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Comparison of the bioactivity of Tulsi and Neem plant extracts with their related endophytic actinomycetes against ESBL producers

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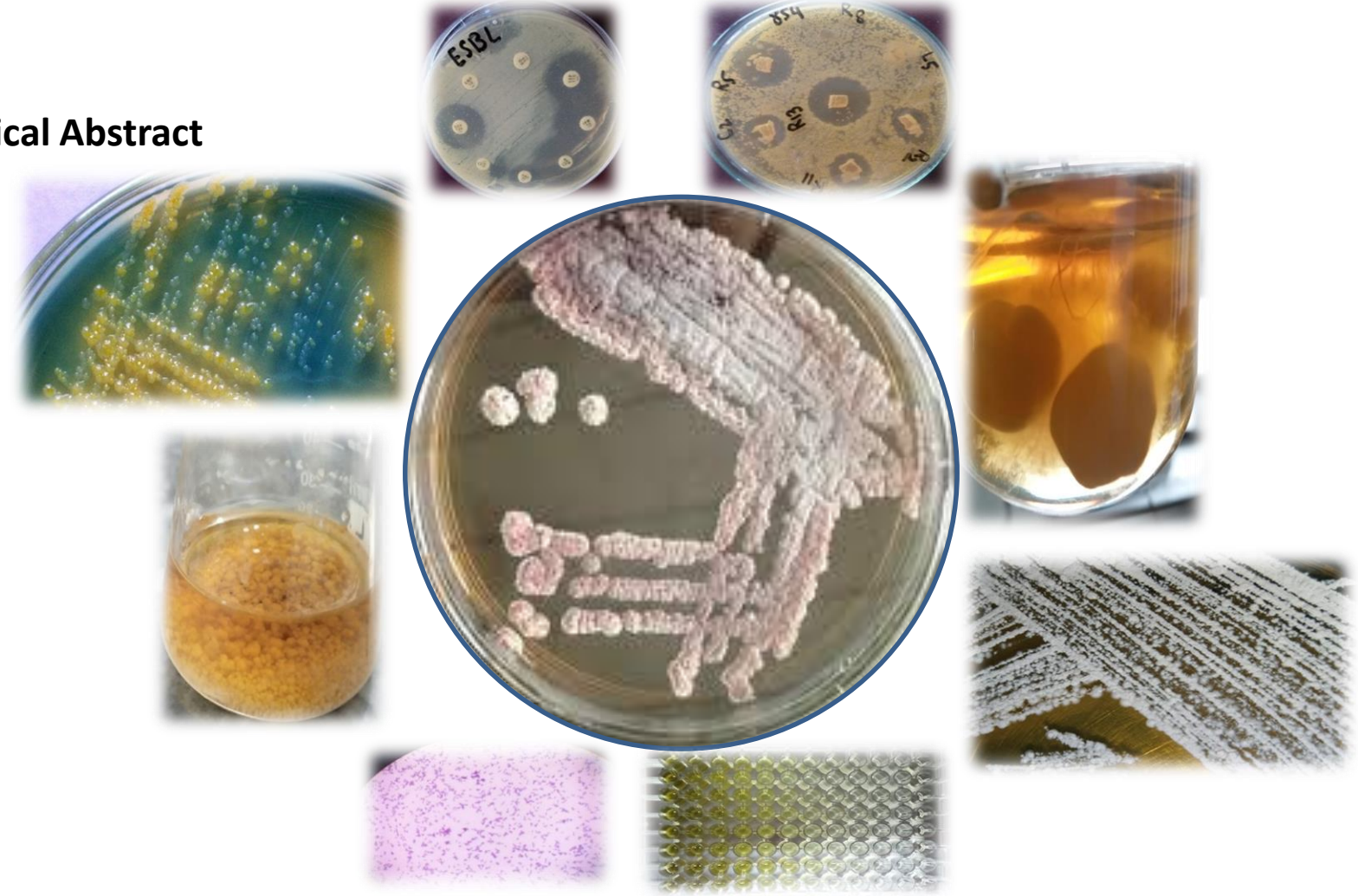
Graphical Abstract



Abstract:
Urinary tract infections (UTIs) are one of the most frequently acquired infections. Due to the emergence of drug resistance mechanisms in the organisms involved, they are becoming extremely difficult to treat. The emergence of drug resistance due to the production of extended-spectrum beta-lactamases (ESBL) is also very common in UTI nowadays. Medicinal plants *Ocmium teniflorum* (Tulsi) and *Azadirachta indica* (Neem) are well-known for their medicinal uses in Unani and Ayurveda medicinal systems. Endophytic actinomycetes confer fitness to the host plant by producing a variety of bioactive metabolites. They are also known to mimic the compounds produced by the plant or produce more potent metabolites due to their exposure to the plant environment. Therefore, the aim of the present study was to compare the antimicrobial activity of endophytic actinomycetes inhabiting tulsi and neem plants with those of the plant extracts against ESBL uropathogens. Preliminary screening displayed 12 endophytes with prominent bioactivity. Interestingly, biological screening of their concentrated broth exhibited excellent inhibitory activity against all ESBL uropathogens i.e. *E. coli* and *Klebsiella pneumoniae.* Further minimum inhibitory concentration (MIC) and minimum bactericidal concentration (MBC) also confirmed the endophytic actinomycetes to be producing more potent antibiotics against ESBL producers as compared to the plant extracts. They inhibited the ESBL producers at a concentration of 100mg/ml. Our study indicated that the endophytic actinomycetes inhabiting tulsi and neem plants are possibly producing more bioactive compounds than the plant itself against ESBL producers involved in urinary tract infections.

Keywords: Actinomycetes; Endophytes; Urinary tract infections

Introduction

- Traditional Medicine
  - Essential role in the healthcare systems
  - In Asian countries
    - 80% of the population is dependent on medicinal practices [1]

- Medicinal Plants
  - Attractive targets for discovering novel therapeutic agents [1]

[1] Miller KI, Ingrey SD, Alvin A, Sze MYD, Roufogalis BD, Neilan BA. Endophytes and the microbial genetics of traditional medicines. Microbiology Australia. 2010;31(2):60-

Introduction(Cont.)

- *Ocimum teniflorum*

- Locally known as ‘Tulsi’
- Widespread throughout the Southeast Asian tropics[2]
- Clinical pharmacological properties of oils and extracts from leaves
  - Antibacterial, anti-inflammatory, anti-pyretic, anti-diabetic, antifungal and anti stress effects [3]

Ocimum teniflorum growing in the nursery of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. Photo courtesy: Ms. Hafsa Shahzadi

[2] Warrier P, Nambiar V, Ramankutty C. Indian Medicinal Plants: A Compendium of 500 Species, vol. III, Orient Longman Pvt. Ltd, Anna Salai, Chennai, India. 1995:38-42.
[3] Singh E, Sharma S, Dwivedi J, Sharma S. 2012. Diversified potentials of Ocimum sanctum Linn (Tulsi): An exhaustive survey. J Nat Prod Plant Resour. 2(1): 39-48.

Introduction(Cont.)

- *Azadirachta indica*

- Locally known as ‘Neem’
- Native to India, Pakistan, Bangladesh, Burma, Malaysia and Sri lanka [4]
- Clinical pharmacological properties
  - Antibacterial, anti inflammatory, antiulcer, antiviral, anti-diabetic and sedative properties [5]

Azadirachta indica growing in the nursery of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. Photo courtesy: Ms. Hafsa Shahzadi

[4] Babu KS, Naik VKM, Latha J, Ramanjaneyulu K. 2016. Pharmacological review on natural products (*Azadirachta indica* Linn). IJCS. 4(5): 01-04.

[5] Agrawal D. Medicinal properties of neem: new findings. History of Indian science and. 2001.

Introduction (Cont.)

- Actinomycetes
  - Greek ‘aktis’ (a ray) and ‘mykes’ (fungus)
  - Producers of a broad array of secondary metabolites
    - Useful applications in veterinary and human medicine [6]

Endophytic actinomycetes strain EH-11 isolated from *Ocimum teniflorum* (Tulsi). Photo courtesy: Ms. Hafsa Shahzadi

- Endophytic Actinomycetes
  - Starting platform
  - Antibiotics, enzyme, anticancer agents, immunomodulators, anthelminthic agents,
  - Long-held alliance, plants and endophytic microorganisms develop good information transfer [7]

[6] Janso JE, Carter GT. 2010. Biosynthetic potential of phylogenetically unique endophytic actinomycetes from tropical plants. J Appl Environ Microbiol 76(13): 4377-4386.
[7] Zhao K, Penttinen P, Guan T, Xiao J, Chen Q, Xu J, Lindström K, Zhang L, Zhang X, Strobel GA. 2011. The diversity and anti-microbial activity of endophytic actinomycetes isolated from medicinal plants in Panxi plateau, China. Curr Microbiol.
62(1): 182-190.

Introduction (Cont.)

- ESBL Producers from Urinary Tract Infections
  - Most common bacterial infection
  - Becoming tough to treat [8]

- Members of Enterobacteriaceae
  - Of particular concern are *Escherichia coli and Klebsiella pneumoniae*
  - These have acquired plasmids that encodes extended-spectrum β-lactamases (ESBLs) [8]

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

Results and discussion

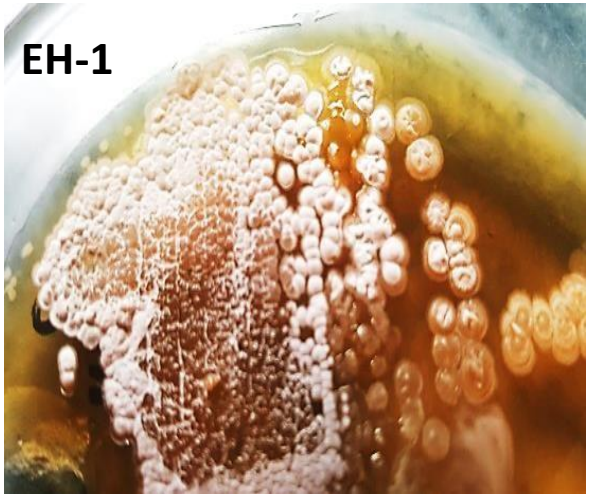
- Total 28 endophytic actinomycetes were isolated from the previous project [8]
- Selected isolates were sub-cultured on Glucose yeast extract malt extract (GYM) agar plates

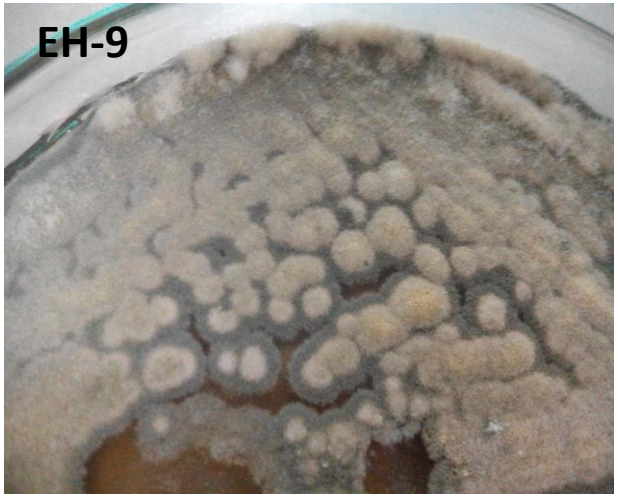
Endophytic actinomycetes from medicinal plants of Punjab

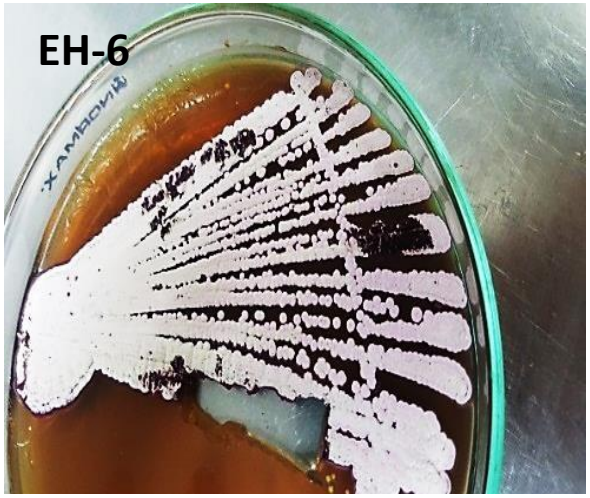
Groups	No. of Isolates
Azadirachta indica (Neem)	6
Ocimum teniflorum (Tulsi)	12

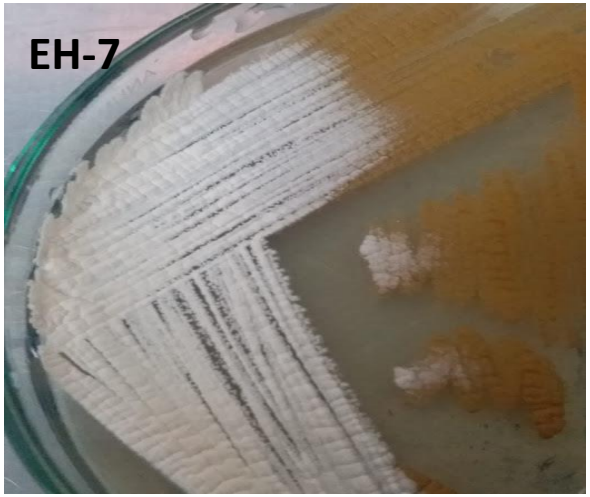
[8] Tanvir, R., I. Sajid, and S. Hasnain, *Screening of endophytic Streptomyces isolated from Parthenium hysterophorus L. against nosocomial pathogens.* Pak J Pharm Sci, 2013. **26**: p. 277-283

Results and discussion (Cont.)









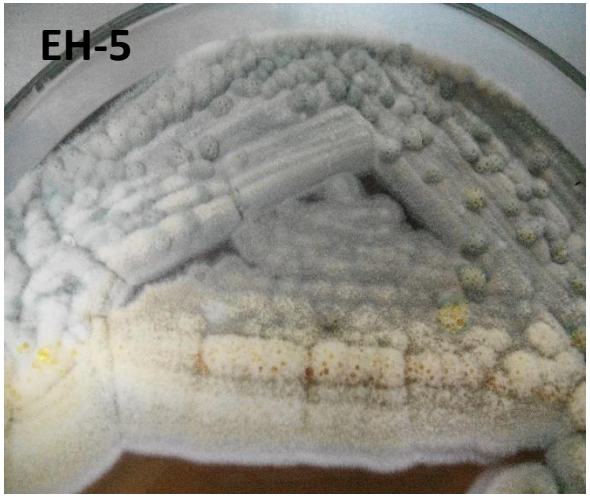
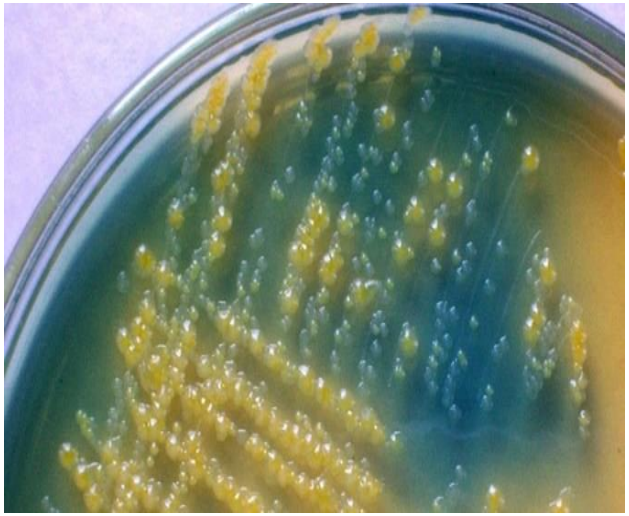


Photo courtesy: Ms. Hafsa Shahzadi

Results and discussion (Cont.)

- Isolation and Identification of ESBL producers
  - Lactose fermenter colonies were obtained on CLED media
  - Purified growth on MacConkey agar



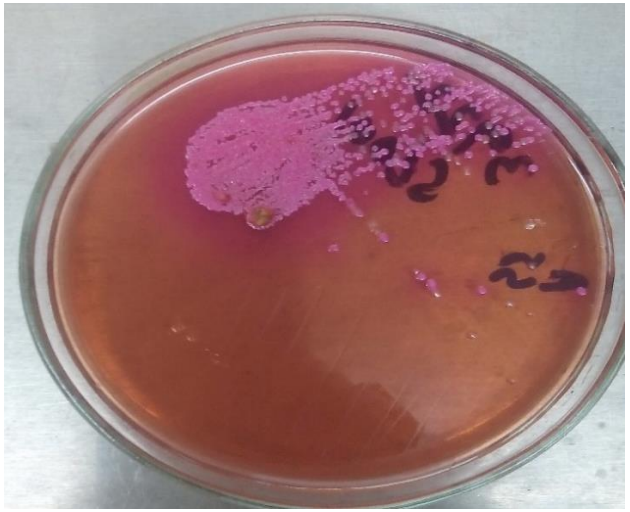


Photo courtesy: Ms. Hafsa Shahzadi

Results and discussion (Cont.)

- Antibiotic profiling for ESBL Producers
  - Antibiotic susceptibility testing
  - 100% of the isolates were resistant to at least one of the tested antimicrobials

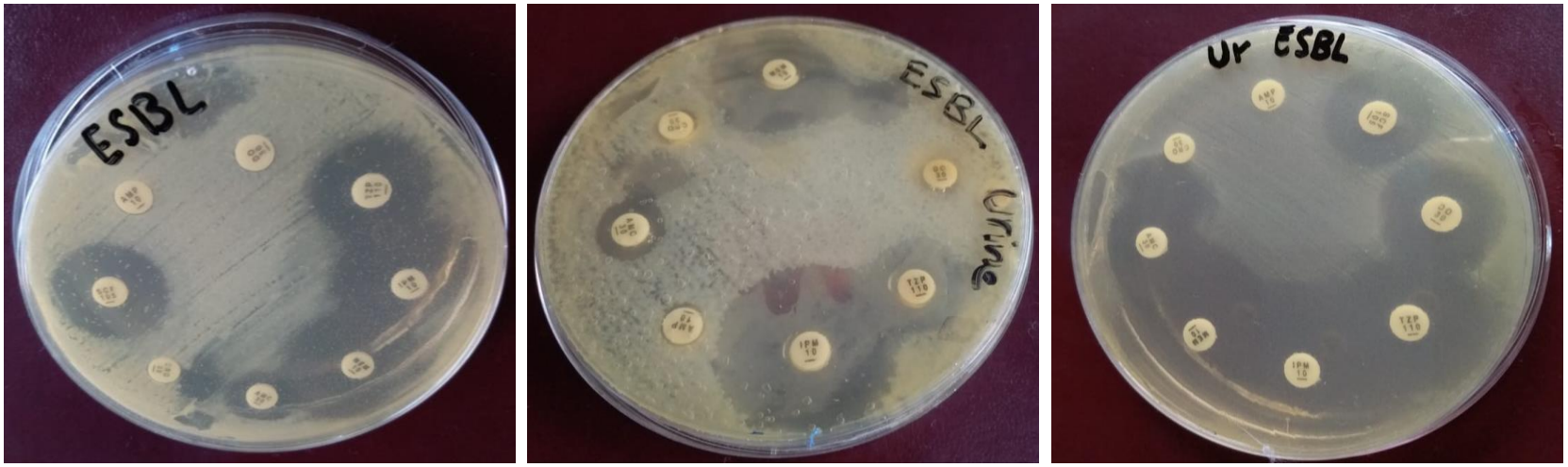


Photo courtesy: Ms. Hafsa Shahzadi

Results and discussion (Cont.)

- Screening of endophytic actinomycetes
  Concentrated Broth culture [9]
    - 100 mg/ml concentration
    - Extracts prepared in 0.1% DMSO

- Biological screening
    - Agar plug method [10]
    - Agar well diffusion method [11]
        - ESBL producers

[9] 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.
[10] Balouiri M, Sadiki M, Ibnsouda SK. Methods for in vitro evaluating antimicrobial activity: A review. Journal of pharmaceutical analysis. 2016;6(2):71-9.
[11] Gebreyhannes, 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.)

- Preliminary antimicrobial activity against ESBL producers from urinary tract infections using agar plug method
  - Broad spectrum activity
  - Maximum zones of inhibition of 25mm

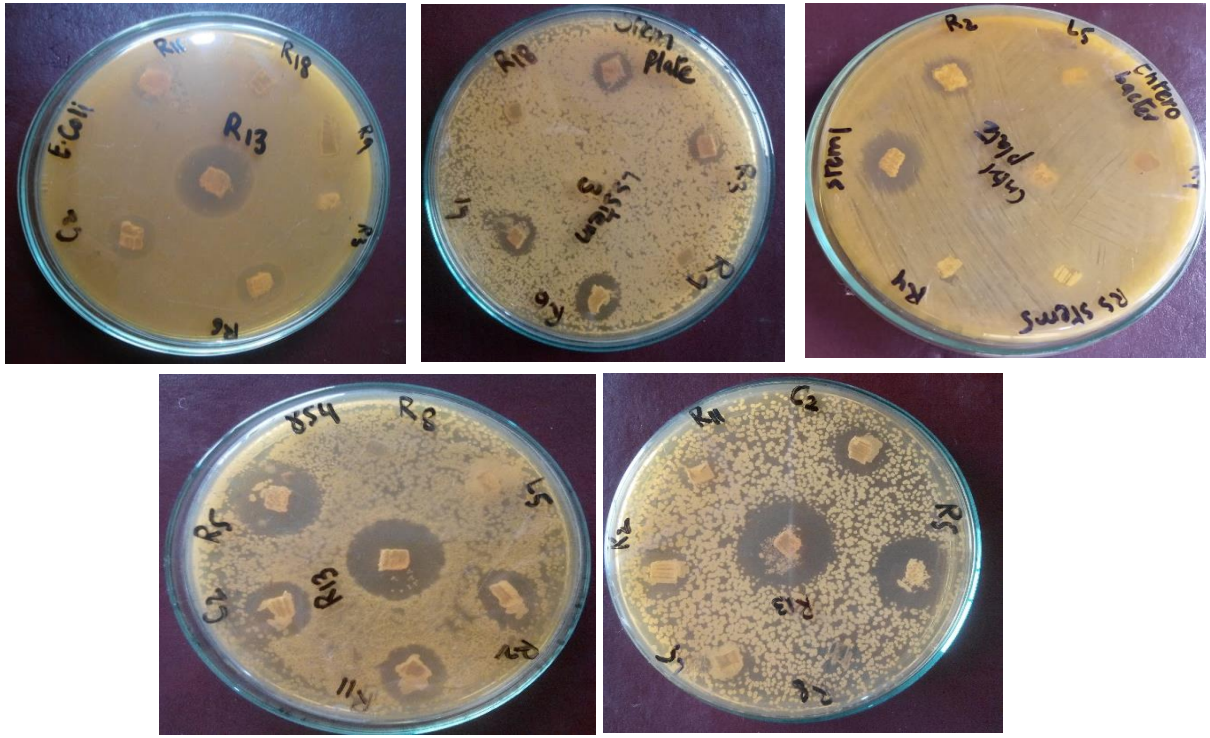


Photo courtesy: Ms. Hafsa Shahzadi

Results and discussion (Cont.)

- Antimicrobial activity against ESBL producers from urinary tract infections using agar well method
  - Broad spectrum antibacterial activity
  - Maximum zones of inhibition of 16mm

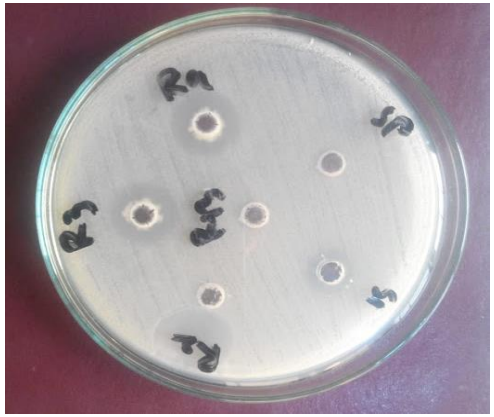


Photo courtesy: Ms. Hafsa Shahzadi

Results and discussion (Cont.)

Antimicrobial activity of endophytic actinomycetes concentrated broth by agar well diffusion method

Endophytic actinomycetes strain code (100 mg/ml)	Zone of Inhibition (mm) against ESBL producers							
	UO-1	UO-2	UO-3	UO-4	UO-5	UO-6	UO-7	UO-8
EH-1	_	10	6	7	8.6	10	_	6.4
EH-3	9	_	13	8	10	7	11	10
EH-9	_	_	15	8.3	8	8.7	9	8
EH-10	_	9	_	6	_	13	_	9
EH-15	13	16	11	10	12	15	13	11

Key: UO-1=*K. pneumoniae*; UO-2= *E. aerogenes*; UO-3= *E. coli*; UO-4= *E. coli*; UO-5= *K. pneumoniae*; UO-6= *K. pneumoniae*; UO-7= *E. coli*; UO-8= *E. coli*; (-) = no zone of inhibition; values are means of triplicate studies

Results and discussion (Cont.)

- Antimicrobial activity against ESBL producers from urinary tract infections using agar well method
  - Maximum zones of inhibition of 12.9mm

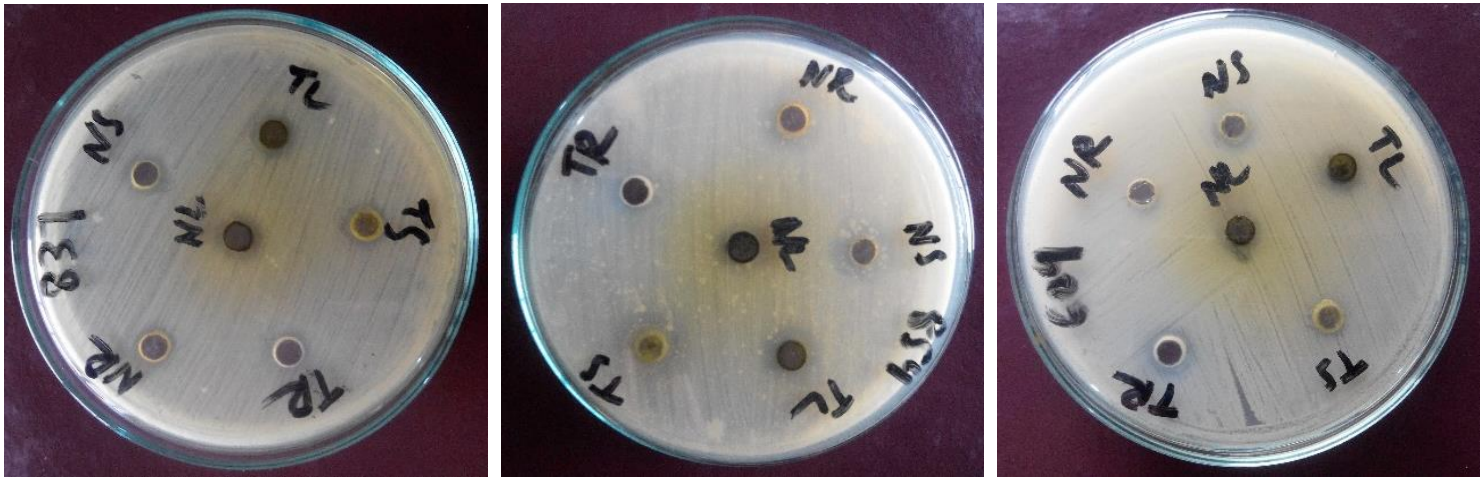


Photo courtesy: Ms. Hafsa Shahzadi

Results and discussion (Cont.)

Antimicrobial activity of plant extracts of *Ocimum teniflorum* (Tulsi) and *Azadirachta indica* (Neem) by agar well diffusion method

Plant type	Extracts of plant parts	Zone of Inhibition against ESBL producers							
		UO-1	UO-2	UO-3	UO-4	UO-5	UO-6	UO-7	UO-8
Azadirachta indica (Neem)	Leaves	8.1	10	11	8.4	6.8	11	_	10
	Roots	9	_	11.3	6	_	11.4	_	7.6
	Shoots	10.3	11.3	6.3	7.1	_	11.4	7.1	10.3
Ocimum teniflorum (Tulsi)	Leaves	8	10	12	8.2	9	12.1	10	9.5
	Roots	9.4	9	11.4	_	8.5	9.6	6.8	7.1
	Shoots	_	7.4	_	8	_	12.9	9	9.2

Key: UO-1=*K. pneumoniae*; UO-2= *E. aerogenes*; UO-3= *E. coli*; UO-4= *E. coli*; UO-5= *K. pneumoniae*; UO-6= *K. pneumoniae*; UO-7= *E. coli*; UO-8= *E. coli* ;(-) = no zone of inhibition; values are means of triplicate studies

Results and discussion (Cont.)

- Minimum inhibitory concentration (MIC) of plant extracts (Tulsi, Neem) against extended spectrum beta-lactamase (ESBL) producing strains
- MIC of plant extracts was determined for a range of concentrations from 0 to 256 µg/ml

Plant type	Extract of plant parts	MIC against ESBL producers							
		UO-1	UO-2	UO-3	UO-4	UO-5	UO-6	UO-7	UO-8
Azadirachta indica (Neem)	Leaves	64	64	64	64	128	32	256	128
	Shoots	8	8	16	16	16	8	16	16
	Roots	16	8	16	16	16	16	16	16
Ocimum teniflorum (Tulsi)	Leaves	16	16	16	16	32	32	64	4
	Shoots	8	8	8	8	8	8	8	8
	Roots	32	8	8	32	16	8	16	4

Key: UO-1=*K. pneumoniae*; UO-2= *E. aerogenes*; UO-3= *E. coli*; UO-4= *E. coli*; UO-5= *K. pneumoniae*; UO-6= *K. pneumoniae*; UO-7= *E. coli*; UO-8= *E. coli*

Results and discussion (Cont.)

- Minimum inhibitory concentration (MIC) of endophytic actinomycetes concentrated broth against extended spectrum beta-lactamase (ESBL) producing strains
- MIC of endophytic actinomycetes was determined for a range of concentrations from 0 to 256 µg/ml

Endophytic actinomycetes strain code	MIC against ESBL producers							
	UO-1	UO-2	UO-3	UO-4	UO-5	UO-6	UO-7	UO-8
EH-1	2	2	8	4	8	4	2	2
EH-3	4	4	8	4	8	8	4	4
EH-9	4	4	4	0	4	2	4	2
EH-10	4	2	4	4	4	4	2	2
EH-15	4	8	8	4	8	8	4	2

Key: UO-1=*K. pneumoniae*; UO-2= *E. aerogenes*; UO-3= *E. coli*; UO-4= *E. coli*; UO-5= *K. pneumoniae*; UO-6= *K. pneumoniae*; UO-7= *E. coli*; UO-8= *E. coli.*

Results and discussion (Cont.)

- Minimum bactericidal concentration (MBC) of *Azadirachta indica* (Neem) and *Ocimum teniflorum* (Tulsi) extracts against extended spectrum beta-lactamase (ESBL) producing strains
  - More bacteriostatic effects than bactericidal effect

Plant type	Extract of plant parts	MBC against ESBL producers							
		UO-1	UO-2	UO-3	UO-4	UO-5	UO-6	UO-7	UO-8
Azadirachta indica (Neem)	Leaves	2*	2*	4*	2*	2*	2*	2*	2*
	Shoots	2*	2*	4*	2*	4**	2*	2*	2*
	Roots	2*	2*	4*	2*	4*	2*	2*	2*
Ocimum teniflorum (Tulsi)	Leaves	2*	2*	2*	2*	4**	2*	2*	2*
	Shoots	2*	2*	4*	2*	4*	2*	2*	2*
	Roots	2*	2*	4*	2*	4*	2*	2*	2*

Key: UO-1=*K. pneumoniae*; UO-2= *E. aerogenes*; UO-3= *E. coli*; UO-4= *E. coli*; UO-5= *K. pneumoniae*; UO-6= *K. pneumoniae*; UO-7= *E. coli*; UO-8= *E. coli;* MBC was determined for a range of concentrations from 0 to 8µg/ml, 0= no inhibition at any range; * =bacteriostatic; **=bactericidal

Results and discussion (Cont.)

- Minimum bactericidal concentration (MBC) of endophytic actinomycetes concentrated broth culture against extended spectrum beta-lactamase (ESBL) producing strains
  - More bactericidal potential than plant extracts
  - Significant bactericidal activity against *K. pneumoniae*

Endophytic actinomycetes strain code	MBC against ESBL producers							
	UO-1	UO-2	UO-3	UO-4	UO-5	UO-6	UO-7	UO-8
EH-1	4*	8*	4**	4*	4**	8*	8*	4*
EH-3	4*	4*	2*	8*	4*	2*	2*	2*
EH-9	2*	4*	4*	4*	4*	4*	2*	2*
EH-10	4*	4**	4*	8*	2*	4*	4*	4*
EH-15	4*	4*	4**	4*	8**	4*	4**	2*

Key: UO-1=*K. pneumoniae*; UO-2= *E. aerogenes*; UO-3= *E. coli*; UO-4= *E. coli*; UO-5= *K. pneumoniae*; UO-6= *K. pneumoniae*; UO-7= *E. coli*; UO-8= *E. coli;* MBC was determined for a range of concentrations from 0 to 8µg/ml, 0= no inhibition at any range; * =bacteriostatic; **=bactericidal

Conclusions

- Endophytic actinomycetes from *Ocmium teniflorum* (Tulsi) and *Azadirachta indica* (Neem)
  - Rarely studied

- Our study
  - Endophytic actinomycetes inhabits medicinal plants of Pakistan
  - Biological screening revealed
    - Broad spectrum bioactivity
    - Bioactive against ESBL producers involved in Urinary tract infections
      - *E.coli, Klebsiella pneumoniae* and *Enterobacter aerogenes*

Conclusions

- Comparison between extracts revealed
  - Endophytic actinomycetes are producing more bioactive compounds than the plants
  - Potent against ESBL producers

- Further exploration of these strains
  - Antiviral activity particularly against Covid-19
  - HPLC-MS and NMR for determination of active compounds

Acknowledgment

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