Bioprospecting of Asteraceae medicinal plants of Pakistan for their associated bioactive endophytic actinomycetes for new drug targets

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Graphical Abstract
Abstract:
Since the beginning of mankind, plants have been used as the source of medicinal agents thereby becoming a major course to discovering new drugs. The practice of using traditional medicine is prevalent in Pakistan that has a rich history of herbal plants being used by Hakims in folk medicine (Unani medicine). The Asteraceae family is the largest plant family in Pakistan, with plants of considerable medicinal importance. Endophytes include all organisms that symptomlessly colonize the living internal tissues of their hosts during a variable period of their lifetime. There they produce a broad variety of bioactive secondary metabolites with unique structure that are advantageous for the plant. Endophytic actinomycetes also colonize the internal tissues of plants without causing any visible changes or damage. They exploit an unusual habitat and considering this, this may enable them to possess the potential to produce bioactive compounds as similar to their host plant. Our study explores the bioprospecting potential related to endophytic actinomycetes of Asteraceae medicinal plants of Pakistan. After isolation and identification the endophytes were screened for their bioactive metabolites potential for new drug targets. This included extensive biological and chemical screening. The bioactive compounds were purified through column chromatography and final identification was done through HPLC-MS and NMR. The purified compounds were observed to be extremely potent with promising antimicrobial potential against major pathogens including algae and fungal strains as well as possessing antioxidant and cytotoxic potential.

Keywords: Actinomycetes; Asteraceae; Endophytes; Medicinal plants; Pakistan
Introduction

• Natural products
  • Compounds acquired from natural sources
    • Such as from plants, animals and microorganisms [1]

• Medicinal plants as sources of new natural products
  • Such products are
    • Easily accessible and having less side effects, toxicity, and better biodegradability
    • Cheaper, safer and environmentally friendly [2]
    • Structurally unique active compounds
    • Potential for new drug discovery [3]

Introduction (Cont.)

• Pakistan
  • Rich in medicinal herbs
    • Traditionally used to prepare herbal medicines by Hakims (Traditional physicians)
  • Used for centuries in the Unani system of medicine (folk medicine)
  • About 80% of the population is dependent on it
    • Particularly in villages and rural areas [4]

• Asteraceae plant family
• Dicotyledonous flowering plants
• Largest plant family in Pakistan
  • Over 650 species distributed in 15 tribes [5]


Introduction (Cont.)

- **Ageratum conyzoides** L.
  - Neeli booti [6]
- Folk medicines
  - Ulcers, pneumonia, diarrhea
  - Burn wounds
  - Leprosy, gynecological diseases [7]
- Anti larvicidal activity
  - *Culex quinquefasciatus*
  - *Aedes aegypti*
  - *Anopheles stephensi* [8]
- Phytochemical screening
  - Alkaloids, resins, saponins, tannins, glycosides and flavonoids [9]

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Introduction (Cont.)

• *Sonchus oleraceus* L.
  - Local name, ‘Dodak’

• Folk medicines
  - Anticancer agent, anti diarrheal
  - Anti inflammatory, blood purifier
  - Gynecological aid, heart medicine
  - Hepatic diseases, vermicide [10]
  - Rich in
    - Antioxidants
    - Polyphenols
    - vitamin C
    - Four times more antioxidant activity than blueberry extracts [11]

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Endophytes
- Microorganisms that reside intra and/or intercellular in the tissues of plants
- Do not cause any symptom
- Produce bioactive secondary metabolites with unique structure
- Antibiotics, immunosuppressants, antioxidants, and anticancer agents [12]

Advantages to the plant
- Fitness enhancements
  - Increased resistance to herbivores
  - Parasitism
  - Drought
  - Growth enhancements [13]

Endophytic actinomycetes
- Gram positive filamentous bacteria
- Reside within the plants
- Exploit an unusual habitat
- Novel bioactive compounds [14]

Results and discussion

- 145 endophytic actinomycetes were isolated
  - *Ageratum conyzoides* (AG) 52
  - *Sonchus oleraceus* (SO) 93

- Frequency of isolation
  - Roots RS = 139
  - Shoots SS = 2
  - Leaves LS = 4

<table>
<thead>
<tr>
<th>Plant sample</th>
<th>Plant parts</th>
<th>Isolates</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Ageratum conyzoides</em></td>
<td>Cut roots</td>
<td>AGRS 1, AGRS 2, AGRS 3, AGRS 4, AGRS 5, AGRS 6, AGRS 7, AGRS 8, AGRS 9, AGRS 10, AGRS 11, AGRS 12, AGRS 13, AGRS 14, AGRS 15, AGRS 16, AGRS 17, AGRS 18, AGRS 19, AGRS 20, AGRS 21, AGRS 22, AGRS 23, AGRS 24, AGRS 29, AGRS 33, AGRS 34, AGRS 35, AGRS 36, AGRS 37, AGRS 38, AGRS 39, AGRS 40, AGRS 41, AGRS 42, AGRS 43, AGRS 44, AGRS 45, AGRS 46, AGRS 47, AGRS 48, AGRS 49, AGRS 50, AGRS 51, AGRS 52, AGRS 53</td>
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<tr>
<td></td>
<td>Cut shoots and leaves</td>
<td>AGL 1, AGLS 1, AGLS 2, AGLS 3, AGSS 1, AGSS 2</td>
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<tr>
<td><em>Sonchus oleraceus</em></td>
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<tr>
<td></td>
<td>Cut shoots and leaves</td>
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</table>
Results and discussion (Cont.)

- Preliminary antimicrobial activity test
- GYM broth [15] with actinomycetes growth tested
- Prominently bioactive against *Bacillus subtilis, Staphlococcus aureus, Escherichia coli, Pseudomonas* and *Enterobacter* sp.

Results and discussion (Cont.)

• Screening of endophytic actinomycetes
  • Selected endophytic actinomycetes with most preliminary bioactivity
  • Crude extraction
    • Sonication and 1:1 ethyl acetate [16]
    • Biological and chemical screening

• Biological screening
  1. Antimicrobial activity testing
    • Agar well diffusion method [17]
      • ATCC standard organisms
      • Multi drug resistant pathogens
      • Clinically isolated fungi
      • Microalgae

## Results and discussion (Cont.)

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<td>10</td>
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</table>

Key: B. subtilis Bacillus subtilis; Ent. spp., Enterobacter spp.; Pseudo spp., Pseudomonas spp.; S. aureus Staphlococcus aureus; E. coli Escherichia coli; K. pneumoniae Klebsiella pneumoniae; MRSA Methicillin Resistant Staphlococcus aureus; S. cerevisiae, Saccharomyces cerevisiae; C. tropicalis Candida tropicalis, C. vulgaris Chlorella vulgaris

(-) no inhibition

60μl crude extract was added in each well

Values are means of triplicates studies
Results and discussion (Cont.)

Photo courtesy: Dr. Rabia Tanvir
2. Brine shrimp cytotoxicity assay [18]
   • Crude extract
   • *Artemia salina* nauplii
   • 10, 50, 100, 150, 500, 1000µg/ml
     • Increase in cytotoxicity with increased concentration

### Results and discussion (Cont.)

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>% mortality under the concentration studied (μg/mL⁻¹)</th>
<th>| LC₅₀ (μg/mL⁻¹)</th>
<th>95% confidence interval</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>10</td>
<td>50</td>
<td>100</td>
</tr>
<tr>
<td>DMSO (1% in seawater)</td>
<td>0</td>
<td>0</td>
<td>0</td>
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<tr>
<td>AGRS1</td>
<td>10.4</td>
<td>23.3</td>
<td>28.0</td>
</tr>
<tr>
<td>AGRS14</td>
<td>33.7</td>
<td>34.7</td>
<td>59.3</td>
</tr>
<tr>
<td>AGRS44</td>
<td>54.1</td>
<td>56.6</td>
<td>60.6</td>
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<tr>
<td>AGRS49</td>
<td>36.6</td>
<td>51.6</td>
<td>59.9</td>
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<td>SORS4</td>
<td>21.8</td>
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<td>SORS26</td>
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<td>53.3</td>
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<td>58.6</td>
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<td>29</td>
<td>37.9</td>
<td>42.9</td>
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<td>SORS82</td>
<td>32.1</td>
<td>33.3</td>
<td>36.6</td>
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<td>SORS95</td>
<td>41.3</td>
<td>53.5</td>
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<td>SORS106</td>
<td>34.1</td>
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<tr>
<td>SORS146</td>
<td>41</td>
<td>42.3</td>
<td>47.7</td>
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</tbody>
</table>

Values are means of triplicate studies, n = 15 (number of nauplii used). Score for LC₅₀: Highly toxic -<20(μg/mL⁻¹), toxic – up to 1000 μg/mL⁻¹ and non toxic –>1000μg/mL⁻¹, LC₅₀ was calculated through Probit.
Results and discussion (Cont.)

Disintegration of body parts observed

Photo courtesy: Dr. Rabia Tanvir
3. Antioxidant activity test

   - Using the 2, 2’-diphenyl-1-picrylhydrazyl free radical (DPPH) assay [18]

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>DPPH Scavenging activity (%) under the concentration studied (µg/mL⁻¹)</th>
<th>EC₅₀ (µg/mL⁻¹)</th>
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<tr>
<td></td>
<td>100</td>
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<td>Ascorbate standard</td>
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<td>AGRS1</td>
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<td>55.4</td>
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<td>SORS146</td>
<td>27.5</td>
<td>28.3</td>
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</table>

Values are means of triplicate studies
Results and discussion (Cont.)

Ascorbic acid standard

Endophytic actinomycetes crude extract

Endophytic actinomycetes crude extract

Photo courtesy: Dr. Rabia Tanvir
4. Embryotoxicity test

- ‘Desi breed’ chicken embryo
- Inoculated with extracts
- incubated at 37°C for 7 days

<table>
<thead>
<tr>
<th>Strain no.</th>
<th>Embryotoxicity against chicken embryo (%a) under the concentration studied (µg/mL⁻¹)</th>
</tr>
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<tr>
<td>Control</td>
<td>Normal embryo formation</td>
</tr>
<tr>
<td>AGRS8</td>
<td>No embryo formation. Coagulated protein, blackened with H₂S gas production</td>
</tr>
<tr>
<td>AGRS49</td>
<td>No embryo formation. Coagulated protein, blackened with H₂S gas production</td>
</tr>
<tr>
<td>SORS106</td>
<td>No embryo formation. Coagulated protein, blackened and greenish with H₂S gas production</td>
</tr>
<tr>
<td>SORS119</td>
<td>No embryo formation. Coagulated protein, blackened and greenish</td>
</tr>
</tbody>
</table>

Five eggs were used for each strain; ten un-inoculated eggs were used as control.
Results and discussion (Cont.)

- Chemical screening
  - Thin Layer Chromatography (TLC)
  - 365 nm and 254nm
  - Diverse metabolites production

Photo courtesy: Dr. Rabia Tanvir
Results and discussion (Cont.)

- Bioautography

- Polar, medium polar and non polar bands
- Yellow area indicated antimicrobial activity against *E.coli*
### Results and discussion (Cont.)

<table>
<thead>
<tr>
<th>Isolates</th>
<th>No. of nucleotides sequenced (bp)</th>
<th>% Similarity with</th>
<th>Gen Bank accession numbers</th>
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</thead>
<tbody>
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<td>AGRS 8</td>
<td>1238</td>
<td>*Streptomyces albovinaceus</td>
<td>KC191695</td>
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<tr>
<td>SORS45</td>
<td>666</td>
<td>Streptomyces diastatochromogenes</td>
<td>99%</td>
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<tr>
<td>SORS106</td>
<td>1166</td>
<td>*Streptomyces badius</td>
<td>KC191702</td>
</tr>
</tbody>
</table>


Results and discussion (Cont.)

- High Performance Liquid Chromatography Mass Spectrometry Diode Array Detector (HPLC-MS-DAD)
  - DAD-UV vis database search in the natural substance library [19]
  - UV ranges of 230, 260, 280, 360 and 435nm
  - A peak similarity observed between compounds in the database

Results and discussion (Cont.)

Total UV absorption spectra of AGRS 44 extract with UV absorption spectra at 230, 260, 280, 360, 435nm
Results and discussion (Cont.)

- $^1$H NMR (600 MHz)
  - Structures were drawn using the software ChemDraw®
  - Comparison of the identified compound with the reference compound
  - Long chained fatty acid amide derivatives
    - Widely bioactive
Results and discussion (Cont.)

Tetradecanamide

N-Hexadecanamide

9- Octadecenamide

13- Docosenamide
Conclusions

• *Asteraceae* plants used in the study
  • Medically important in a wide variety of aliments
  • Evidence of the production of the active metabolites by the endophytic actinomycetes residing within these plants

• Endophytes of *Asteraceae* plant family
  • Diversity of endophytic actinomycetes isolated from different plant parts
  • Biological screening revealed
    • Wide spectrum of biotechnologically significant compounds
      – Antimicrobial, cytotoxic, antioxidant and embryotoxic agents
      – Dose dependent response
Conclusions (Cont.)

• Chemical screening revealed
  • Producing non polar, medium polar and polar bioactive compounds

• HPLC-MS-DAD
  • Interesting matches but with slight precision

• $^1$H NMR
  • Fatty acid amides and derivatives
  • None reported from endophytes
  • Reported as anti tumor, anti infective, cardiovascular, nervous system agent, anti-inflammatory, immune agent, and enzyme inhibitor [20]

• Further exploration of this novel ecological niche
  • Novel metabolites
  • Biotechnological industries

Acknowledgments

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- Dr. Yi Zhang at the Guangdong Ocean University China provided the *E. coli* K-12 strain for which the authors are greatly thankful