoids: + indicates < 0.8 mg, ++ indicates 0.8–1.0 mg, +++ indicates > 1.0 mg

diseases reveals the deteriorative reaction of active oxygen and free radicals [29]. However, the antioxidant ability of the isolates may be used to treat the human body for oxidative damages caused by free radicals and active oxygen. Antioxidant activity is represented by the EC_{50} value, which is a widely used parameter to measure free-radical scavenging activity. A lower EC_{50} value indicates a higher antioxidant activity [30].

Phenolics or polyphenols and flavonoids are secondary plant metabolites that are ubiquitously present in plants and are observed among isolated plant-associated bacteria. The presence of such metabolites enhances the level of antioxidant [31-32] and antagonistic activities [33-34] of endophytic and epiphytic bacteria, making them good candidates for the isolation and characterization of bioactive compounds.

Endophytic bacteria showed significantly higher antagonistic activity against the tested pathogens compared to epiphytic bacteria. This may be the reason that these two micro-populations colonize different micro-environments on the host. Endophytes indicate a possibility of mimicking mechanisms to produce similar metabolites as their host plants. Among the epiphytic bacteria, *Exiguobacterium indicum* CC8 and *Pantoea eucalypti* CEN7E showed good antioxidant activities but relatively poor antagonistic activity, which may be the reason for their inability to enter the internal tissues.

The genetic screening of the NRPS gene increases the likelihood of isolating plant-associated bacteria, which biosynthesize novel pharmaceutical compounds independent of the host plant. In the present study, both endophytic and epiphytic bacteria revealed the presence of the NRPS gene. The isolates showing positive for the NRPS gene also indicated relatively higher antioxidant activity than others. Overall, the endophytic bacteria revealed bioactivity superior to that of the epiphytic bacteria. These findings suggest plant-associated bacterial endophytes as potential sources of natural compounds of therapeutic relevance that could replace their hosts in providing bio-natural products and prevent the exploitation of medicinal plants. Further research is required to optimize and identify compounds that aid in antioxidant and antagonistic properties of endophytic bacteria.

Conclusions

This study identified medicinal plants found to harbor endophytes and epiphytes with bioactive potential, which could be targeted in future investigations. The antioxidant and antimicrobial ability of the isolates could be due to the presence of phenolics, flavonoids, or NRPSs. The antioxidant ability of the isolates may be used to reduce the oxidative damage caused by free radicals and to treat diseases caused by lipid peroxidation. The antimicrobial ability and the presence of NRPSs among the isolates has revealed new antimicrobial agents and could be used in maintaining public health, as drug-resistant pathogens spread rapidly. The study holds significance in the light of the problem facing the future of endophyte biology and natural product discovery related to the rapid depletion of rainforests together with the disappearance of traditionally used medicinal plants, which are the greatest potential resource for acquiring novel microorganisms and their products.

Acknowledgements

SRJ acknowledges DBT, Govt of India and FMWN acknowledges UGC, Govt of India for financial assistance in the form of research project and fellowship(F. 17-113/08 (SA-I) dated 18 May 2012). Dr. CS Rao is thankfully acknowledged for his help in the collection and identification of plants.

References

1. Kusari S, Spiteller M (2011) Are we ready for industrial production of bioactive plant secondary metabolites utilizing endophytes? Nat Prod Rep 28: 1203-1207.
2. Hynniewta SR, Kumar Y (2008) Herbal remedies among the Khasi traditional healers and village folks in Meghalaya. Indian J Tradit Know 7: 581-586.
3. Aruoma OI, Spencer JPE, Rossi R, Aeschbach R, Khan A, Mahmood N, Munoz A, Murcia A, Butler J, Halliwell B (1996) An evaluation of the antioxidant and antiviral action of extracts of rosemary and provencal herbs. Food Chem Toxicol 34: 449-456.
4. McCarthy TL, Kerry JP, Kerry JF, Lynch PB, Buckley DJ (2001) Evaluation of the antioxidant potential of natural food/plant extracts as compared with synthetic antioxidants and vitamin E in raw and cooked pork patties. Meat Sci 57: 45-52.
5. Gu L, Weng X (2001) Antioxidant activity and components of *Salvia plebeia R. Br*. a Chinese herb. Food Chem 73: 299-305.
6. Stierle A, Strobel G, Stierle D (1993) Taxol and taxane production by *Taxomyces andreanae*, an endophytic fungus of pacific yew (*Taxus brevifolia*). Science 260: 214-216.
7. Strobel G, Daisy B, Castillo U, Harper J (2004) Natural products from endophytic microorganisms. J Nat Prod 67: 257-268.

8. Taylor JLS, Rabe T, McGaw LJ, Jäger AK, van Staden J (2001) Towards the scientific validation of traditional medicinal plants. Plant Growth Regul 34: 23-37.
9. Hallmann J, Quadt-Hallmann A, Mahaffee WF, Kloepper JW (1997) Bacterial endophytes in agricultural crops. Can J Microbiol 43: 895-914.
10. Sauer M, Lu P, Sangari R, Kennedy S, Polishook J, Bills G, An Z (2002) Estimating polyketide metabolic potential among non-sporulating fungal endophytes of Vaccinium macrocarpon. Mycol Res 106: 460-470.
11. Burns BP, Seifert A, Goh F, Pomati F, Jungblut AD, Serhat A, Neilan BA (2005) Genetic potential for secondary metabolite production in stromatolite communities. FEMS Microbiol Lett 243: 293-301.
12. Ehrenreich IM, Waterbury JB, Webb EA (2005) Distribution and diversity of natural product genes in marine and freshwater cyanobacterial cultures and genomes. Appl Environ Microbiol 71: 7401-7413.
13. Nongkhlaw FMW, Joshi SR (2014) Micrographical assessment of antifungal effect of endophytic bacteria. Proc Natl Acad Sci India Sect B Biol Sci doi 10.1007/s40011-014-0321-z.
14. Nongkhlaw FMW, Joshi SR (2014) Distribution pattern analysis of epiphytic bacteria on ethnomedicinal plant surfaces: A micrographical and molecular approach. J Microsc Ultra 2: 34-40.
15. Nongkhlaw FMW, Joshi SR (2014) Epiphytic and endophytic bacteria that promote growth of ethnomedicinal plants in the subtropical forests of Meghalaya, India. Rev Biol Trop 62: 1295-1308.
16. Heuer H, Krsek M, Baker P, Smalla K, Wellington E (1997) Analysis of actinomycete communities by specific amplification of genes encoding 16S rRNA and gel-electrophoretic separation in denaturing gradients. Appl Environ Microbiol 63: 3233-3241.
17. Das M, Royer TV, Leff LG (2007) Diversity of fungi, bacteria, and actinomycetes on leaves decomposing in a stream. Appl Environ Microbiol 73: 756-767.
18. Chun J, Lee JH, Jung Y, Kim M, Kim S, Kim BK, Lim YW (2007) EzTaxon: a web-based tool for the identification of prokaryotes based on 16S ribosomal RNA gene sequences. Int J Syst Evol Microbiol 57: 2259-2261.
19. Tamura K, Dudley J, Nei M, Kumar S (2007) MEGA4: Molecular evolutionary genetics analysis (MEGA) software version 4.0. Mol Biol Evol 24: 1596-1599.
20. Singleton VL, Orthofer R, Lamuela-Raventos RM (1999) Analysis of total phenols and other oxidation substrates and antioxidants by means of Folin-Ciocalteu reagent. Method Enzymol 299: 152-178.
21. Brand-Williams W, Cuvelier ME, Berset C (1995) Use of a free radical method to evaluate antioxidant activity. Food Sci Technol Lebensm Wiss 28: 25-30.
22. Oyaizu M (1986) Studies on product of browning reaction prepared from glucose amine. Jap J Nutr 44: 307-315.
23. Dinis TCP, Madeira VMC, Almeida LM (1994) Action of phenolic derivatives (acetoamino phen, salicylate and 5-aminosalycilate) as inhibitors of membrane lipid peroxidations and a peroxyl radical scavenger. Arch Bioch Bioph 27: 511-532.
24. Olurinola PF (1996) A laboratory manual of pharmaceutical microbiology. Abuja: National Institute for Pharmaceutical Research and Development, Nigeria pp.130
25. Mukherjee PK, Balsubramanian R, Saha K, Pal M, Saha BP (1995) Antibacterial efficiency of *Nelumbo nucifera* (Nymphaeaceae) rhizome extract. Indian Drugs 32: 274-276.
26. Neilan BA, Dittmann E, Rouhiainen L, Bass RA, Schaub V, Sivonen K, Börner T (1999) Nonribosomal peptide synthesis and toxigenicity of cyanobacteria. J Bacteriol 181: 4089-4097.
27. Osawa T (1994) Novel natural antioxidants for utilization in food and biological systems. In Uritani I, Garcia VV, Mendoza EM, editors. Postharvest biochemistry of plant food-materials in the tropics. Tokyo Japan Scientific Societies Press. 241-251.
28. Yagi K (1987) Lipid peroxides and human disease. Chem Phys Lipids 45: 337-341.
29. Halliwell B (1991) Reactive oxygen species in living systems: source, biochemistry, and role in human disease. Am J Med 9: 14-22.
30. Maisuthisakul P, Suttajit M, Pongsawatmanit R (2007) Assessment of phenolic content and free radical-scavenging capacity of some Thai indigenous plants. Food Chem 100: 1409-1418.
31. Rice-Evans C, Miller NJ, Paganga G (1996) Structure-antioxidant activity relationships of flavonoids and phenolic acids. Free Radic Biol Med 20: 933-956.
32. Velioglu YS, Mazza G, Gao L, Oomah BD (1998) Antioxidant activity and total phenolics in selected fruits, vegetables, and grain products. J Agr Food Chem 46: 4113-4117.
33. Sousa A, Ferreira IC, Calhelha R, Andrade PB, Valent P, Seabra R, Estevinho L, Bento A, Pereira JA (2006) Phenolics and antimicrobial activity of traditional stoned table olives 'alcaparra'. Bioorg Med Chem 14: 8533-8538.
34. Pereira JA, Oliveira I, Sousa A, Valento P, Andrade PB, Ferreira IC, Ferreres F, Bento A, Seabra R, Estevinho L (2007) Walnut (*Juglans regia L.*) leaves: phenolic compounds, antimicrobial activity and antioxidant potential of different cultivars. Food Chem Toxicol 45: 2287-2295.

Corresponding author
Santa Ram Joshi
Microbiology Laboratory, Department of Biotechnology and Bioinformatics
North-Eastern Hill University, Shillong
793022, India
Phone: +91 3642722405

Conflict of interests: No conflict of interests is declared.

Supplementary Items

Supplementary Figure 1. Antagonistic activity using agar well diffusion method against the tested human pathogens. A: MTCC183, B: MTCC1925, C: MTCC96, and D: MTCC730 were observed by some bacterial extracts.