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ORIGINAL RESEARCH PAPER

Biotechnological potential of endophytic actinomycetes associated with Asteraceae plants: isolation, biodiversity and bioactivities

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Abstract Endophytic actinomycetes from five Asteraceae plants were isolated and evaluated for their bioactivities. From *Parthenium hysterophorus*, *Ageratum conyzoides*, *Sonchus oleraceus*, *Sonchus asper* and *Hieracium canadense*, 42, 45, 90, 3, and 2 isolates, respectively, were obtained. Of the isolates, 86 (47.2 %) showed antimicrobial activity. Majority of the isolates were recovered from the roots ($n = 127$, 69.7 %). The dominant genus was *Streptomyces* ($n = 96$, 52.7 %), while *Amycolatopsis*, *Pseudonocardia*, *Nocardia* and *Micromonospora* were also recovered. Overall, 36 of the 86 isolates were significantly bioactivity while 18 (20.9 %) showed strong bioactivity. In total, 52.1 and 66.6 % showed potent cytotoxicity and antioxidant activities. The LC_{50} for 15 strains was <20 μg/ml. Compared to the ascorbate standard (EC_{50} 0.34 μg/ml), all isolates gave impressive results with notable EC_{50} values of 0.65, 0.67, 0.74 and 0.79 μg/ml.

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Introduction

With the continuing search for novel natural products, it has become evident that the rate of re-isolation of known compounds has increased; therefore new microbial habitats need to be examined. One relatively overlooked but biologically important niche is the inner tissues of higher plants (Qin et al. 2009). All plants retain a microbiota (Bascom-Slack et al. 2009) and organisms that establish an endosymbiotic relationship with plants are defined as endophytes. Actinomycetes are saprophytes well known for producing a wide range of secondary metabolites. However, very little information is available to us regarding the biodiversity, tissue distribution, and biosynthetic potential of endophytic actinomycetes residing in wild, native plants (Janso and Carter 2010).

The Asteraceae family includes some of the most valued and oldest medicinal plants (Jeschke et al. 2009). *Ageratum conyzoides* L. is widely distributed all over Pakistan (Singh et al. 2012) and locally known as 'Neeli Booti' (Zafar et al. 2006). It has been used in traditional medicine for the treatment of innumerous disorders. However, no report of the in vitro anticancer

activity of the plant has been evaluated or published (Adebayo et al. 2010).

Sonchus asper is another important herbal plant used as an alternative in the treatment of various diseases. It is a rich source of phenolic compounds, flavonoids, carotenoids, sesquiterpene lactones glycosides, ascorbic acid, and ω-3 fatty acids. (Khan et al. 2010).

The aim of the study was to explore the diversity and analyze the endophytic actinomycetes isolated from parts of medicinal plants belonging to Asteraceae family for specific biological activities. Actinomycetes were isolated from the medicinal plants from several of its natural habitats in Pakistan. Keeping in mind there is a lack of data on the antioxidant and cytotoxicity studies of endophytic actinomycetes from these plants therefore this study provides new and useful information.

Materials and methods

Plant material and collection site

Healthy plants of *Parthenium hysterophorus* were collected from the agricultural lands at the department of microbiology and molecular genetics (MMG) located at the University of the Punjab, Lahore. *Ageratum conyzoides, Sonchus oleraceus*, *Sonchus asper* and *Hieracium canadense* were collected from shady, moist areas around the department (31.49°N, 74.29°E, elevation ~210 m).

Sample treatment and isolation

Each plant sample was thoroughly washed in running tap water to remove soil particles and adhered epiphytes. After drying in sterile conditions, tissues surfaces were divided into 0.5 cm segments and surface sterilized by the five step sequential process as described by Tanvir et al. (2013) and plated.

Test organisms and antibiosis assay

For detection of antimicrobial activity, *Staphylococcus aureus* (ATCC 25923), *Escherichia coli* (ATCC 25922), *Klebsiella pneumoniae* (ATCC 706003), Methicillin-resistant *Staphylococcus aureus* (MRSA), *Pseudomonas*, *Enterobacter*, *Bacillus*, *Candida tropicalis*, and *Chlorella vulgaris* were used. The minimum inhibitory concentration (MIC) of the extracts obtained from endophytic actinomycetes were tested by broth dilution method as described by Andrews (2001). Extraction of fermentation broth of endophytic strains was done with ethyl acetate which was dried on a rotary evaporator to yield crude extracts.

Brine shrimp lethality test

For brine shrimp cytotoxicity assay, the extracts were dried and dissolved in DMSO to obtain the desired concentrations. The final concentration of DMSO in the assay volume was kept at 1 % (v/v) to prevent possible false effects originating from DMSO toxicity. Actinomycin D was used a positive control and artificial sea water was used as a negative control for the assay. Toxicity of the crude extracts was tested at 10, 50, 100, 150, 500, 1,000 µg/ml in seawater containing 1 % (v/v) DMSO. The mortality rate M was calculated using the formula:

$$M = \left(\frac{(A - B - N)}{(G - N)}\right) \times 100$$

where M is the percent of the dead nauplii after 24 h; A is the number of the dead nauplii after 24 h; B is the average number of the dead nauplii in the blind samples after 24 h; *N* is the number of dead nauplii before starting the test; G is the total number of nauplii.

Cytotoxicity was considered significant if the LC_{50} value was less than 20–30 µg/ml (Geran et al. 1972).

Free radical scavenging activity assay using diphenylpicrylhydrazyl (DPPH)

Antioxidant activity was tested according to the procedure by Pajero et al. (2000) with slight modifications. Antioxidant activity of the crude extracts was tested at 100, 500, 1,000, 5,000 and 10,000 µg DPPH/ml dissolved in methanol. An ascorbate standard was used as a positive control and DPPH stock was used as a negative control. The DPPH radical scavenging activity of the sample was calculated as:

$$\left[Abs_{control} - Abs_{sample}\right] / Ab_{sample} \times 100$$

The antioxidant activity was expressed in terms of EC_{50}, which was defined as the dilution of the crude

extract needed to decrease initial DPPH chemiluminescence intensity by 50 %.

Statistical analysis

All experiments were performed in triplicates and results are presented as mean values. The calculations for LC_{50} were done through probit regression analysis and the determination of EC_{50} was done through dose response curve. Analysis was done using statistical package SPSS version 20.0 (SPSS, USA).

Results and discussion

A variety of plant species have been explored for the isolation of endophytic actinobacteria ranging from different woody tree species, ferns, club mosses even crop plants, like rice, wheat, carrots, potato, tomato and citrus (Qin et al. 2011a, b). However, there are few reports on bioactive compounds from actinomycetes of such origin (Radhakrishnan et al. 2010). This situation encouraged us to explore and investigate the endophytic actinomycetes community of medicinal plants in family Asteraceae. Therefore this study is one of its kind to describe the biodiversity and bioactivities of endophytic actinomycetes associated with the plants described.

Our study revealed that the tissues of these plants harbor a variety of endophytic actinomycetes. A total of 182 isolates were obtained from the stem, root and leaf of the plants. Most isolates were identified as *Streptomyces* spp. after cultural characterization. Of these 182 isolates, a majority were recorded from the roots ($n = 127$, 69.7 %) then shoots ($n = 32$, 17.5 %) and leaves ($n = 23$, 12.6 %). The *Streptomyces* genus was dominant ($n = 96$, 52.7 % of all isolates), followed by *Micromonospora* ($n = 3$, 1.5 %), *Amycolatopsis* ($n = 2$, 1 %) and *Pseudonocardia*, *Nocardia* ($n = 1$, 0.5 %) (Table S1).

Among the isolates, various subgroups were identified and among *Streptomyces* spp., the subgroup *Streptomyces rochei* ($n = 3$, 7.1 %) was also isolated. Apart from our previous study (Tanvir et al. 2013), no other study has reported its isolation from Asteraceae plants. The roots of Asteraceae plants harbored besides *Streptomyces* spp., other genera like *Amycolatopsis*, *Nocardia*, *Pseudonocardia,* and *Micromonospora*. There are reports of isolation of *Micromonospora tulbaghia* sp. nov., from the leaves of *Tulbaghia violacea* (Kirby and Meyers 2010), and *Pseudonocardia sichuanensis* sp. nov., from the root of *Jatropha curcas* L. (Qin et al. 2011a, b).

Considering these findings, it is apparent that a selected group of actinomycetes namely *Streptomyces*, *Amycolatopsis*, *Nocardia*, *Pseudonocardia,* and *Micromonospora* are able to form endophytic associations with a wide range of plant hosts.

Eighty-six of the isolates (47.2 %) showed growth inhibitory activity against pathogenic strains of microorganisms and among them 36 (41.8 %) isolates had significant broad spectrum antimicrobial activity while 25 (29.0 %) showed strong bioactivity. The isolates RT 6, 13, 18, 36, 49, 56, 67 and AGRS8, SORS80 (all isolated from the roots) were further characterized by 16S rRNA sequencing (Table S2).

Streptomyces strains, such as RT 46, 49, 54 and 63, exhibited major MIC values ranging from 4–32 μg/ml against all nosocomial pathogens (Table 1). One of the isolate AGRS8 with 99 % homology to *Streptomyces albovinaceus* showed the most significant MIC value of 2 μg/ml against all multidrug resistant isolates whereas another strain AGLS2 identified as

Table 1 MIC of the endophytic actinomycetes isolates against nosocomial pathogens

General	Isolates	X4	M9	S2	E4
Streptomyces spp.	RT-37	0	32	32	32
	RT-40	32	0	0	0
	RT-41	32	0	0	0
	RT-43	32	0	0	0
	RT-44	0	32	0	0
	RT-46	32	8	32	8
	RT-49	32	8	0	0
	RT-54	8	32	0	0
	RT-55	4	0	0	0
	RT-63	32	32	8	8
	RT-64	32	32	0	8
	AGRS8	4	4	4	8
Pseudonocardia spp.	AGLS2	8	8	8	8
Amycolatopsis spp.	SORS80	8	8	8	8

X4 Pseudomonas spp., *M9 Enterobacter* spp., *S2 Enterobacter* spp., *E4 Enterobacter* spp.

MIC was determined for a range of concentrations from 0 to 256 μg/ml

0 no inhibition at any range

Table 2 Minimal inhibitory concentration (MIC) for determining the antimicrobial, antifungal, and antialgal potential of the endophytic actinomycetes from Asteraceae plants

Genera	Isolates	Tissue source	*B. subtilis*	*S. aureus ATCC 25923*	*E. coli ATCC 25922*	*K. pneumonia ATCC 706003*	*MRSA*	*C. tropicalis*	*C. vulgaris*
Streptomyces spp.	RT-6	PH roots	8	16	16	0	32	8	0
	RT-13	PH shoots	4	8	8	16	8	8	8
	RT-18	PH shoots	8	16	16	0	32	16	0
	RT-36	PH roots	8	16	16	0	32	32	0
	RT-46	PH roots	0	16	16	0	16	32	0
	RT-49	PH roots	16	0	0	0	0	0	0
	RT-50	PH roots	8	8	16	0	8	8	0
	RT-53	PH roots	8	16	16	32	16	8	0
	RT-56	PH roots	8	16	16	32	32	8	0
	RT-57	PH roots	0	16	16	32	32	32	32
	RT-59	PH roots	8	16	16	–	32	32	32
	RT-60	PH roots	8	16	16	32	32	16	16
	RT-63	PH roots	16	0	0	0	0	0	0
	RT-64	PH roots	16	0	0	0	0	32	0
	RT-67	PH roots	8	16	16	32	32	16	16
	AGRS8	AC roots	2	2	2	4	2	2	4
Pseudonocardia spp.	AGLS2	AC leaves	4	8	0	0	4	4	8
Amycolatopsis spp.	SORS80	SO roots	4	8	8	8	8	8	0

MIC was determined for a range of concentrations from 0 to 256 μg/ml

B. subtilis Bacillus subtilis, *S. aureus Staphylococcus aureus*, *E. coli Escherichia coli*, *K. pneumoniae Klebsiella pneumoniae*, *MRSA* methicillin resistant *S. aureus*, *C. tropicalis Candida tropicalis*, *C. vulgaris Chlorella vulgaris*, *PH Parthenium hysterophorus*, *AC Ageratum conyzoides*, *SO Sonchus oleraceus*, *0* no inhibition at any range

Pseudonocardia carboxydivorans and SORS80 identified as *Amycolatopsis japonicum* also showed 4–8 μg/ml MIC values against all test organisms as shown in Table 2.

Exposure of brine shrimp to extracts also revealed interesting results: in case of strains RT 13, 50, 59 and 67 the percentage mortality after 24 h ranged from 96.6 % at 100 μg/ml to 100 % at 500, 1 and 5 mg/ml. A high mortality rate was also observed in the isolates RT 18, 36 and 53 which gave 100 % mortality at 100 μg/ml which was the lowest concentration for these extracts tested (Fig. S1). The LC_{50} value for the extracts was very potent at 20 μg/ml, which is considered extremely toxic when compared to values provided by Geran et al. (1972). Overall, 15 of the 33 isolates selected for cytotoxicity showed an extremely potent cytotoxicity value of LC_{50} 10 μg/ml whereas two isolates showed LC_{50} values between 23 and 50 μg/ml which is also very toxic (Table 3).

It is evident from our study that the endophytic actinomycetes residing in the plants of Asteraceae family have significant capability of producing cytotoxic compounds. Previous reports also support our results with novel antibiotics such as coronamycin from endophytic *Streptomyces* sp. with cytotoxicity of IC_{50} 5–10 μg ml (Ezra et al. 2004). It establishes the fact that endophytes are excellent source for the isolation of novel cytotoxic compounds.

In light of the previous reports regarding the antioxidant activity for the *Streptomyces*, the activity for endophytic actinomycetes was quantified using the DPPH free-radical scavenging activity assay. As compared to the ascorbate standard for which the EC_{50} was 0.34 μg/ml, the EC_{50} of all of the actinomycetes strains gave impressive results. The EC_{50} of the isolates SORS64b, SORS124, AGRS16, AGLS2, AGRS19, gave EC_{50} values of 0.55, 0.57, 0.62, 0.65, and 0.67 μg/ml, respectively, with 0.552 and 0.567 μg/ml

Table 3 Cytotoxicity results of endophytic actinomycetes from *Parthenium hysterophorus*, *Ageratum conyzoides, Sonchus oleraceus and Hieracium canadense* after 24 h

Strain no.	% Mortality under the concentration studied (μg/ml)						LC_{50} (μg/ml^{-1})
	10	50	100	150	500	1,000	
DMSO (1 % in sea water)	0	0	0	0	0	0	0
AGRS8	42.8	61.6	72.1	89.2	100	100	10
AGRS16	65.8	69.9	77.9	80.7	83.9	84.6	23
AGRS19	79.9	86.4	90	93.3	93.3	93.3	10
AGSS1	47.4	55.2	72.3	72.3	79.5	79.9	50
SORS24	54	59.6	65	67.8	82.7	100	10
CR3	60.7	64.2	70	73	73	92.5	10
RT-6	–	–	50	–	60	100	10
RT-13	–	–	96.6	–	100	100	10
RT-18	–	–	100	–	100	100	10
RT-36	–	–	100	–	100	100	10
RT-50	–	–	96.5	–	100	100	10
RT-53	–	–	100	–	100	100	10
RT-56	–	–	70	–	73.3	100	10
RT-57	–	–	58.6	–	100	100	10
RT-59	–	–	73.3	–	93.3	96.6	10
RT-60	–	–	66.6	–	80	100	10
RT-67	–	–	96.5	–	96.6	100	10

Dried eggs of *Artemia salina* (0.5 g) were hatched in a tank containing 500 ml artificial sea water (Kester et al. 1967). The tank was well aerated with the help of an air pump; light source of 1,000–4,000 lux was also provided. The nauplii hatched within 24–36 h at 30–35 °C. Briefly, with the help of a pipette, 15 shrimp nauplii were collected and transferred to a deep well microtitre plate filled with 0.2 ml of artificial sea water. The numbers of dead nauplii were counted as value *N*, and then 100 μl of each extract was added. The plates were incubated at room temperature in the dark for 24 h. A parallel series of tests with the positive control Actinomycin D of 100 % mortality rate (10 μg/ml) were carried out and the negative control was also included. After 24 h, surviving nauplii were counted using a stereo microscope. Then the surviving nauplii were killed by addition of 0.5 ml methanol so that the total number G could be calculated

Values are means of triplicate studies; $n = 15$ (number of nauplii used); Score for LC_{50}: Highly toxic <20 μg/ml, toxic up to 1,000 μg/ml and non toxic >1,000 μg/ml; LC_{50} was calculated through Probit; (–) not analyzed

Table 4 Total antioxidant activity of different concentrations of endophytic actinomycetes

Strain no.	DPPH scavenging activity (%) under the concentration studied (μg/ml)					EC_{50} (μg/ml)
	100	500	1,000	5,000	10,000	
Ascorbate standard	100	100	100	100	100	0.338
AGRS16	57.2	73.7	77.2	81.7	96.2	0.621
AGRS19	52.0	65.3	73.5	91.2	97.0	0.695
AGRS49	58.1	59.5	70.7	95.5	99.1	0.718
AGSS1	37.8	41.4	49.0	51.0	72.8	0.751
AGLS2	40.5	74.1	76.0	76.7	98.3	0.670
SORS64b	28.3	37.1	38.7	41.4	43.7	0.552
SORS95	16.2	17.5	27.4	35.8	39	0.797
SORS124	21.7	38.4	40	42.1	43.7	0.567
RT-18	26.8	33.3	48.7	50.7	53.4	0.654
RT-50	10.5	33.2	70.7	–	–	0.740
RT-56	20.4	69.1	89.7	–	–	0.790
RT-67	13.3	30.8	62.2	66.7	93.1	0.665

Fresh 1 mM stock of DPPH was prepared in methanol and its OD was set at 0.9 ± 2 at 517 nm. Then reaction mixture was prepared in a 96 well microtitre plate containing 50 μl of extract and 150 μl of the stock solution of DPPH and incubated for 30 min at room temperature. After incubation, the quenching at an absorbance of 517 nm was measured by ELISA plate reader

Values are means of triplicate studies; (–) not determined

being the highest EC_{50} value (Table 4). The remaining isolates also gave good results with EC_{50} value of 0.7 ± 2 μg/ml when compared to the ascorbate standard. According to our study, the antioxidant activity of the crude extracts increased with the increase in concentration which is similar to the results of Kekuda et al. (2010) who reported that with higher concentration the scavenging activity of the *Streptomyces* was also increased.

Summary

This study suggests that the Asteraceae plant family harbors endophytic actinomycetes that produce a wide range of novel natural products with pharmaceutical potential. As such to the best of our knowledge, there is a lack of data on exploring the diversity of endophytic actinomycetes residing in the plant tissue of Asteraceae plant family, therefore this study not only describes an untapped environment but also highlights the potential usage of these isolates in biotechnological, medical and pharmaceutical applications.

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Conflict of interest The authors declare they have no conflict of interest.

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