4th International Electronic Conference on Medicinal Chemistry
1-30 November 2018
chaired by Dr. Jean Jacques Vanden Eynde
sponsored by pharmaceuticals

The effects of polar and non polar compounds from endophytic actinomycetes in *Ocmium tenuiflorum* (Tulsi) and *Azadirachta indica* (Neem) on veterinary and human pathogens

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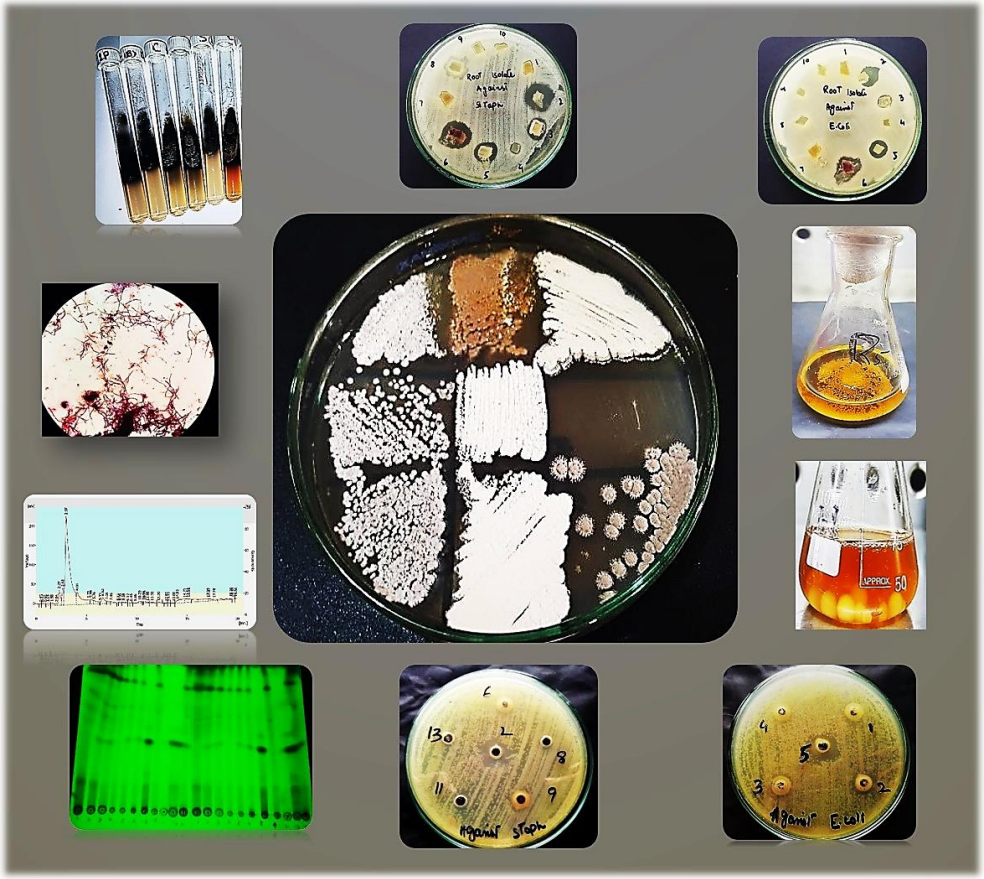
The effects of polar and non polar compounds from endophytic actinomycetes in *Ocmium tenuiflorum* (Tulsi) and *Azadirachta indica* (Neem) on veterinary and human pathogens

Graphical Abstract



Abstract:

Ocmium teniflorum (Local name: Tulsi, Family: Lamiaceae) is a plant well known for its medicinal uses in unani and ayuveda medicine. It is called the 'queen of herbs' for its antimicrobial, antiinflammatory, hypotensive, hypoglycemic antipyretic and analgesic activities. *Azadirachta indica* (Local name: Neem, Family: Meliaceae) is a medicinal plant best known for its antimicrobial, antiinflammatory, anticancer and antiviral activities. It is a fact that the individual exudates of each plant attracts a specific specie of microbes and induces them to produce possible novel compounds under the influence of the plant environment. With this idea in mind, we investigated the antimicrobial activity of endophytic actinomycetes inhabiting tulsi and neem plants. The preliminary screening was done using agar plug method and it displayed 12 isolates with prominent bioactivity. Further biological screening of their active metabolites showed that the compounds were most active against *Salmonella enteritidis, Campylobacter jejuni* and *Proteus mirabilis.* The thin layer chromatography (TLC) and high performance liquid chromatography-UV (HPLC-UV) displayed UV absorbing polar and non polar compounds. Our study reveals tulsi and neem plant microenvironment as an unexplored niche harboring endophytes that are prominently bioactive against multidrug resistant (MDR) poultry and human pathogens.

Keywords: Actinomycetes; Endophytes; Neem*;* Tulsi

Introduction

- Traditional medicine
  - Essential role in the healthcare systems
  - In Asian countries
    - 80% of the population 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):60-3.

Introduction (Cont.)

- *Ocmium teniflorum*

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

Ocmium tenuiflorum **growing in the nursery of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. Photo courtesy: Ms. Fatima Nawaz**

[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* A. Juss

- Locally known as ‘Neem’
- Native to Pakistan, India, Bangladesh, Burma, Malaysia and Sri Lanka [4]
- Clinical pharmacological properties
  - Antifungal, antibacterial, anti-diabetic, antiviral, anthelmintic and sedative properties [5]

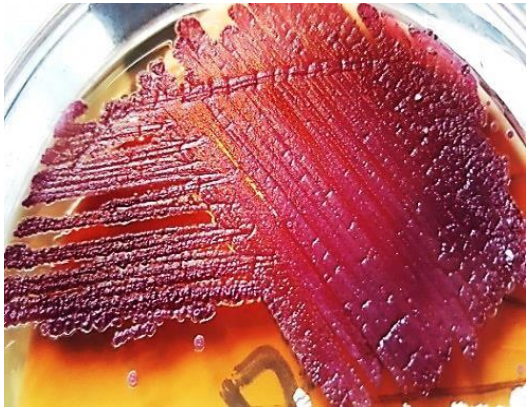
Azadirachta indica **growing in the nursery of University of Veterinary and Animal Sciences (UVAS), Lahore Pakistan. Photo courtesy: Ms. Fatima Nawaz**

[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 FHK-6 isolated from *Ocmium tenuiflorum* (Tulsi). Photo courtesy: Ms. Fatima Nawaz

- 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.

Results and discussion

- Total 28 endophytic *actinomycetes* were isolated [8]

- Frequency of isolation
  - *Ocimum tenuiflorum* (Tulsi)
    - Roots = 03
    - Roots slurry= 10
    - Shoots = 02
    - Shoot slurry = 06
    - Leaves slurry = 01

    - Total = 22

[8] 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.)

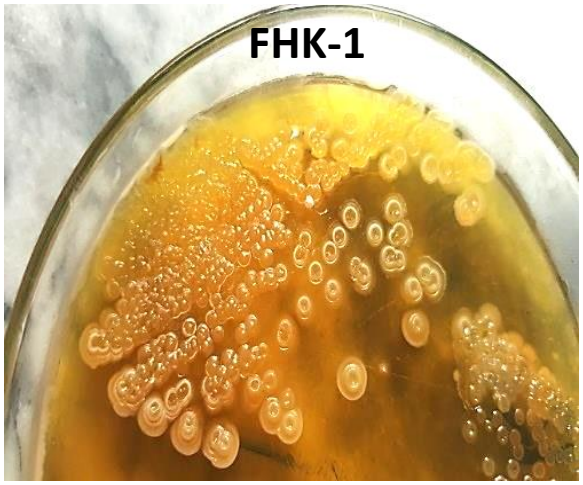
- Total 28 endophytic *actinomycetes* were isolated [8]

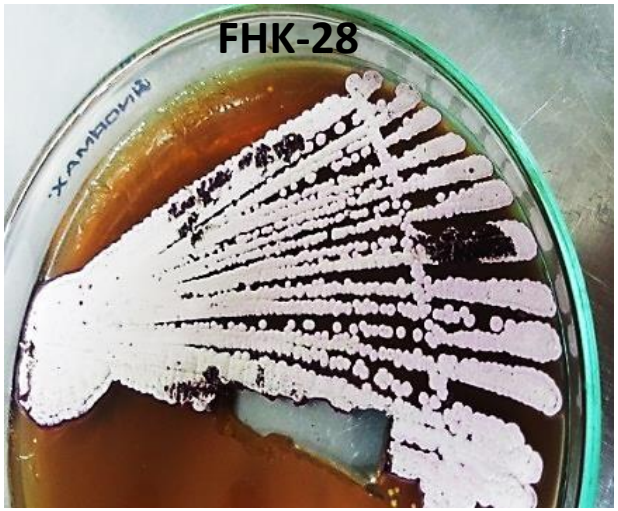
- Frequency of isolation
  - *Azadirachta indica* (Neem)
    - Roots slurry= 05
    - Shoot slurry = 01

    - Total = 06

[8] 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.)





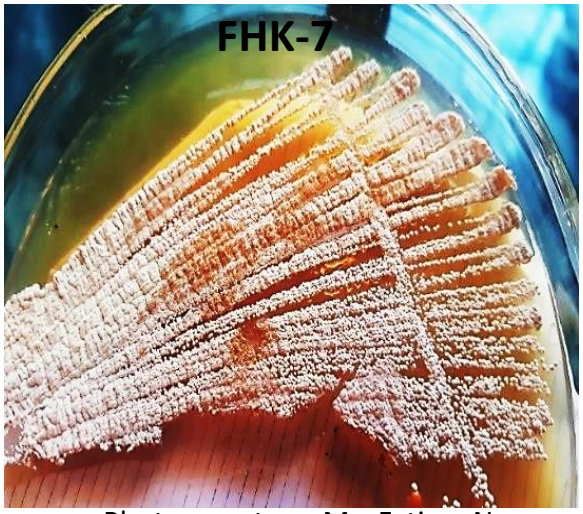


Photo courtesy: Ms. Fatima Nawaz

Results and discussion (Cont.)

- Physiological characterization
  - Melanin production test [8]
    - Melanin producers = 15
    - Non melanin producers = 11

Photo courtesy: Ms. Fatima Nawaz

[8] 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
  - Utilization of sugars and similar compounds [8]
    - Strains able to utilize 6 sugars = 11

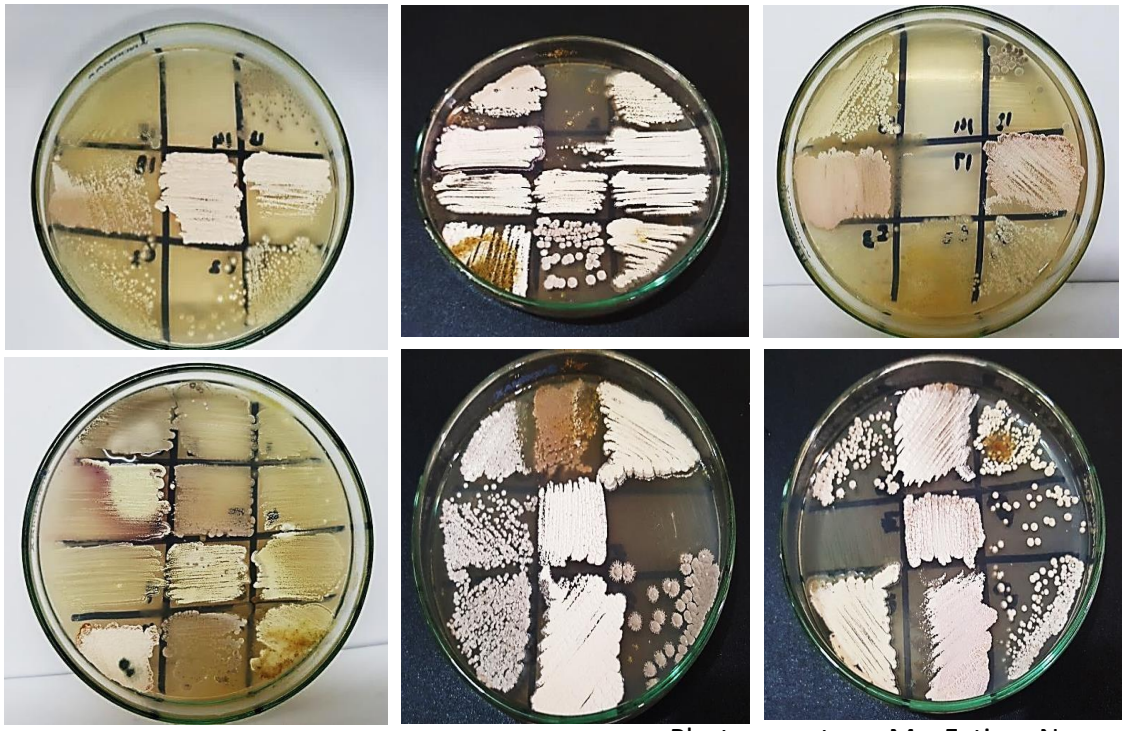


Photo courtesy: Ms. Fatima Nawaz

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Results and discussion (Cont.)

- Screening of endophytic actinomycetes
  - Crude extraction
    - 1:1 ethyl acetate [9]
    - Extracts stored in absolute methanol

- Biological screening
    - Agar plug method [10]
    - Agar well diffusion method [11]
      - MDR Veterinary and human pathogens

[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 Gram positive and Gram negative bacteria using agar plug method
  - Broad spectrum activity
  - Maximum zones of inhibition of 20mm

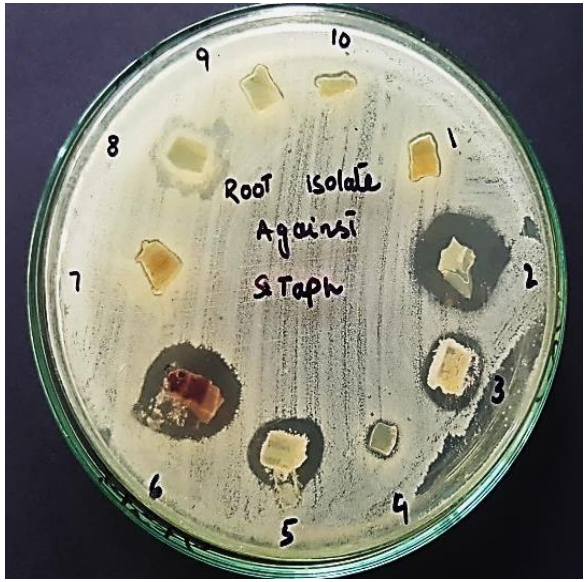


Photo courtesy: Ms. Fatima Nawaz

Results and discussion (Cont.)

- Antimicrobial activity against veterinary and human pathogens using agar well method
  - Maximum zones of inhibition of 18mm

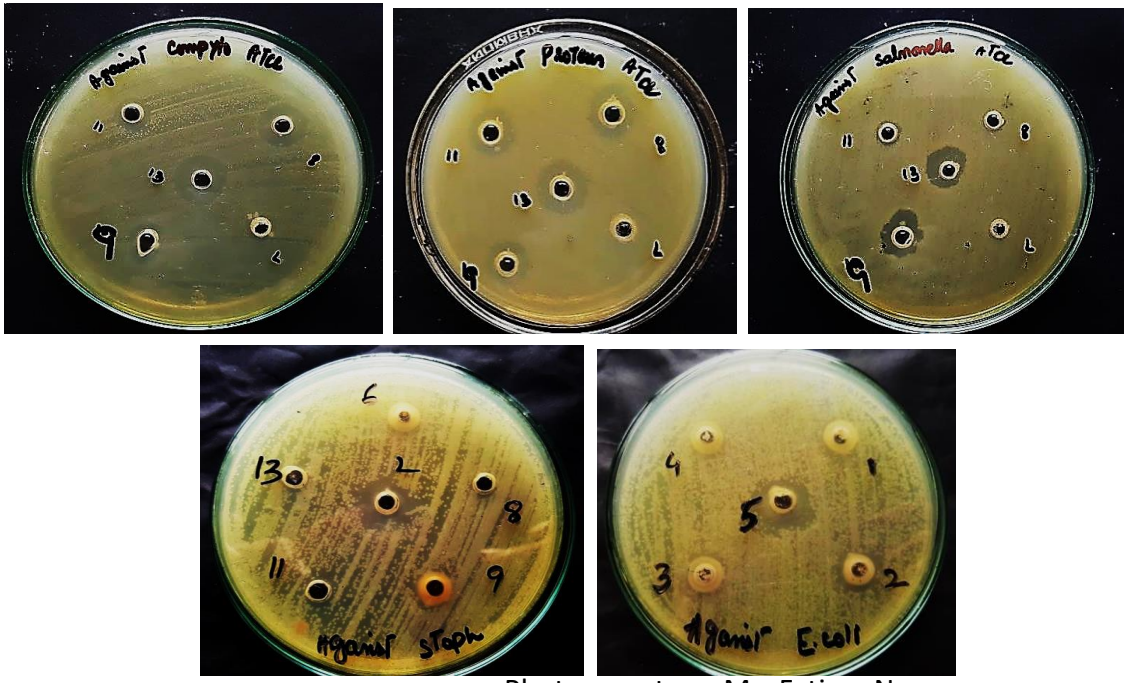


Photo courtesy: Ms. Fatima Nawaz

Results and discussion (Cont.)

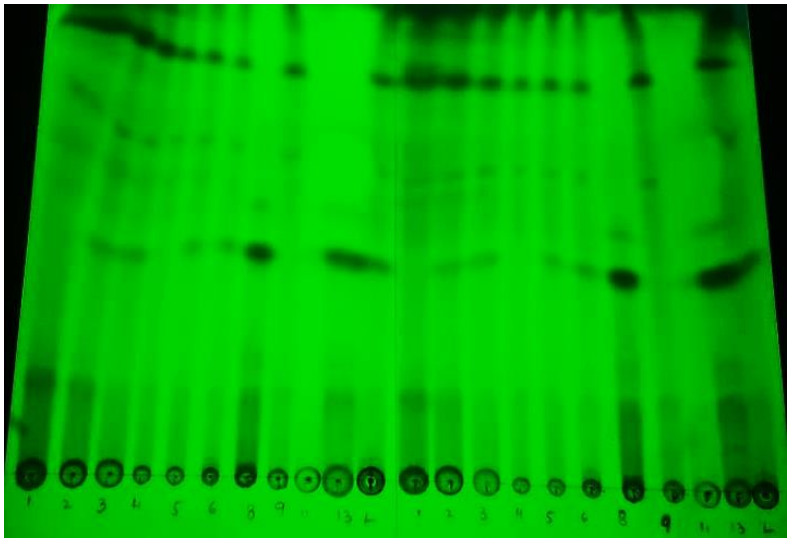
Antimicrobial testing for polar and non polar compounds in crude extracts

Strain code	Zone of inhibition (mm)					
	E. coli	*C. jejunii*	*P. mirabilis*	*Staph. aureus*	*S. enteritidis*	*A. baumanii*
FHK-1	-	-	-	11.6	10.6	-
FHK-2	6.3	-	6.3	10	17.6	-
FHK-3	6.3	11	8.6	11	13	6
FHK-4	5.3	9.6	8.3	9	17	6
FHK-5	6.6	10.6	6.6	6.6	10.3	-
FHK-6	10.6	13.6	8.3	8.3	12.3	-
FHK-7	4	6	6.6	6.6	5.3	5
FHK-8	6	10	11.3	11.3	7.6	4.3
FHK-9	11.3	9.6	8.6	8.6	9.6	11.3
FHK-11	6.6	9.6	10	10	8	6.6
FHK-13	3.6	12.3	12.6	12.6	10.6	5.6
FHK-28	6	3	-	17	10.3	7.6

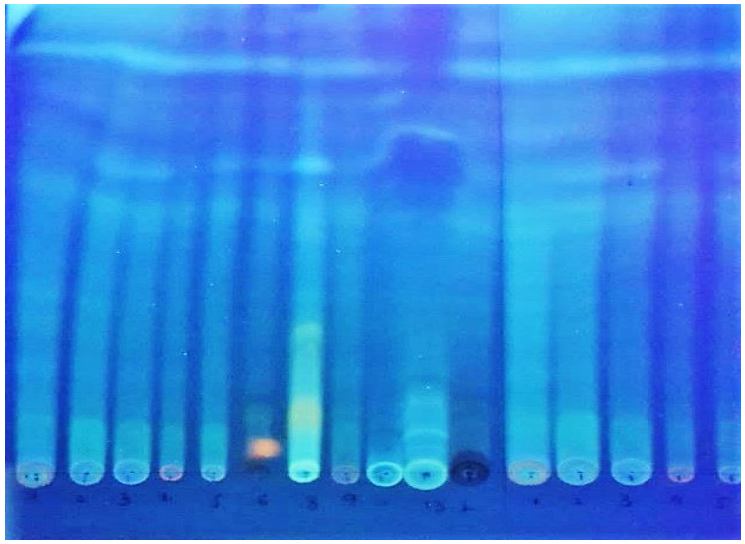
Key: *E.coli= Escherichia coli*; *C. jejuni = Campylobacter jejuni; P.mirabilis = Proteus mirabilis; Staph aureus = Staphylococcus aureus; S. enteritidis = Salmonella enteritidis*; *A. baumanii = Acinetobacter baumanii*

Results and discussion (Cont.)

- Bioactive compound diversity through chemical screening
  - Thin Layer Chromatography (TLC) [8]
  - 366 nm and 254nm
  - Polar, medium polar and non polar compounds



(a) 254nm

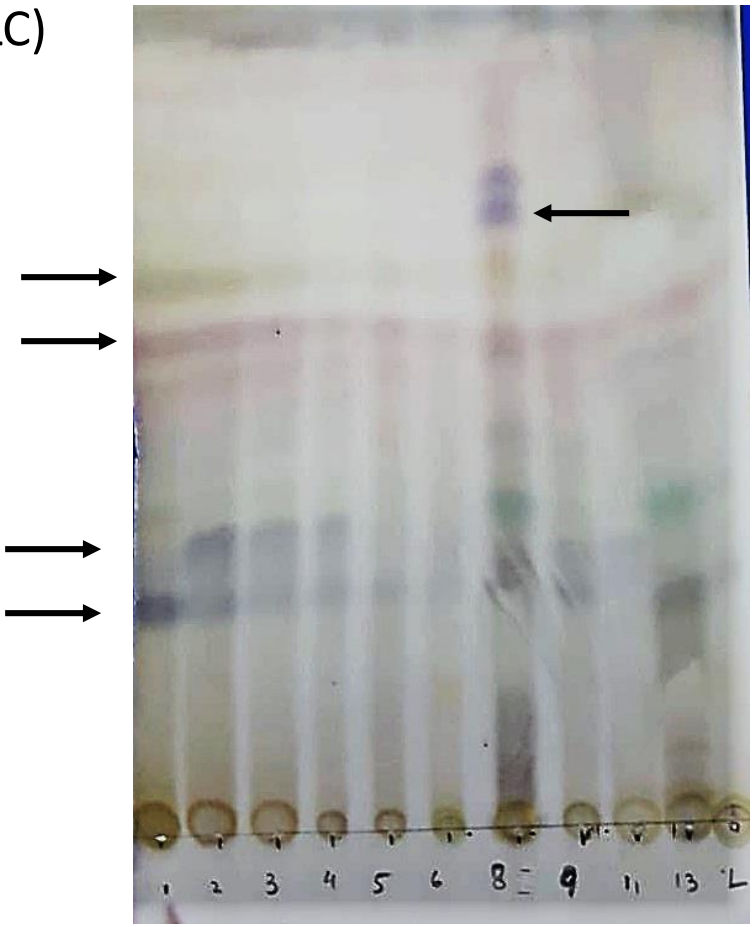


(b) 366nm

[8] 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.)

- Bioactive compound diversity through chemical screening
  - Thin Layer Chromatography (TLC)
  - Anisaldehyde/H_2SO_4 reagent staining [8]
  - Purple and red spot (Indoles), yellow spot (N-heterocycles)



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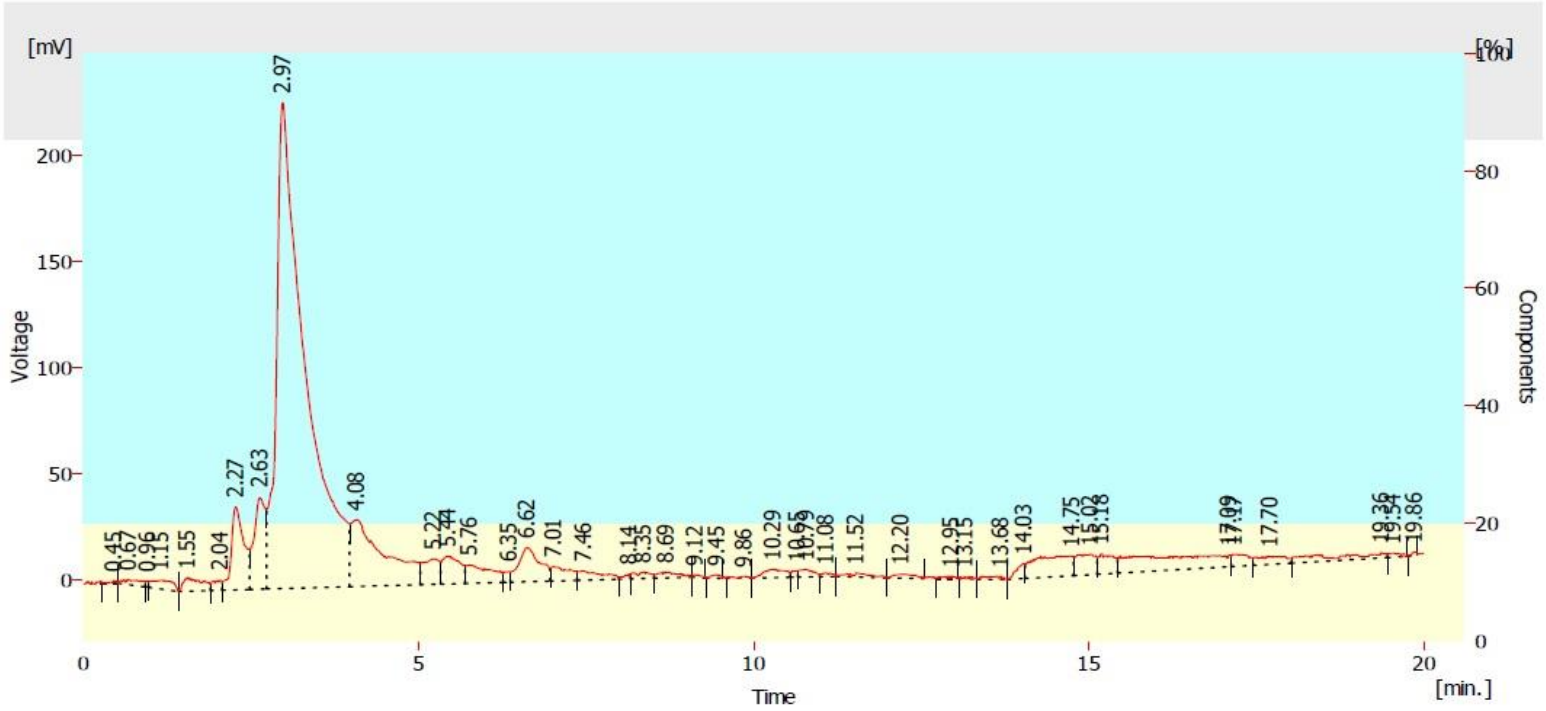
Results and discussion (Cont.)

- High Performance Liquid Chromatography (HPLC-UV) [8]
  - UV absorbance of 254nm
  - A prominent peak of non polar compound at Rt 2.97



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Results and discussion (Cont.)

- High Performance Liquid Chromatography (HPLC-UV)
  - Different peak observed at UV absorbance of 254nm

Result Table (Uncal - FATIMA-13)

	Reten. Time [min]	Area [mV.s]	Height [mV]	Area [%]	Height [%]	W05 [min]
1	0.452	7.175	1.201	0.1	0.2	0.07
2	0.672	47.508	2.534	0.4	0.5	0.28
3	0.956	8.612	3.142	0.1	0.6	0.05
4	1.152	93.469	4.127	0.7	0.8	0.40
5	1.552	124.252	6.323	1.0	1.2	0.43
6	2.040	43.301	4.832	0.3	0.9	0.18
7	2.272	509.637	39.050	4.0	7.5	0.24
8	2.632	505.630	43.181	3.9	8.3	0.21
9	2.972	6834.531	228.864	53.0	44.1	0.39
10	4.076	1108.921	31.435	8.6	6.1	0.46
11	5.224	202.600	11.982	1.6	2.3	0.30
12	5.440	244.553	13.148	1.9	2.5	0.36
13	5.760	226.926	8.612	1.8	1.7	0.57
14	6.348	31.431	5.076	0.2	1.0	0.11
15	6.620	355.158	16.037	2.8	3.1	0.41
16	7.012	130.118	6.898	1.0	1.3	0.40
17	7.460	109.295	4.389	0.8	0.8	0.58
18	8.144	14.543	2.264	0.1	0.4	0.09
19	8.352	50.804	3.039	0.4	0.6	0.24
20	8.692	59.036	2.773	0.5	0.5	0.42
21	9.124	10.689	1.227	0.1	0.2	0.14
22	9.448	14.047	1.579	0.1	0.3	0.17
23	9.856	10.042	0.780	0.1	0.2	0.13
24	10.292	87.518	3.882	0.7	0.7	0.42
25	10.652	18.635	2.985	0.1	0.6	0.11
26	10.788	54.238	3.549	0.4	0.7	0.29

Conclusions

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

- Our study
  - Diversity of actinomycetes reside in different plant parts
  - Biological screening revealed
    - Broad spectrum activity
    - Bioactive against veterinary and human pathogens
      - » *Campylobacter jejuni; Proteus mirabilis; Staphylococcus aureus; Salmonella enteritidis*; *Acinetobacter baumanii*

Conclusions

- Metabolically diverse compounds
  - UV absorbing
  - Polar, medium polar and non polar
  - Indoles and N-heterocycles

- Further exploration of these strains
  - Large scale study
  - Further bioactivity screening
  - HPLC-MS and NMR