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Diversity of bioactive endophytic *Streptomyces* sp. residing in a common weed, *Parthenium hysterophorus* (*Asteraceae*: *Heliantheae*)

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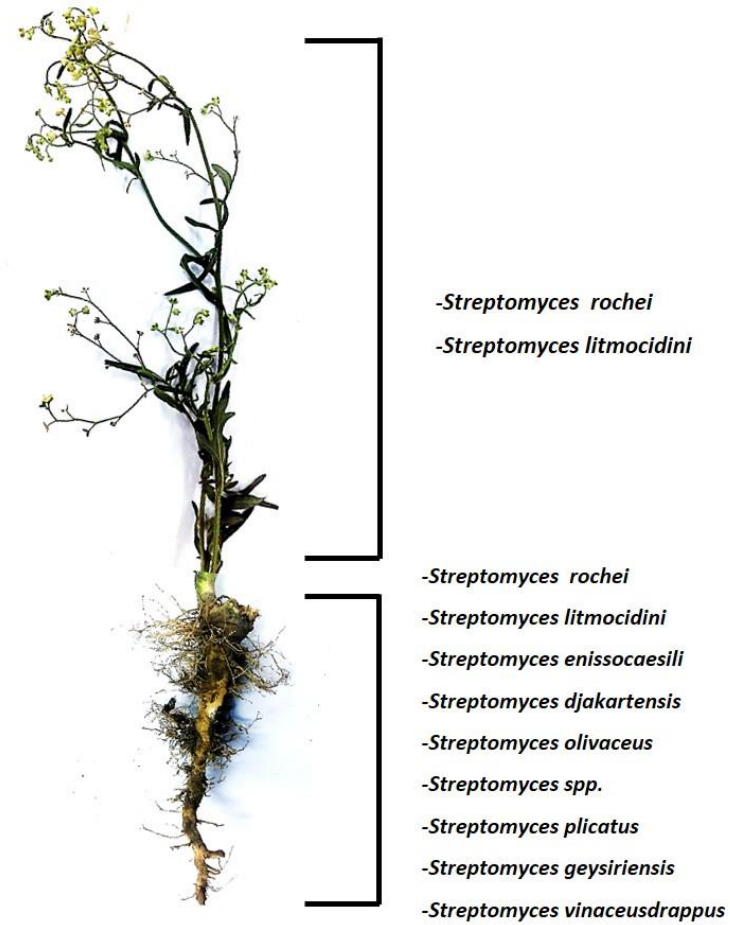
Diversity of bioactive endophytic *Streptomyces* sp. residing in a common weed, *Parthenium hysterophorus* (*Asteraceae*: *Heliantheae*)

Graphical Abstract



Parthenium hysterophorus

Abstract:
The sunflower family (Asteraceae) comprises of over 650 species making it the largest plant family in Pakistan. Members of this family are extremely diverse including weed plants such as *Parthenium hysterophorus*. The word endophyte means 'in the plant', the term includes the microbial colonizers which take up residence in the inner tissues of plants. Recently, *Streptomyces* species have been described in the plant tissues. It is noted that the possibility of re-isolation of compounds from soil actinomycetes has increased making it crucial that unexplored habitats be pursued for the search for new compounds. Considering this idea, we explored the diversity of endophytic *Streptomyces* residing in the common weed, *Parthenium hysterophorus*. A variety of *Streptomyces* sp. were identified through 16S gene sequencing that were not reported in prior studies to the best of our knowledge. The isolates were screened for their antimicrobial potential particularly against multidrug resistant (MDR) pathogens and for the diversity of their secondary metabolites through thin layer chromatography (TLC) and high performance liquid chromatography-UV (HPLC-UV). The staining of the TLC plates by reagents such as Ehrlich's reagent and anisaldehyde /H_2SO_4 revealed indols and N-heterocycles whereas the HPLC-UV chromatograms revealed peaks of diverse compounds.

Keywords: Asteraceae; Endophytes; *Streptomyces;* Weeds

Introduction

- *Asteraceae* plant family
- Largest flowering plant family in Pakistan
  - Over 650 species in 15 tribes [1]

- Endophytes
- Group of microbial colonizers
- Take up residence in the inner tissues of plants [2]

[1] Hussain, Javid, Zia Muhammad, Riaz Ullah, Farman Ullah Khan, I Ullah, Naeem Khan, Javed Ali, and Saleem Jan. "Evaluation of the chemical composition of *Sonchus eruca* and *Sonchus asper*." *J. Am. Sci* 6, no. 9 (2010): 231-35.
[2] Bascom-Slack, C. A., Ma, C., Moore, E., Babbs, B., Fenn, K., Greene, J. S., Hann, B. D., Keehner, J., Kelley-Swift, E. G., Kembaiyan, V., Lee, S. J., Li, P., Light, D. Y., Lin, E. H., Schorn, M. A., Vekhter, D., Boulanger, L.-A., Hess, W. M., Vargas, P. N., Strobel, G. A. and Strobel, S. A. 2009. Multiple, novel biologically active endophytic actinomycetes isolated from upper Amazonian rainforests. *Microbiol. Ecol.* **58:** 374–383.

Introduction (Cont.)

- *Parthenium hysterophorus* (*Asteraceae*: *Heliantheae*)
- Native herbaceous plant from tropical and subtropical America [3]
- Common names
- Parthenium weed (Australia), bitter weed, carrot weed; broom-bush and congress weed, false ragweed and ragweed

P. hysterophorus growing behind the department of Microbiology and Molecular Genetics, University of the Punjab, Lahore Pakistan. Photo courtesy: Dr. Rabia Tanvir

[3] Romero, A., Carrion, G. and Rico Gray, V. 2001. Fungal latent pathogens and endophytes from leaves of *Parthenium hysterophorus* (Asteraceae). *Fungal Diversity.* **7:** 81-87.

Introduction (Cont.)

- Actinobacteria
- Gram positive, high Guanine-Cytosine (G+C) content in their genomes [4]
- Hyphal (fungal-like) morphology [5]
- Form more intimate associations with plants and colonize their internal tissues [4]

- Streptomycetes
- Saprophytic, soil-dwelling organisms [4]
- Astonishing diversity in terms of morphology, ecology, genome size, genomic G+C content, and the number of coding sequences in the genome [6]

Purified endophytic *Streptomyces* strains on GYM agar plates. Photo courtesy: Dr. Rabia Tanvir

[4] Coombs, J. T. and Franco, C. M. M. 2003. Isolation and identification of actinobacteria from surface-sterilized wheat roots. *Appl. Environ. Microbiol.* **69(9):** 5603-5608.

[5] Taechowisan, T., Peberdy, J. F. and Lumyong, S. 2003. Isolation of endophytic actinomycetes from selected plants and their antifungal activity. *World J. Microbiol. Biotechnol.* **19:** 381–385.

[6] Alam, M. T., Merlo, M. E., Takano, E. and Breitling, R. 2010. Genome based phylogenetic analysis of *Streptomyces* and its relatives. *Mol. Phylogenet. Evol.* **54:** 763–77.

Results and discussion

- 42 endophytic *Streptomyces* were isolated [7]
- Frequency of isolation
  - Roots = 34
  - Shoots and leaves = 2
  - Slurry = 6

PLANT SAMPLE	PLANT PART	CONDITION OF THE PLANT	ISOLATES
Parthenium hysterophorus	Cut roots	Fresh	RT-6, RT-7, RT-36, RT-37, RT-38, RT-39, RT-40, RT-41, RT-42, RT-43, RT-44, RT-45, RT-46, RT-47, RT-48, RT-49, RT-50, RT-51, RT-52, RT-53, RT-54, RT-55, RT-56, RT-57, RT-58, RT-59, RT-60, RT-61, RT-62, RT-63, RT-64, RT-65, RT-66, RT-67
	Cut shoots and leaves	Fresh	RT-10, RT-11
	Slurry of roots, shoots and leaves	Fresh	RT-13, RT-14, RT-15, RT-16, RT-17 and RT-18

[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (cont.)

- Morphological characterization
- Isolated colonies were sub-cultured on GYM agar plates [8]
  - Size
  - Shape
  - Margin
  - Presence of aerial mycelium, substrate mycelium
  - Production of soluble pigments
  - Texture [9]

[8] Shirling, E. B. and Gottlieb, D. 1966. Methods for characterization of *Streptomyces* species. *Int. J. Syst. Bacteriol.* **16:** 313-340.
[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening for type I polyketide synthases genes of endophytic Streptomycetes isolated from Parthenium hysterophorus L.* Molecular Genetics, Microbiology and Virology, 2013. **28**(1): p. 32-39.

Results and discussion (cont.)

Table 1. Morphological characteristics of the selected endophytic *Streptomyces* used in the study

Strain	Colony size, mm	Colony shape	Margin	Color of sub-strate	Color of aerial mycelium	Texture	Pigmentation
RT-6	3	Circular	Undulate	Light brown	Light grey	Dry	None
RT-7	2	Circular	Undulate	Light brown	Dark grey	Dry	None
RT- 10	3	Circular	Errose	Light brown	Light brown	Dry	None
RT-11	3	Circular	Entire	Dark brown	Light brown	Dry	None
RT-12	2	Circular	Entire	Dark brown	White	Dry	None
RT-13	1	Circular	Entire	Dark brown	Grey	Dry	None
RT-14	3	Circular	Entire	Light brown	Light pink	Dry	None
RT-15	1	Irregular	Errose	Light yellow	White	Dry	None
RT-18	3	Circular	Undulate	Brown	Light brown	Dry	None
RT-34	3	Irregular	Undulate	Light brown	White	Dry	None
RT-36	3	Circular	Entire	Brown	Grey	Dry	None
RT-37	2	Circular	Entire	Brown	Light brown	Dry	None
RT-38	2	Circular	Entire	Light brown	White	Dry	None
RT-39	5	Circular	Entire	Brown	Light brown	Dry	None
RT-40	1	Circular	Entire	Brown	Grey	Dry	None
RT-41	2	Circular	Entire	Brown	Grey	Dry	None

[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening for type I polyketide synthases genes of endophytic Streptomycetes isolated from Parthenium hysterophorus L.* Molecular Genetics, Microbiology and Virology, 2013. **28**(1): p. 32-39.

Results and discussion (cont.)

RT-43	3	Circular	Entire	Brown	Light grey	Dry	None
RT-44	3	Circular	Entire	Light brown	Light brown	Dry	None
RT-46	3	Circular	Errose	Black	Grey	Dry	None
RT-47	3	Circular	Undulate	Brown	Light grey	Dry	None
RT-48	3	Circular	Undulate	Brown	Grey	Dry	None
RT-49	3	Circular	Entire	Light brown	Grey	Dry	None
RT-50	1	Circular	Entire	Brown	Light Grey	Dry	None
RT-51	1	Circular	Entire	Light brown	White	Dry	None
RT-52	2	Circular	Entire	Brown	Light pink	Dry	None
RT-53	3	Circular	Undulate	Dark brown	Light brown	Dry	None
RT-54	4	Circular	Entire	Dark brown	Light brown	Dry	None
RT-55	3	Circular	Entire	Dark brown	Light Grey	Dry	None
RT-56	.3	Circular	Entire	Brown	LightGrey	Dry	None
RT-57	3	Circular	Entire	Brown	Light Grey	Dry	None
RT-58	1	Circular	Entire	Brown	Light Grey	Dry	None
RT-59	1	Circular	Entire	Brown	White	Dry	None
RT-60	2	Circular	Entire	Brown	White	Dry	None
RT-61	4	Circular	Errose	Yellow	Light yellow	Dry	None
RT-62	1	Circular	Entire	Dark brown	Light pink	Dry	None
RT-63	4	Circular	Errose	Light Yellow	Yellow	Dry	None
RT-64	2	Circular	Errose	Light Yellow	LightGrey	Dry	None
RT-65	3	Circular	Undulate	Brown	Light brown	Dry	None
RT-66	3	Circular	Entire	Brown	Grey	Dry	None
RT-67	3	Circular	Entire	Brown	Grey	Dry	None

[9] Tanvir, R., I. Sajid, and S. Hasnain, *Screening for type I polyketide synthases genes of endophytic Streptomycetes isolated from Parthenium hysterophorus L.* Molecular Genetics, Microbiology and Virology, 2013. **28**(1): p. 32-39.

Results and discussion (cont.)

- Physiological characterization [7]
  - Melanin production

Streptomyces strain	Formation of melanin	Streptomyces strain	Formation of melanin
RT-6	+	RT-48	+
RT-7	+	RT-49	+
RT- 10	+	RT-51	+
RT-11	+	RT-52	+
RT-12	+	RT-53	+
RT-13	+	RT-54	+
RT-14	+	RT-55	+
RT-15	+	RT-56	+
RT-16	+	RT-57	+
RT-18	+	RT-58	+
RT-34	+	RT-59	+
RT-36	+	RT-60	+
RT-37	+	RT-61	+
RT-38	+	RT-62	+
RT-39	+	RT-63	+
RT-40	+	RT-64	+
RT-41	+	RT-65	–
RT-43	+	RT-66	+
RT-44	+	RT-67	+
RT-46	+		
RT-47	+		

[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (cont.)

- Physiological characterization [7]
  - Utilization of sugars and similar compounds

Strain no.	Glu	Fru	Raf	Rha	Ara	Man	Lac	Gal	Suc
RT-6	+	+	+	–	–	+	–	+	+
RT-7	+	+	+	+	+	+	+	+	+
RT- 10	+	+	–	+	–	+	–	+	–
RT-13	+	–	–	–	–	–	–	–	–
RT-14	+	+	–	–	–	+	–	+	+
RT-18	+	+	+	+	+	+	+	+	+
RT-36	+	+	+	+	–	+	+	+	+
RT-37	+	+	+	+	–	+	+	+	+
RT-38	+	+	+	–	+	+	+	+	+
RT-39	+	+	+	+	+	+	+	–	+
RT-40	+	+	+	+	+	+	+	+	+
RT-41	+	+	+	+	+	+	+	+	+
RT-43	+	+	+	+	+	+	+	+	+
RT-44	+	–	+	+	+	+	+	+	+
RT-46	+	–	+	+	–	–	–	+	–
RT-47	+	+	+	+	–	+	+	–	+
RT-49	+	+	+	+	+	–	+	+	+
RT-50	+	–	+	–	+	–	+	–	+
RT-53	+	–	–	–	–	–	–	–	–
RT-54	+	+	+	+	–	+	+	+	+
RT-55	+	+	+	–	–	–	+	+	–
RT-56	+	–	+	+	+	+	+	+	+
RT-57	+	+	+	+	+	+	+	+	+
RT-58	+	+	+	+	–	+	+	+	+
RT-59	+	+	+	+	–	–	+	–	–
RT-60	+	+	+	+	+	+	+	+	+
RT-61	+	–	–	–	–	–	–	–	–
RT-63	+	+	–	–	–	–	–	+	–
RT-64	+	+	+	+	+	+	+	+	+
RT-65	+	–	–	–	–	–	–	–	–
RT-67	+	–	+	+	–	+	–	–	+

[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (cont.)

- 16S gene characterization [7]
  - DNA extraction [10]
  - PCR by using forward (27f) and reverse primer (1522r)
  - BLAST analysis of 16S rRNA gene (1.5kb)
  - Sequence data submitted in Genbank database

[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283
[10] Sajid, I., Shaaban, K. A., Yao, C. B. F., Hasnain, S. and Laatsch, H. 2009. Antifungal and antibacterial activities of indigenous streptomycetes isolated from saline farmlands, prescreening, ribotyping and metabolic diversity. *World J. Microbiol. Biotechnol.* **25:** 601-610.

Results and discussion (cont.)

Table 2: Results of 16S rRNA gene sequencing of the endophytic streptomycetes

Streptomyces strains	No. of Nucleotides sequenced (bp)	*Streptomyces Spp.*	% Homology	Gen bank accession No.
RT-6	1425	*Streptomyces rochei*	99%	HQ909753
RT-13	1442	*Streptomyces litmocidini*	99%	HQ909754
RT-18	1444	*Streptomyces rochei*	99%	HQ909755
RT-36	1445	*Streptomyces rochei*	99%	HQ909756
RT-46	1445	*Streptomyces enissocaesili*	99%	HQ909757
RT-49	1444	*Streptomyces djakartensis*	99%	HQ909758
RT-54	1445	*Streptomyces olivaceus*	99%	HQ909759
RT-56	1442	*Streptomyces spp.*	99%	HQ909760
RT-57	1445	*Streptomyces plicatus*	99%	HQ909761
RT-63	1444	*Streptomyces geysiriensis*	99%	HQ909762
RT-64	1443	*Streptomyces spp.*	99%	HQ909763
RT-67	1444	*Streptomyces vinaceusdrappus*	99%	HQ909764

[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (cont.)

- Screening of endophytic *Streptomyces*
  - Crude extraction
    - Sonication and 1:1 ethyl acetate [11]
    - Biological and chemical screening

- Biological screening
    - Agar well diffusion method [12]
      - Multi drug resistant pathogens
      - Nosocomial pathogens

[11] Tanvir, Rabia, Imran Sajid, Shahida Hasnain, Andreas Kulik, and Stephanie Grond. "Rare actinomycetes *Nocardia caishijiensis* and *Pseudonocardia carboxydivorans* as endophytes, their bioactivity and metabolites evaluation." *Microbiological Research* 185 (2016): 22-35.
[12] Gebreyohannes, Gebreselema, Feleke Moges, Samuel Sahile, and Nagappan Raja. "Isolation and characterization of potential antibiotic producing actinomycetes from water and sediments of Lake Tana, Ethiopia." *Asian Pacific Journal of Tropical Biomedicine* 3, no. 6 (2013): 426-35.

Results and discussion (cont.)

- Antimicrobial activity against MDR pathogens [7]
  - Significant activity with maximum zones of inhibition of 25mm

Table 3: Antimicrobial activity of the endophytic streptomycete isolates against nosocomial pathogens

Strain No.	*Zone of Inhibition (mm)							
	Biofilm formers					Clinical isolates		
	Bacillus	*X4*	*M9*	*S2*	*E4*	*Staph. aureus*	*E. coli*	*C. albicans*
RT-6	18	13	15	18	15	15	15	22
RT-13	20	12	19	16	16	15	13	21
RT-18	18	15	14	18	15	16	15	17
RT-36	15	15	20	15	15	19	15	21
RT-46	20	15	11	12	11	15	14	12
RT-50	17	18	10	10	10	10	10	16
RT-53	18	16	25	14	17	20	15	12
RT-56	20	13	15	10	15	10	15	22
RT-57	17	13	10	10	14	15	10	10
RT-59	15	12	15	10	14	16	10	16
RT-60	16	20	15	10	14	10	10	22
RT-67	15	10	14	10	15	10	10	15

X4, *Pseudomonas spp.*; M9, *Enterobacter; S2, Enterobacter; E4, Enterobacter, Staph. aureus, Staphlococcus aureus; E. coli, Escherichia coli; C. albicans, Candida albicans*

* The zone of inhibition greater than 10mm was considered significant

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Results and discussion (cont.)

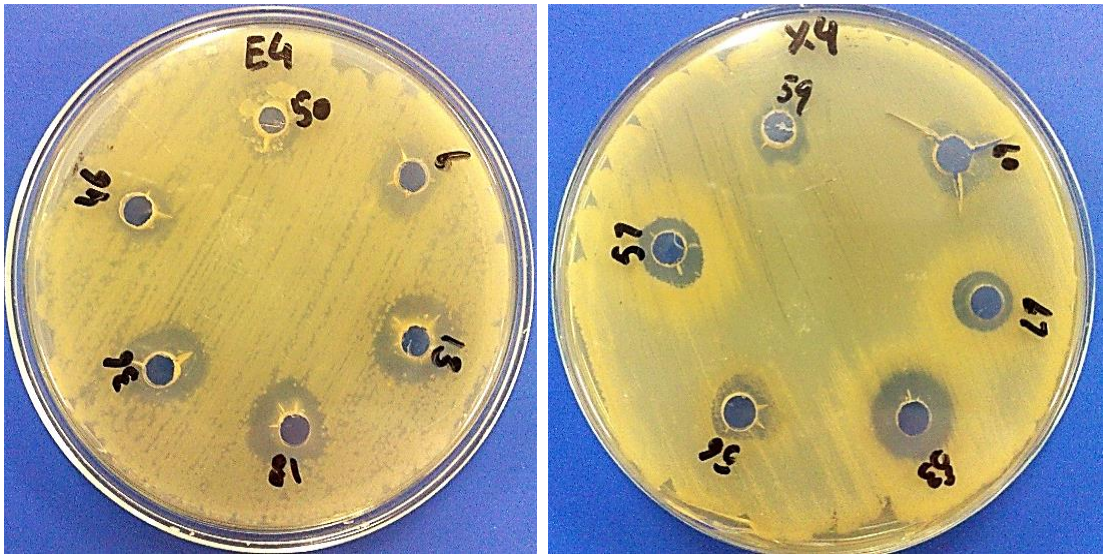
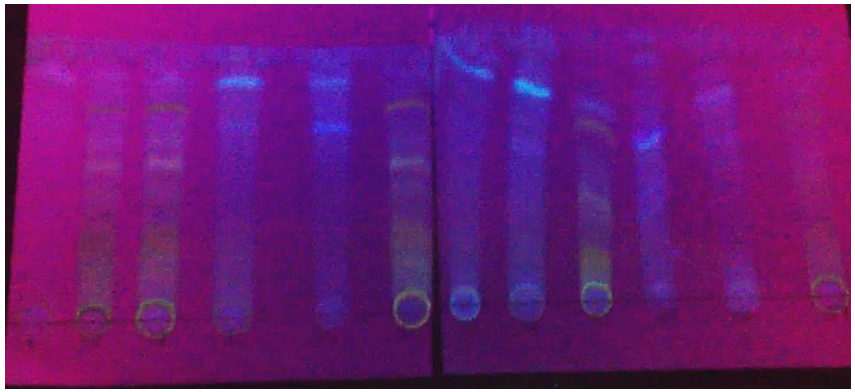


Photo courtesy: Dr. Rabia Tanvir

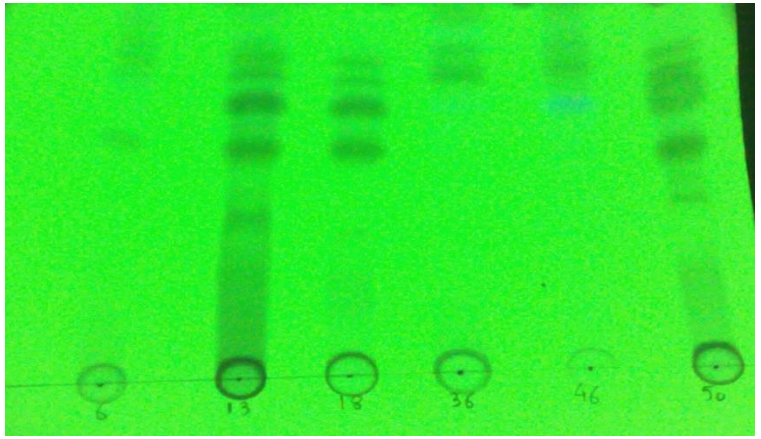
[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (cont.)

- Chemical screening [7]
  - Thin Layer Chromatography (TLC)
  - 366 nm and 254nm
  - Diverse metabolites production



(a) 366nm



(b) 254nm

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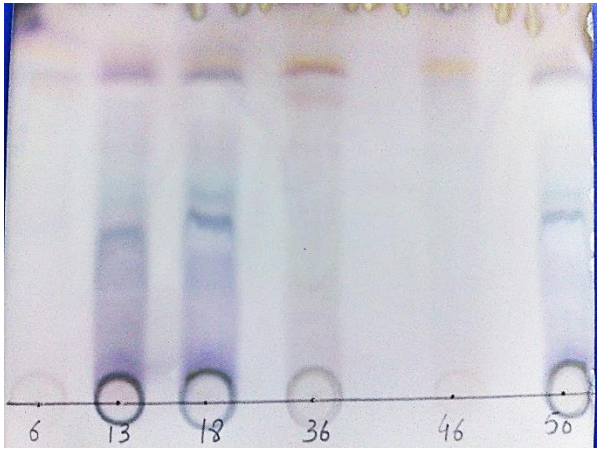
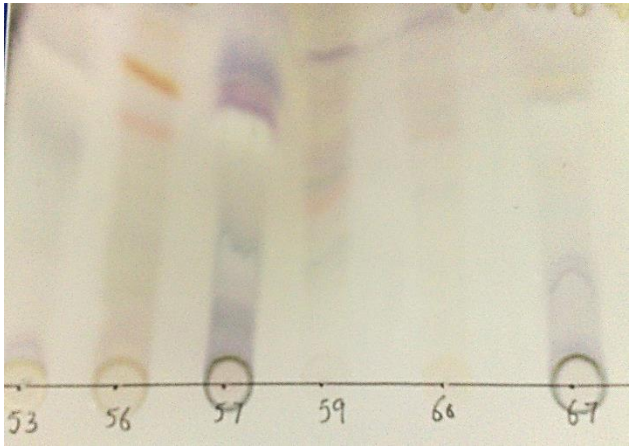
Results and discussion (cont.)

- Chemical screening [7]
  - Thin Layer Chromatography (TLC)
  - Purple and red spot (Indoles), yellow spot (N-heterocycles)



(a) Staining with anisaldehyde /H_2SO_4 reagent



(b) Staining with Ehrlich's reagent

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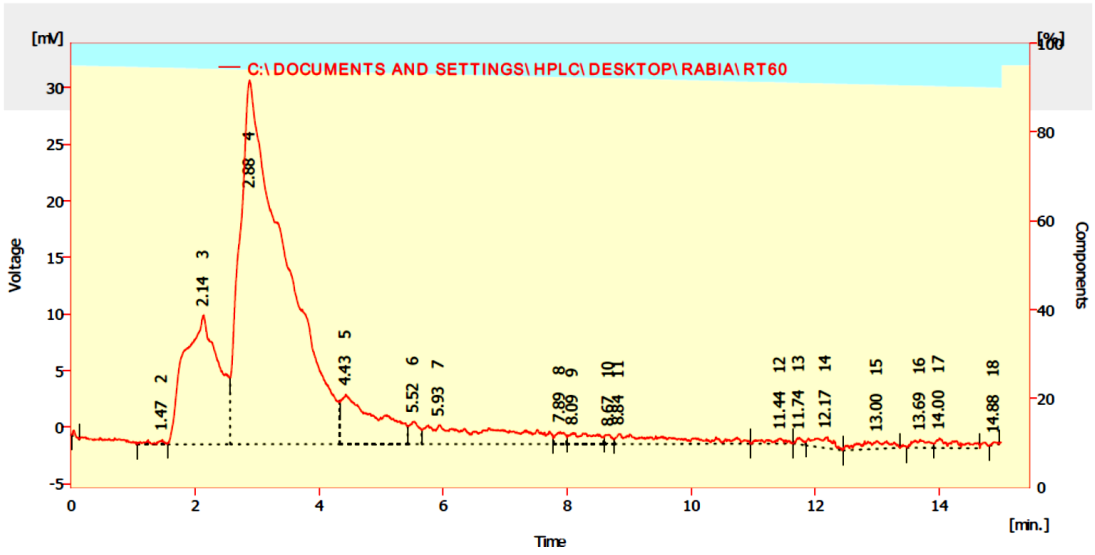
Results and discussion (cont.)

- High Performance Liquid Chromatography (HPLC-UV) [7]
  - Different peak observed at UV range of 254nm of different compounds

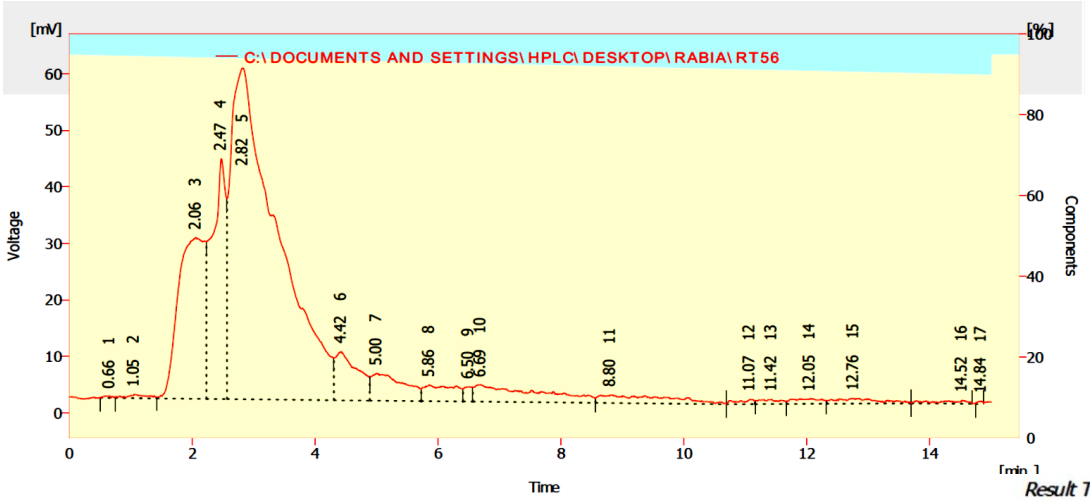


Result Table (Uncal - C:\|DOCUMENTS AND SETTINGS\|HPLC\|DESKTOP\|RABIA\|RT60)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	0.044	1.435	0.540	0.1	0.9	0.04
2	1.472	4.707	0.408	0.2	0.7	0.08
3	2.136	442.414	11.370	16.8	18.7	0.85
4	2.880	1632.739	32.187	62.1	52.9	0.80
5	4.428	182.071	4.374	6.9	7.2	0.61
6	5.524	22.649	1.993	0.9	3.3	0.22
7	5.932	146.981	1.686	5.6	2.8	0.77
8	7.888	11.493	1.093	0.4	1.8	0.22
9	8.088	27.590	0.931	1.0	1.5	0.60
10	8.672	6.828	0.838	0.3	1.4	0.17
11	8.836	58.693	0.874	2.2	1.4	0.35
12	11.440	9.794	0.453	0.4	0.7	0.10
13	11.744	4.278	0.588	0.2	1.0	0.11
14	12.172	20.046	0.933	0.8	1.5	0.42
15	13.000	23.501	0.687	0.9	1.1	0.16
16	13.688	12.103	0.725	0.5	1.2	0.35
17	14.000	20.673	0.875	0.8	1.4	0.13
18	14.884	1.339	0.264	0.1	0.4	0.10
	Total	2629.335	60.819	100.0	100.0	

[7] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomycetes isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (cont.)



Result Table (Uncal - C:\DOCUMENTS AND SETTINGS\HPLC\DESKTOP\RABIA\RT56)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	0.656	3.376	0.350	0.1	0.2	0.15
2	1.052	15.104	0.687	0.3	0.4	0.28
3	2.060	847.289	28.570	15.1	18.0	0.51
4	2.472	671.893	42.519	12.0	26.8	0.34
5	2.824	3086.538	58.632	54.9	36.9	0.84
6	4.424	222.781	8.649	4.0	5.4	0.54
7	5.000	180.860	4.868	3.2	3.1	0.78
8	5.856	102.819	2.913	1.8	1.8	0.68
9	6.496	24.192	2.583	0.4	1.6	0.16
10	6.692	219.864	3.022	3.9	1.9	0.86
11	8.804	113.917	1.434	2.0	0.9	1.36
12	11.072	14.369	0.815	0.3	0.5	0.14
13	11.424	19.135	0.796	0.3	0.5	0.51
14	12.048	28.939	0.925	0.5	0.6	0.65
15	12.756	46.357	0.959	0.8	0.6	0.81
16	14.524	19.128	0.586	0.3	0.4	0.15
17	14.836	2.893	0.480	0.1	0.3	0.13
	Total	5619.455	158.784	100.0	100.0	

Conclusions

- Endophytic *Streptomyces*
- Isolated from a variety of plants and extensively studied

- *P. hysterophorus*
- Very less work has been done

- Endophytes
  - Diversity of endophytic *Streptomyces* isolated from different plant parts
  - Biological screening revealed
    - Antimicrobial against MDR pathogens
    - Significant zones of inhibitions

Conclusions (cont.)

- Chemical screening revealed
  - Indoles and N-heterocycles
- HPLC-UV
  - Interesting peaks at different retention times
  - UV absorbing non polar bioactive compounds
- Further exploration of this novel ecological niche
  - Novel metabolites
  - Biotechnological industries