



## Full Length Research Article

Advancements in Life Sciences – International Quarterly Journal of Biological Sciences

## ARTICLE INFO

Open Access



Date Received:

27/02/2022;

Date Revised:

21/03/2023;

Date Published Online:

30/06/2023;

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## How to Cite:

Hassan A, Ponomariva LV, Thorson JS, Shaaban K, Sajid I (2023). Bioactivity and Metabolomics Profiling of Endophytic Actinobacteria Isolated from Roots of the Medicinal Plants Dominant in South Asian Region. Adv. Life Sci. 10(2): 200-209.

## Keywords:

Endophytic Actinobacteria; Metabolomic Profiling; Natural Products; Antimicrobial Compounds; Anticancer Compounds

# Bioactivity and Metabolomics Profiling of Endophytic Actinobacteria Isolated from Roots of the Medicinal Plants Dominant in South Asian Region

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## Abstract

**Background:** Plant-derived endophytic actinobacteria are the center of attention due to their capacity to produce diverse antimicrobial and anticancer compounds and their metabolites influence plant growth.

**Methods:** In this study, 40 endophytic actinobacteria strains were isolated from the roots of eight medicinal plants used as folk medicine in South Asian region. The isolates were characterized morphologically, biochemically and physiologically and the genus level identification of the selected strains was done by 16SrRNA gene sequencing. In small scale cultivation (50ml broth), the isolates were grown in A-medium to prepare the crude extracts. These crude extracts were subsequently evaluated for their antimicrobial, anticancer and antioxidant activity and the metabolomics profile of each of the extract was determined by TLC and HPLC-UV/MS.

**Results:** The taxonomic studies showed that the isolates belong to the group actinobacteria based on their morphological and physiological characteristics and the 16SrRNA gene sequencing of the selected strains identified the genera including *Streptomyces*, *Micromonospora* and *Nocardia*. Cumulatively, 53% of extracts exhibited anti-Gram-(+) activity, 47% exhibited anti-Gram-(-) activity, 32% exhibited antifungal activity and 30% were cytotoxic to PC3 and A549 cancer cell lines and most of the extracts have shown antioxidant activity greater than 50%. The metabolomics analysis predicted the presence of an array of low molecular weight metabolites and indicated the promising isolates in collection for further studies for novel bioactive metabolite isolation and structure elucidation.

**Conclusion:** Overall the study provides an overview of the endophytic actinobacteria residing in the roots of the selected medicinal plants prevalent in south Asian region and their potential to produce the medicinally and biotechnologically useful compounds.



## Introduction

Plant derived bioactive compounds and their metabolic byproducts, or the secondary metabolites produced by the residential endophytic microbes (microbes living inside the plant tissues) have been the rich source of bioactive metabolites. The root exudates secreted by the plants attract microbes from their surroundings [1]. The endophytic microbial flora benefit plants with respect to ecological and evolutionary perspective [2]. Their mutualistic association influences the fitness of the plants by various mechanisms, including the induction of systemic resistance [3], and improves the plant health [4]. Secondary metabolites produced by endophytic bacteria also contribute to the heterogeneity of the phytochemical profile of the plants [5]. The soil harbors enormous diversity of various microbial groups, among them actinobacteria are the most abundant soil dwelling bacteria and are present in almost all types of soils. The association of the actinobacteria with roots is common as compared to other plant parts due to the close proximity of the roots with soil microbes (rhizospheric soil population) [6] and due to the chemical cues secreted by plants [7]. The increasing focus on the screening of actinobacteria from diverse and unusual environments has made them attractive targets for medicinally useful compounds. For example, endophytic actinobacteria from mangrove plants have been reported to produce 84 new compounds including salinosporamides, xiamycin and novel indolocarbazoles [8]. Xu et al. [9] hypothesized endophytic actinobacteria to provide a greater percentage of novel metabolites and revealed 73 novel compounds from such sources. Among the endophytic actinobacteria, the genus *Streptomyces* accounts for the abundant genus in plant tissues followed by various other genera [10]. The endophytic *Glycomyces* and *Streptomyces* have proved to be the producer of bioactive compounds against penicillin resistant *S. aureus* [11]. The plant growth promoting compounds produced by the endophytic actinobacteria can enhance the growth of host plant [12]. In 2002, Igarashi et al. [13] reported 398 endophytic actinomycetes having antagonistic activity against phytopathogens. The 2 new novobiocin antibiotics were obtained from the endophytic *Streptomyces* isolated from the *Aucuba japonica* [14]. Two novel butyrolactone antibiotics (cedarmycin A and B) were derived from the endophytic *Streptomyces* isolated from *Cryptomeria japonica* [15]. Alnumycin, a new naphthoquinone antibiotic was reported from the endophytic *Streptomyces* isolated from the *Alnus glutinosa* [16]. Endophytic actinobacteria not only produce bioactive secondary metabolites but also show positive effect on plant growth.

The isolates from chickpeas have shown not only positive effect on its growth but also protected the plant from pathogen and produced industrially important enzymes (chitinase, cellulase and protease) [17]. Similarly, the endophytic strain *Jishengella endophytica* have been reported to produce the 1-hydroxy-Beta-carboline against influenza virus type A subtype H1N1 [18].

In this study, the root associated endophytic actinobacteria were isolated from 8 selected medicinal plants. A comprehensive identification strategy including morphological, physiological, biochemical and genomic characterization of the isolates was adopted. The crude extracts of the isolated strains exhibited promising antimicrobial activity against the bacterial test strains, also high cytotoxicity/anticancer activity against the human cancerous cell lines and significant antioxidant activity was observed in *in-vitro* assays. Similarly, the extracts exhibited impressive metabolomic profiles and presence of low molecular weight compounds by TLC HPLC-UV/MS analysis. Based on the bioactivity and chemical profiling, 7 isolates were prioritized including PU-Aml3, PU-LG3, PU-Aki2, PU-Aki3, PU-Aki4, PU-Kach5 and PU-Kach15 for future scale-up fermentation, compounds purification and structure elucidation.

## Methods

### Sample Collection

The root samples were collected in sterile plastic bags from various locations in the botanical garden and adjoining areas at the campus of University of the Punjab, Lahore, Pakistan and were transferred to the laboratory for further processing. The sampled plants included *Cassia fistula*, *Phyllanthus emblica*, *Bauhinia variegata*, *Cassia occidentalis*, *Cymbopogon*, *Ocimum tenuiflorum*, *Moringa oleifera* and *Tamarindus indica*.

### Surface Sterilization and Selective Isolation of the Endophytic Actinobacteria

The root samples of plants were surface sterilized using chemical sterilant of different concentrations with a slight modification to the original surface sterilization protocol by Cao *et al.* [19]. The collected roots were thoroughly washed with tap water and then sequentially sterilized by i) 70% ethanol for 5 min and rinsed with sterile water ii) 0.1% sodium hypochlorite solution for 10min and rinsed with sterile water and iii) 70% ethanol for 5 min, iv) rinsed (x4) with sterile water. The surface sterilized roots were fragmented into small pieces of 2-3cm in size using sterilized scalpel and the fragments were placed slightly embedded on actinomycetes isolation agar (Difco), starch-casein-KNO<sub>3</sub> agar and raffinose-histidine agar. The efficacy of the surface sterilization procedure was evaluated by

negative control media plate which was inoculated with 60 µl of last wash of water used in sterilization protocol and incubated at 28°C for 3-4 weeks. The colonies of actinobacteria that appeared on the isolation agar were further sub-cultured on A-media. The preliminary phenotypic characterization was done by following the guidelines as mentioned in Bergey's manual of systematic bacteriology Volume II for initial identification of the isolates [20].

#### Taxonomic Identification of the Endophytic Actinobacteria

The endophytic actinobacteria retrieved from the roots embedded on the agar were distinguished from the fungus by visualizing the plates under stereomicroscope. The phenotypic identification of isolates included macroscopic characterization (color of substrate and aerial mycelium, pigment production and texture of the colonies) and microscopic examination included gram staining, while the biochemical characterization was done by determining, utilization of various carbon and nitrogen sources, melanin production, esculin hydrolysis [21,22]. The production of amylase, pectinase and cellulase enzymes was determined following the protocols of Minotto et al. [23]. The optimal conditions for the growth of endophytic actinobacteria were determined under a range of temperature (28°C, 37°C and 45°C) and pH conditions [7-9, 11]. The indole production was analyzed by the protocol of Yandigeri et al. [24], and phosphate solubilization activity was determined on Pikovskaya medium [25]. The 16S rRNA gene sequences from forward and reverse primers were assembled and the resultant contigs were analyzed for similarity to the 16S rRNA sequences in NCBI. The sequences were submitted to GenBank for accession numbers of the gene. A phylogenetic tree of all the isolates was constructed by neighbor joining method at 1000 bootstrap value using Mega software (version 6.06).

#### Laboratory Scale Cultivation and Extraction of the Endophytic Actinobacteria

The crude extracts of the pure cultures were prepared by cultivating each isolate in A-medium. The strains were inoculated in 50ml of A-media in 250ml flasks and the flasks were incubated on rotary shaker for 10 days at 28°C at 210 rpm. After 10 days the cultures were harvested, amberlite XAD-16 (4% w/v) was added in the culture flasks, which were kept on shaker for 4 hours under same incubation conditions. The flasks were removed from the shaker, and the mixture was transferred to 50 ml vials and was centrifuged at 4 °C at 4000 rpm for 15 min. The supernatant was removed and the XAD resin was washed with demineralized water 2-3 times to remove the residual media components and salts *etc.* Then 20ml methanol was

added in each vial and the mixtures were vortexed and were kept at room temperature overnight. The tubes were centrifuged to separate the methanol from the XAD resin, and the methanolic extract was evaporated on speed vacuum and the dry extracts were obtained. The dilutions of the dried extracts were prepared in methanol and in DMSO, which were used for biological activities (antimicrobial activity, cytotoxicity/antitumor activity) and chemical profiling by TLC and HPLC-UV/MS.

#### Determination of Antibacterial, Antifungal Activity and Cytotoxicity Assays

The antibacterial activity was determined against ATCC panel of test organisms while antifungal activity was determined against *Saccharomyces cerevisiae* ATCC 204508, and cytotoxicity was determined against the cancer cell lines A549 (non-small cell lung) and PC3 (prostate) following the previously described protocols (Resazurin fluorescence assay) [26-29]. The positive control included kanamycin and ampicillin (*S. aureus*, *S. enterica* and *E. coli*), amphotericin B (*S. cerevisiae*), actinomycin D and/or H<sub>2</sub>O<sub>2</sub> (A549 and PC3).

#### Determination of DPPH (2,2-diphenyl-1-picrylhydrazyl-hydrate) Radical Scavenging Activity

The DPPH assay was performed to determine the antioxidant potential of the crude extracts in a microtiter plate. The crude extracts (10mg/ml) were dissolved in DMSO and the 0.04mM solution of DPPH radical solution in DMSO was prepared. The 100 µl crude extracts were dispensed in the wells of the microtiter plate and 100µl of DPPH radical solution was also added in each of the well, the microtiter plate was incubated at room temperature for 30 min in the dark. After incubation, the absorbance was measured at 517nm in spectrophotometer. The radical scavenging activity was calculated by the following formula:

$$\text{Percentage of inhibition} = \frac{\text{Absorbance control} - \text{Absorbance sample}}{\text{Absorbance control}} \times 100$$

The positive control was ascorbic acid while DMSO was used as the negative control.

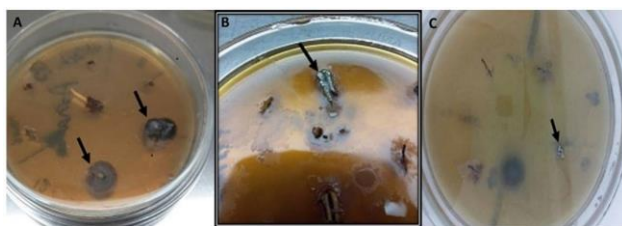
#### Thin Layer Chromatography (TLC) and HPLC-UV/MS analysis

The thin layer chromatography was performed by using glass TLC plates (TLC Silica gel 60 F<sub>254</sub>; 10 x 20cm). The extracts were spotted on the plate by a capillary tube and air dried. TLC plates were developed with the solvent system 7% methanol:dichloromethane visualized under UV at 254nm and 366nm and then stained with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> spraying reagent and heated. HPLC-UV/MS analyses was performed on the Agilent Infinity Lab LC/MSD mass spectrometer. The

samples were prepared by mixing the dried extracts in 100µl of the HPLC graded methanol.

## Results

The 40 endophytic actinobacterial strains were selectively isolated from the roots of 8 different medicinal plants. Among the three different selective isolation media used, the actinobacteria colonies mainly appeared on actinomycetes isolation agar (AIA) (Figure 1). The endophytic actinobacteria n=12 was recovered from *Cassia fistula*, while the lowest or minimum number (n=1) of isolates was recovered from the roots of the plant *Tamarindus indica*. The actinobacteria colonies appeared on the isolation agar were observed under stereomicroscope and in general the colonies with the compact, hard and embedded growth on the agar were selected. Among the 40 selected strains, a few strains seemed redundant due to the same morphology on the agar surface, however all the strains were preceded for identification and further investigation.

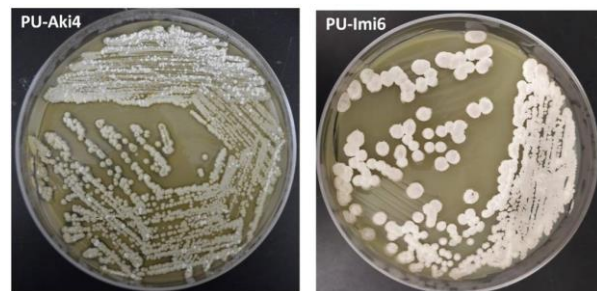


**Figure 1:** Isolation plates of the Actinomycetes isolation agar(AIA)arrows indicating the colonies of actinobacteria on the roots embedded in the agar A: AIA media plate for the plant *Cassia fistula* B: AIA media plate for the plant *Cassia occidentalis* C: AIA media plate for the plant *Tamarindus indica*.

### Morphological Characteristics of the Selected Endophytic Actinobacteria

The morphology of the isolates was observed and documented after 7-10 days growth at 28 °C on the GYM agar. The growth pattern of most of the isolates varies, as some isolates were slow grower and growth appeared on the media after 8-10 days while some colonies appeared within 5 days of incubation (Figure. 2).The strains produced a variety of colored rounded to irregular colonies ranging in various size, with convex elevation, hard texture and dry or powder like aerial spores. The gram staining showed the typical gram-positive filamentous pattern in the form of chain while observed on the glass slide at 100X using oil immersion. Such as, among the strains originated from *Cassia occidentalis*, the strain PU-Aki2, produced off white growth on A- media with embedded colonies, the strain PU-Aki3 produced creamy off-white growth, similarly the strain PU-Aki4 produced off white regular colonies. The strains isolated from the plant *Bauhinia variegata* exhibited characteristic actinobacteria like

features such as, the strain PU-Kach5 and the strain PU-Kach15 produced off-white growth, regular colonies. The strain isolated from *Phyllanthus emblica* PU-Aml3 produced creamy greenish slightly filamentous colonies. The isolate of *Cymbopogon* (Lemongrass) produced creamy whitish regular colonies when grown on A-media under the specified growth conditions.



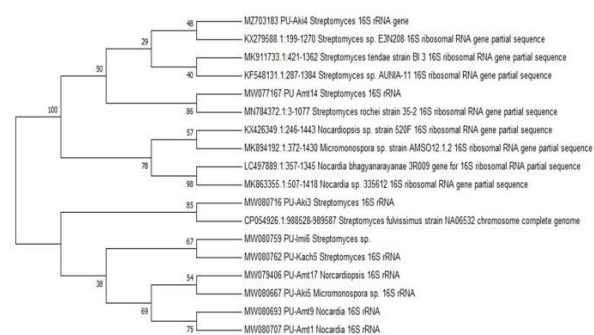
**Figure 2:** Endophytic actinobacteria isolates PU-Aki4 and PU-Imi6 cultured on the GYM media at 28 °C for 7-10 days. The less dense spore growth was observed on PU-Aki4 and light brown pigment was produced in the surrounding medium while PU-Imi6 showed dense spore growth and light pinkish coloured pigment was produced in the medium.

### Biochemical and Physiological Characteristics of the Selected Endophytic Actinobacteria

Among all the isolates 73% exhibited the production of melanin (tyrosine hydrolysis, while 80% of the isolates showed esculin hydrolysis. The utilization of various sugars as carbon source was investigated and almost all the strains showed flourishing growth on glucose, fructose, mannose, arabinose, galactose, and sucrose, while moderate and less growth was observed on rhamnose, raffinose and myo-inositol. All the isolates utilized organic acid (sodium malonate) and turned the media from greenish to blue. The enzymatic hydrolysis was positive for all isolates, almost all the strains exhibited the ability to produce cellulase, pectinase and amylase enzymes. In hemolysis test all the isolates showed transparent zones around the colonies while grown on bloods supplemented media. The whole cell analysis for DAP (Diaminopimelic acid) and carbohydrates revealed the presence of both isomers *meso* and L-DAP and smear of amino acid and sugars were observed on the stained TLC based on colors. The indole acetic acid (IAA) production was exhibited by all the strains as indicated by the appearance of pink color in the culture broth by adding Salkowski's reagent except PU-Amt1, PU-Tul1 and PU-Mor2. Similarly, phosphate solubilization was observed in case of almost all the isolates, which is another indicative of the plant growth is promoting ability of the endophytic actinobacteria strains along with the IAA production.

### 16S rRNA Gene Sequencing of the Selected Endophytic Actinobacteria Strains

The selected isolates based on the activity and/or metabolic LCMS profiles were identified using molecular genetics approach by 16SrRNA gene sequencing and the % similarity at genus level was determined, on the basis of which a neighbor joining phylogenetic tree was constructed by using MEGA 6.0 at 1000 bootstrap replicates (Figure 3). The majority of the strains belonged to the genus *Streptomyces* which includes PU-Aki3 GenBank accession number; MW080716, PU-Aki4 (MZ703183), PU-Kach5 (MW080762), PU-LG1 (MW080760), PU-Imi6 (MW080759), PU-Amt17 (MW079406), PU-Amt14 (MW077167), PU-LG2 (MW080715). While the strains PU-Aki5 (MW080667) belonged to the genus *Micromonospora*, PU-Amt1 (MW080707) and PU-Amt9 (MW080693) belonged to the genus *Nocardia*.



**Figure 3:** Neighbour-joining tree based on 16SrRNA gene sequence of the endophytic actinobacteria isolates at 1000 bootstrap replicates using MEGA 6.0.

### Antimicrobial Activity of the Methanolic Crude Extracts

The crude extracts exhibited significant antimicrobial activity against the ATCC test strains by microtiter plate assay. About 53% of the methanolic extracts significantly inhibited the growth of *S. aureus* including the extracts of the strains PU-Mor1, PU-Kach5, PU-Aki2, PU-Amt14, PU-LG3, PU-Imi6, PU-LG1, PU-Aki1, PU-Amt9, PU-Aml3, PU-Tul4, PU-Kach2, PU-Amt7, PU-Aml1, PU-Kach9, PU-Aki3, PU-Amt15, PU-Amt17. While moderate antimicrobial activity was exhibited by 47% of the extracts against *E. coli*, among which the major inhibition was observed in case of the extract of strains PU-Aki4, PU-Kach5 and PU-Amt17. The antifungal activity was exhibited by 32% of the extracts including the extracts of strains PU-Aki2, PU-LG3, PU-Amt14, PU-Kach14, PU-Aki1, PU-Tul4, PU-Amt7, PU-Kach11, PU-Kach9, PU-Kach6 and PU-Aml6 (Figure. 4).

### Cytotoxicity and *In-vitro* Antitumor Activity of the Extracts

The extracts exhibited significant cytotoxicity or anti proliferative activity against the cancerous cell line PC3

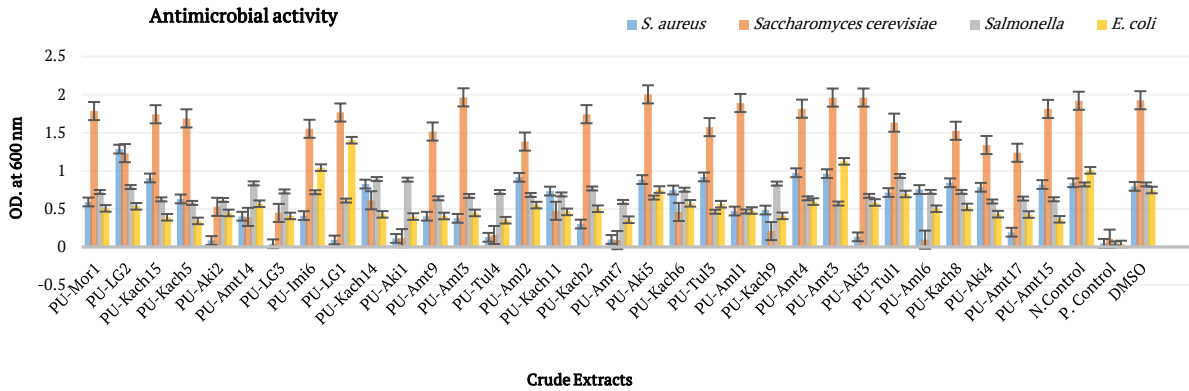
(prostate cancer cell line) as compared to the A549 (non-small lungs carcinoma cell line). A total of 30% of crude extracts exhibited maximum cytotoxicity with percentage viability of cells less than 50%. The extracts of the strains PU-Aki1 and PU-Aki2 exhibited 0% cell viability for both of the cell lines, the extract of strain PU-Aki3 exhibited 20% cell viability for PC3 and less than 10% viability for A549, similarly the extract of strain PU-Aki4 showed 20% viability for A549 and 50% for PC3, while the extract of strain PU-Aki5 showed 50% cell viability for both of the cell lines. The extract of strain PU-Aml6 was more cytotoxic to PC3 (10% cell viability) as compared to A549 (40% cell viability), similarly the extract of strain PU-Amt14 exhibited less than 20% viability of both of the cell lines and PU-LG1 showed about 50% cell viability (Figure 5).

### DPPH Radical Scavenging Activity

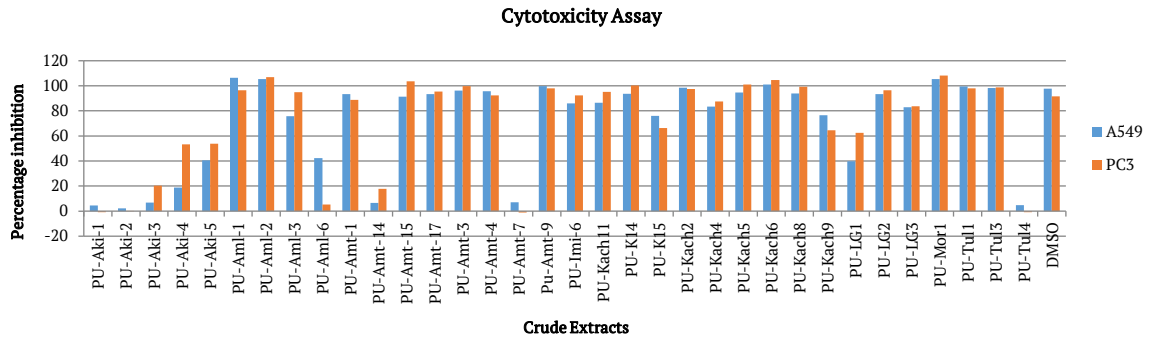
The *in-vitro* DPPH radical scavenging activity was exhibited by most of the crude extracts, and in most of the cases more than 50% radical scavenging activity was observed. The maximum % inhibition of DPPH was exhibited by strains PU-Aki4, PU-Kach5 and PU-Amt 17 as compared to the ascorbic acid (positive control) (Figure 6).

### Metabolomic Profiles of the Selected Endophytic Actinobacteria Strains

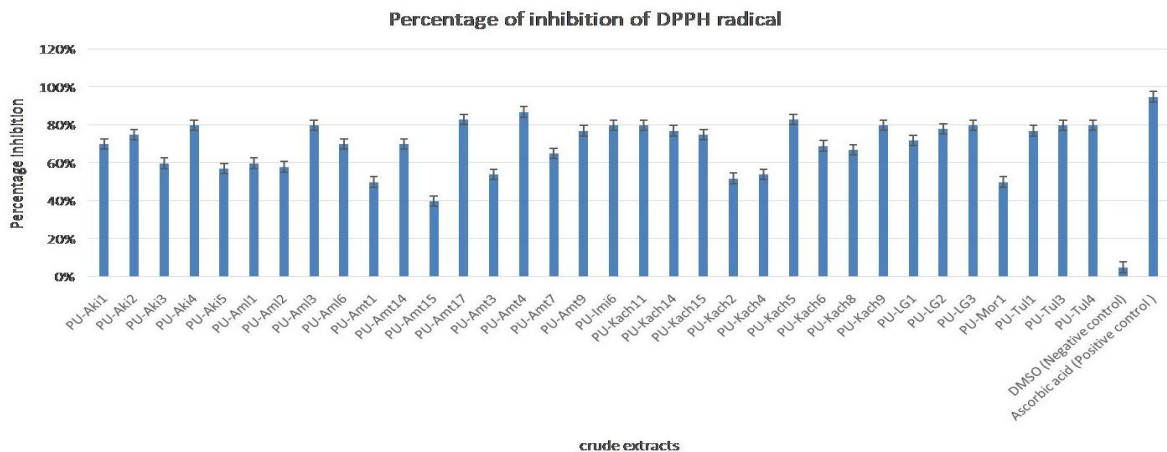
The bioactive metabolites present in the extracts of the strains showed different colored bands on TLC after staining with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> reagent, some prominent bands were observed in case of the extracts of strains PU-Amt17, PU-LG1, PU-Aki2, PU-Aki4, PU-Aki5 and PU-Aml1. The fluorescent spots of red, blue, yellowish, violet and orange-color were seen under UV (366nm). A few of the bands were visualized under short UV (254nm), the prominent UV absorbing bands were observed in case of the extract of strains PU-Aki4, PU-Aki5. After staining the TLC plate with staining with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> reagent; reddish, blue, and light yellowish- and violet-colored bands were observed and were documented. The HPLC-UV/MS metabolic profile of strain PU-Aki4 displayed several bacterial metabolites eluted at different retention ( $t_R$  12 ~ 29 min) with molecular masses ranges  $m/z = 377 \sim 817$ . The crude extracts of the strain PU-Kach5 showed 5 prominent peaks at  $t_R$  12~ 27 min with molecular masses ranges  $m/z=179 \sim 41$ . The crude extracts of the strain PU-Amt17 showed distinctive peaks eluted at retention time  $t_R$  22~ 29 min, and masses of these eluted metabolites were  $m/z = 213 \sim 401$  (Table 1).



**Figure 4:** Comparative bioactivity of the endophytic actinobacteria crude extracts (10mg/ml) against *S. aureus*, *S. cerevisiae*, *S. enterica* and *E. coli*. The bar graph indicated optical density of the strain on Y-axis and crude extracts on X-axis. The short bars with less OD values indicated that growth of the test organism was inhibited by the extracts, while long bars with high OD values indicated that extracts were inactive against the test organisms and the cell viability.



**Figure 5:** Cytotoxicity against A549 (non-small lung) and PC3 (prostate) human cancer cell lines of the crude extracts produced by the endophytic actinobacterial isolates (after 72h). The percentage viability on Y-axis and crude extracts on X-axis. Higher bars indicate that the extracts were less cytotoxic while short bars indicate that the extracts were more cytotoxic.



**Figure 6:** DPPH radical scavenging activity of the crude extracts (10mg/ml) dissolved in DMSO. Crude extracts on X-axis and percentage of inhibition on Y-axis and higher bars indicated that the extracts have more antioxidant potential as compared to others.

Strains	Thin Layer Chromatography (TLC)			HPLC-UV/MS ( $t_R$ /min)	*(+) and (-)-ESI-MS: ( $m/z$ )
	UV Visualization		Staining with Anisaldehyde/H <sub>2</sub> SO		
	254 nm	366 nm			
PU-Amt1	2 bands	6 bands	2light yellow-brown band	-	-
PU-Amt9	1 band	2 bands	1 violet band	-	-
PU-Aml3	5 bands	8 bands	2 light purple bands	17.18 19.47 21.32 22.08 23.83 28.23	511[M+H] <sup>+</sup> ; 509 [M-H] <sup>-</sup> 758[M+H] <sup>+</sup> ; 756 [M-H] <sup>-</sup> 481[M+H] <sup>+</sup> ; 479 [M-H] <sup>-</sup> 465[M+H] <sup>+</sup> ; 463 [M-H] <sup>-</sup> 818[M+H] <sup>+</sup> ; 816 [M-H] <sup>-</sup> 325[M+H] <sup>+</sup>
PU-Aml6	2 bands	2 light bands	1 light brown band at the base line	14.65 17.81 22.98 22.98 24.26 26.37	605 [M+H] <sup>+</sup> ; 601 [M-H] <sup>-</sup> - 422 [M+H] <sup>+</sup> ; 420 [M-H] <sup>-</sup> 655 [M+H] <sup>+</sup> ; 653 [M-H] <sup>-</sup> 813 [M+H] <sup>+</sup> ; 811 [M-H] <sup>-</sup> 464 [M+H] <sup>+</sup> ; 498 [M+Cl] <sup>-</sup>
PU-Amt7	1 light band	1major band	Pale-yellow band	17.88 20.05 24.70 26.96 29.19 31.26	218[M+H] <sup>+</sup> ; 216 [M-H] <sup>-</sup> 527[M+H] <sup>+</sup> ; 525 [M-H] <sup>-</sup> 541[M+H] <sup>+</sup> ; 539 [M-H] <sup>-</sup> 588[M+H] <sup>+</sup> ; 586 [M-H] <sup>-</sup> 511[M+H] <sup>+</sup> ; 509 [M-H] <sup>-</sup> 495[M+H] <sup>+</sup> ; 493 [M-H] <sup>-</sup>
PU-Amt14	5 light bands and 1dark band	3	1 light yellow	14.55 17.23 17.86 26.26 26.83 27.40	704 [M+H] <sup>+</sup> ; 702 [M-H] <sup>-</sup> 691 [M+H] <sup>+</sup> ; 689 [M-H] <sup>-</sup> 213 [M+H] <sup>+</sup> ; 211 [M-H] <sup>-</sup> 244 [M+H] <sup>+</sup> ; 242 [M-H] <sup>-</sup> 544 [M+H] <sup>+</sup> ; 520 [M-H] <sup>-</sup> 472 [(M-H <sub>2</sub> O)+H] <sup>+</sup> ; 488 [M-H] <sup>-</sup>
PU-Amt17	3	7	1 light violet	22.56 24.65 25.33 25.33 27.37 28.81 21.64	337 [M+H] <sup>+</sup> ; 335 [M-H] <sup>-</sup> 315 [M+H] <sup>+</sup> ; 313 [M-H] <sup>-</sup> 315 [M+H] <sup>+</sup> ; 629 [2M+H] <sup>+</sup> 388 [M+H] <sup>+</sup> ; 386 [M-H] <sup>-</sup> 315 402 [M+H] <sup>+</sup> ; 400 [M-H] <sup>-</sup> 402 [M+H] <sup>+</sup> ; 400 [M-H] <sup>-</sup> 213 [M+H] <sup>+</sup>
PU-Aki3	2 bands	5 bands	1 violet, 3 light brown bands		
PU-Aki4	3 bands, smear of 4-5 bands	3 major light bands	2 major light brown bands	12.62 14.82 16.32 19.16 23.81 25.49 28.93 29.51	742 [M+H] <sup>+</sup> ; 740 [M-H] <sup>-</sup> 818 [M+H] <sup>+</sup> ; 816 [M-H] <sup>-</sup> 518 [M+H] <sup>+</sup> 378 [M+H] <sup>+</sup> ; 376 [M-H] <sup>-</sup> 818 [M+H] <sup>+</sup> ; 816 [M-H] <sup>-</sup> 432 [M+H] <sup>+</sup> ; 454 [M+Na] <sup>+</sup> 438 [M+H] <sup>+</sup> 438 [M+H] <sup>+</sup> ; 482 [M+HCOO] <sup>-</sup>
PU-Aki5	4 bands	7 bands	2 light yellow bands	-	-
PU-Kach5	2 bands	2 bands	1 light brown band	12.53 14.53 18.42 26.19	354 [M+H] <sup>+</sup> ; 352 [M-H] <sup>-</sup> 180 [M+H] <sup>+</sup> ; 178 [M-H] <sup>-</sup> 442 [M+H] <sup>+</sup> ; 440 [M-H] <sup>-</sup> 424 [M+H] <sup>+</sup> ; 422 [M-H] <sup>-</sup>
PU-Kach6	2 light bands	2 bands	1 brownish band at the baseline	12.47 17.49 18.52 18.52 24.51	219[M+H] <sup>+</sup> ; 217 [M-H] <sup>-</sup> 637[M+H] <sup>+</sup> ; 635 [M-H] <sup>-</sup> 208[M+H] <sup>+</sup> ; 206 [M-H] <sup>-</sup> 638[M+H] <sup>+</sup> ; 636 [M-H] <sup>-</sup> 291[M+H] <sup>+</sup> ; 323 [M+Cl] <sup>-</sup>
PU-Kach8	--	--	1 brownish band at the baseline	12.50 13.61 14.53 17.47 17.85 19.98 19.98	219[M+H] <sup>+</sup> ; 217 [M-H] <sup>-</sup> 702[M+H] <sup>+</sup> ; 700 [M-H] <sup>-</sup> 180[M+H] <sup>+</sup> ; 178[M-H] <sup>-</sup> 637[M+H] <sup>+</sup> ; 635 [M-H] <sup>-</sup> 336[M+H] <sup>+</sup> 160[M+H] <sup>+</sup> ; 158 [M-H] <sup>-</sup> 512[M+H] <sup>+</sup> ; 510 [M-H] <sup>-</sup>
PU-LG1	4 bands	5 bands	1 light yellow band	-	-
PU-LG2	--	2 bands	3 light brown bands	-	-
PU-lmi6	3 bands	6 bands	1 reddish band, 1 light yellow band	-	-
PU-Aki1	2 bands	3 bands	1 purplish band at the baseline	20.05 21.67 24.67 26.80	527[M+H] <sup>+</sup> ; 525 [M-H] <sup>-</sup> 511[M+H] <sup>+</sup> ; 509 [M-H] <sup>-</sup> 541[M+H] <sup>+</sup> ; 539 [M-H] <sup>-</sup> 525[M+H] <sup>+</sup> ; 523 [M-H] <sup>-</sup>
PU-Aki2	3 bands	6 bands	1 light yellow brownish band	15.17 15.17 21.48 23.83 26.28	443 [M+H] <sup>+</sup> ; 441 [M-H] <sup>-</sup> 601 [M+H] <sup>+</sup> ; 599 [M-H] <sup>-</sup> 430 [(M-2H <sub>2</sub> O)+H] <sup>+</sup> ; 464 [M-H] <sup>-</sup> 373 [M+H] <sup>+</sup> ; 407 [M+Cl] <sup>-</sup> 726 [M+H] <sup>+</sup> ; 724 [M-H] <sup>-</sup>
PU-Aki3	6 bands	8 bands	3 bands	20.78 21.45	166[M+H] <sup>+</sup> 430[M+H] <sup>+</sup> ; 464 [M+Cl] <sup>-</sup>
PU-LG3	2 minor bands	3-5 minor bands	2 major (pink and dark gray bands)	11.54 12.27 16.1 16.35 21.10 14.53	519 [M+H] <sup>+</sup> ; 517[M-H] <sup>-</sup> 546 [M+H] <sup>+</sup> ; 544 [M-H] <sup>-</sup> 231 [M+H] <sup>+</sup> ; 229 [M-H] <sup>-</sup> 292 [M+H] <sup>+</sup> ; 290 [M-H] <sup>-</sup> 362 [M+H] <sup>+</sup> ; 407 [M+HCOO] <sup>-</sup> 1097 [M+H] <sup>+</sup> ; 1095 [M-H] <sup>-</sup>
PU-Kach14	1 band	3 bands	1 light brownish band at the baseline	17.45 22.53 25.35 33.35	637 [M+H] <sup>+</sup> ; 635 [M-H] <sup>-</sup> 197 [M+H] <sup>+</sup> ; 195 [M-H] <sup>-</sup> 163 [M+H] <sup>+</sup> 228 [M+H] <sup>+</sup>
PU-Kach15	4 bands	5 bands	3 bands	14.76 17.62	585 [M+H] <sup>+</sup> ; 583 [M-H] <sup>-</sup> 247 [M+H] <sup>+</sup> ; 245 [M-H] <sup>-</sup>
PU-Tul4	2 bands	5 bands	3 purplish bands	17.88 20.05 24.70 26.96 29.19 31.26	218[M+H] <sup>+</sup> ; 216 [M-H] <sup>-</sup> 527[M+H] <sup>+</sup> ; 525 [M-H] <sup>-</sup> 541[M+H] <sup>+</sup> ; 539 [M-H] <sup>-</sup> 588[M+H] <sup>+</sup> ; 586 [M-H] <sup>-</sup> 511[M+H] <sup>+</sup> ; 509 [M-H] <sup>-</sup> 495[M+H] <sup>+</sup> ; 493 [M-H] <sup>-</sup>

Table 1: Metabolomics profiles of the selected endophytic actinobacteria isolates determined by TLC and HPLC-UV/MS analysis [30].

## Discussion

Currently, in drug discovery, the endophytic actinobacteria are attractive candidates due to the perception that their untapped genome may encode novel bioactive compounds, which have been proved by several studies [31-34]. So it is anticipated that the endophytic actinobacteria are a rich source of the diversified metabolites with respect to structure and function [35]. In this study 40 endophytic actinobacteria strains were isolated following stringent protocols of plant sample's surface disinfection following the methods of Cao et al. [19]. All the isolated and subsequently selected strains showed the typical morphological characteristics of the group actinobacteria as mentioned in Bergey's manual of bacteriology (Volume II) [20]. The biochemical and physiological characterization further confirmed that these isolates are the member of different actinobacterial genera showing the typical actinobacteria like characteristics. For example the pigment production is one of the characteristics feature of the actinobacteria and most of the strains in this study produced xero-pigments and showed the production of melanin. Similarly most of the strains showed the hydrolysis of esculin and utilization of organic acid, while in case of the utilization of the different sugars as sole carbon source [36], isolates in this study utilized most of the tested sugars except L-Rhamnose, L-Raffinose and myo-inositol. Kim et al. [37] observed the cellulase activity of the endophytic actinobacteria isolated from the roots of the plants and a similar outcome has been observed in this study and the strains exhibited cellulose activity which further confirms the origin of these actinobacteria strains from the plant tissue. Borah and Thakur [38] reported that at least one extracellular enzyme is produced by endophytic actinobacteria while in our study; most of the isolates were able to produce cellulase, amylase and pectinase enzymes. The significance of these enzymes lies in the fact that they are responsible for the entry of the endophytic isolates into the plant tissues; however the same ability of these strains can be exploited for the industrial applications and production of such commercially useful compounds.

Several actinobacteria genera have been reported to live as endophytes, however the genus *Streptomyces* is found to be the predominant endophyte [35, 39, 40] a similar outcome was seen in our study as all the prioritized and bioactive strains detected in this study belonged to the genus *Streptomyces*. However the 16S rRNA gene sequencing and subsequent BLAST analysis helped to detect the strains belonging to actinobacterial genera other than *Streptomyces*, such as the strain PU-Aki5 exhibited maximum similarity with the genus *Micromonospora*, the strains PU-Amt9

and PU-Amt1 exhibited maximum similarity with the genus *Nocardia*. This shows that although the *Streptomyces* could be the predominant genus in the endophytic actinobacteria population however members of other actinobacterial genera also invade the plant tissues and develop mutualistic relationships with the plants.

The inhibitory activity of the extracts was observed against both gram-positive and gram-negative test strains, however the prominent inhibition was observed against gram-positive bacteria especially *S. aureus*. Among the 40 endophytic actinobacteria strains, 18 strains originated from *Cassia occidentalis* and *Cassia fistula* showed inhibition of *S. aureus*. The 10 endophytic strains exhibited significant antifungal activity while 15 were active against *E. coli*. In screening studies it has been observed that the activity against Gram negative test strains is usually less as compared to the activity against Gram positive test strains, as reported by Benhadjet al. [41] and Assad et al. [42]. In case of our study the response was same, the activity of the isolated endophytic actinobacteria was more prominent against gram positive bacteria. The *in-vitro* antitumor activity against human cancerous cell lines was exhibited by the extracts between moderate to high cytotoxicity against A549 and PC3 cancer cell lines, the almost similar response by endophytic actinobacteria was reported by Conti et al. [43], where the strains exhibited moderate to high cytotoxicity against the tested cell lines. In general it was observed the endophytic actinobacteria strains recovered from *Cassia occidentalis* showed more prominent antimicrobial and *in-vitro* antitumor activity or high cytotoxicity as compared to the strains retrieved from other medicinal plants investigated in this study. The production of indole acetic acid (IAA) by most of the strains indicate that the endophytic actinobacteria contribute towards the plant growth promotion (PGP) of the host plants. It supports the assumption that the endophytic actinobacteria cause positive effect on the growth of their host as already reported by Passariet al. [44]. The antioxidant potential, as is evident by DPPH radical scavenging activity, show these isolates as the potential source for the isolation and discovery of antioxidant compounds, the antioxidant potential of the endophytic actinobacteria has already been reported in many studies [45].

The metabolomics profiles of the isolated endophytic strains were determined by thin layer chromatography (TLC) and HPLC-UV/MS analysis depicted the presence of variety of metabolites in the extracts of these endophytic strains. For example numerous UV absorbing, fluorescent and colored compounds were observed on TLC, the different colored compounds that



appeared after staining with anisaldehyde/H<sub>2</sub>SO<sub>4</sub> reagent shows the metabolites present in the extracts belong to different structural classes of the bioactive compounds. Similarly, a range of molecular masses of the low molecular weight compounds present in the crude extracts was detected, such as in case of strain PU-Aki4 the molecular masses of the metabolites in extracts were as follows;  $m/z = 741, 817, 518, 377$  and  $438$  Daltons, etc. while in case of strain PU-Amt17, the molecular masses of the compounds were as follow;  $m/z = 336, 314, 387, 401$  and  $213$  Daltons, etc. This detected range of low molecular weight compounds with potent antimicrobial activities indicate the rich diversity of the metabolites produced by the isolated endophytic actinobacteria strains. The chemical analysis in turns further helped to prioritize the strains for media optimization and scale up studies of the priority strains in this collection.

The study revealed that the endophytic actinobacteria present in the tissues of the selected medicinal plants which are frequently used as folk medicines in south Asian region are a rich and untapped source of bioactive and structurally diverse compounds, as is indicated by their potent antimicrobial and cytotoxic/anticancer activities and distinct metabolomics profiles. Among the 40 isolated endophytic actinobacteria strains, the predominant genus was *Streptomyces* however members from other genera such as *Micromonospora* and *Nocardia* were also identified. The 7 strains in this collection were selected as priority strains based on their preliminary screening for scale-up fermentation, compounds purification and structure elucidation. The study shows, the continuous isolation and screening of endophytic actinobacteria from these medicinal plants may yield pharmaceutically useful metabolites and compounds.

## Acknowledgments

This work was supported by Higher Education Commission (HEC) Pakistan grant (HEC-NRPU-Project 2121). The work was also supported by NIH grant R37 AI52218 (JST), the UK Center of Biomedical Research Excellence (COBRE) in Translational Chemical Biology (P20 GM130456) and the University of Kentucky College of Pharmacy and Center for Clinical and Translational Sciences (UL1 TR000117 and UL1 TR001998). The data presented in this manuscript is part of the PhD thesis of author Ashba Hassan.

## Competing Interest

The authors declare that there is no conflict of interest.

## Author Contributions

AH, LVP, IS and KAS performed research and analyzed data; AH, IS and KAS wrote the first draft of the manuscript; AH and IS contributed to plant sampling, strain isolation and sequencing; IS supervised the strains isolation and identification; JST, KAS and IS contributed to the experimental design, project oversight and manuscript preparation. All authors read and approved the manuscript.

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