6.0. EFFECT OF BIOACTIVE SECONDARY METABOLITES FROM ACTINOMYCETES AGAINST HUMAN BACTERIAL PATHOGENS

6.1. Introduction

Human evolution can be viewed both as biological and cultural, with cultural evolution causing the most dramatic changes in human history over the past fifteen thousand years. As we have marched toward the modern world, we have affected the microbial world around us and created many of our own human diseases. Infectious diseases are the world’s leading cause of premature deaths, killing almost 50,000 people every day.

Antimicrobials are natural or synthetic drugs which inhibit or kill bacteria. This capability makes them unique for the control of deadly infectious diseases caused by a large variety of pathogenic bacteria. Today, more than 15 different classes of antimicrobials are known. They differ in chemical structure and mechanism of action. Specific antimicrobials are necessary for the treatment of specific pathogens. Following their 20th century triumph in human medicine, Antimicrobials have also been used increasingly for the treatment of bacterial disease in animals, fish and plants. In addition, they became an important element of intense animal husbandry because of their observed growth-enhancing effect, when added in sub-therapeutic doses to animal feed.
However, the indiscriminate use of these therapeutic entities cause resistance among microorganism. Presently, we face a global public health crisis, as infectious diseases top the list for causes of death worldwide. While it is likely that antibiotic resistance contributes significantly to this problem, data on consumption and resistance to antibiotics are limited for most countries (Saiman, 2002). *Pseudomonas aeruginosa*, *Burkholderia cenocepacia* and *Mycobacterium avium*, are opportunistic pathogens and cause disease mainly in people suffering from immunosuppression or cystic fibrosis (Heise, 1982). Hence, the present study has been undertaken to find out the alternative therapeutics from marine actinomycetes to overcome the problem of resistance and to enhance the production of antimicrobials for the treatment of human disease without causing side effects.

6.2 Materials and Methods

6.2.1 Primary screening

The antagonistic activity of chosen actinomycetes from mangrove environment was tested by following cross streak method (Ellaiah et al., 1997) Single streak of actinomycetes strains were (ACT1 - ACT5) streaked on the surface of modified nutrient agar medium plates and incubated at room temperature 27°C for 5-7 days. On obtaining ribbon like growth, the overnight culture of human pathogenic bacteria *viz.*, *Salmonella paratyphi* (A), *S. paratyphi* (B), *Klebsiella pneumoniae*, *Pseudomonas
*aeruginosa, Staphylococcus aureus, E. coli, Proteus vulgaris, Clostridium sp.* were streaked at perpendicular to the original streak of actinomycetes and incubated at 28±2°C and the inhibition was measured after 24 hours.

**Composition of the modified nutrient agar (g.l⁻¹)**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Glucose</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Peptone</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>3.0 gm</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>5.0 gm</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000 ml</td>
</tr>
<tr>
<td>pH</td>
<td>7±0.2</td>
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</tbody>
</table>

6.2.2. Secondary screening

6.2.2.1 Mass cultivation of antagonistic actinomycetes

Experiment was conducted for the mass cultivation of chosen actinomycetes *viz.*, ACT-1, ACT-2, ACT-3, ACT-4, ACT-5 by using fermentor. Fermentor was thoroughly washed with soap solution and autoclaved without media for 15 minutes at 15 lbs. After sterilising the whole fermentor, the substrate (Bennett media) was transferred into the fermentor under laminar air flow chamber. After that, fermentor was sterilized (autoclaved) for 15 minutes at 15 lbs at 121°C. After the sterilization process, the media was allowed to cool (thawed) to room temperature. A loopful inoculum of morphologically different actinomycetes strains *viz.*, ACT-1, ACT-2, ACT-3, ACT-4, ACT-5 were
further inoculated into 500ml conical flask containing 100ml of Yeast extract-Malt extract broth and kept at 28°C for 72 hours with continuous shaking. Then twenty millilitre of grown culture was transferred into the fermentor containing 1000ml of Bennett medium using sterile syringe and incubated for 7 days under continuous shaking. The parameters for fermentation process were properly set using external controlling device. The temperature was maintained at 24°C, dissolved oxygen about 3, the agitator at 200rpm and pH about 7±0.2. The fermentation process was carried out for 7 days. Continues checking was carried out so as to find out the contamination i.e. other than desired organism.

### Composition of Benett Medium (g.L⁻¹)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
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<tbody>
<tr>
<td>Pancreatic digest casein</td>
<td>2.0gm</td>
</tr>
<tr>
<td>Yeast Extract</td>
<td>1.0gm</td>
</tr>
<tr>
<td>Beef extract</td>
<td>1.0gm</td>
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<tr>
<td>Glucose</td>
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<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
<tr>
<td>pH</td>
<td>7±0.2</td>
</tr>
</tbody>
</table>

6.2.2.2 Extraction of bioactive secondary metabolites from fermented broth

The mass cultivated broth (pH 7.2) was adjusted to pH 5.0 using 1N Hydrochloric acid and filtered by cheese cloth to remove the mycelia
biomass. One litre of the filtrate was mixed with 500ml of ethyl acetate in a separating funnel to extract the bioactive compound. After removing the lower aqueous phase, the upper solvent phase was concentrated crude extract was obtained. This process was repeated for three times to obtain complete extraction of active principles. This crude extract was used for further screening.

6.2.2.3 Minimum Inhibitory Concentration (MIC) (Hammond and Lambert, 1978)

Minimum inhibitory concentration is defined as the lowest concentration of antibiotic that inhibit the growth of a particular microorganism by broth dilution method. 0.1 ml of 24 hours microbial broth of human bacterial pathogens viz., *Salmonella paratyphi A*, *S. paratyphi B*, *Klebsiella pneumoniae*, *Pseudomonas aeruginosa*, *Staphylococcus aureus*, *E. coli*, *Proteus vulgaris*, *Clostridium sp.* were added to the tubes containing various concentrations (10, 20, 30, 40 and 50µg.ml⁻¹) of bioactive secondary metabolites from actinomycetes and 0.5 ml of nutrient broth. Nutrient broth alone served as negative control. Whole setup in duplicate was incubated at 37°C for 48 hours in a thermostat shaker. After incubation, optical density was measured at 620nm by using UV-Visible spectrophotometer (SHIMADZU, JAPAN).
6.2.2.4 Antibacterial sensitivity assay by agar well diffusion method (Pandey et al., 2004)

Antibiotic sensitivity test will be performed by the commonly used agar diffusion method which is designed to determine the smallest amount of the bioactive secondary metabolites needed to inhibit the growth of the microorganism. The medium of choice is Muller – Hinton agar with a pH of 7.2 to 7.4, which is poured into plates to a uniform depth of 5mm and allowed to solidify. Prior to use, the plates are transferred to an incubator at 37˚ C for 10 to 20 minutes to dry off the moisture that develops on the agar surface. Overnight growth of chosen bacterial broth culture was swabbed on the surface of the agar media and further well was prepared by using well cutter. To each well, 50µg bioactive secondary metabolites from actinomycetes was added and incubated in a thermostat incubator for 24 hrs. After, incubation, the zone of inhibition around the well was calculated and expressed as zone of inhibition in millimeter in diameter.

Composition of Muller Hinton Agar (g.l⁻¹)

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Amount</th>
</tr>
</thead>
<tbody>
<tr>
<td>Meat infusion</td>
<td>300.0gm</td>
</tr>
<tr>
<td>Casein acid hydrolysate</td>
<td>17.5gm</td>
</tr>
<tr>
<td>Starch</td>
<td>1.5gm</td>
</tr>
<tr>
<td>Agar</td>
<td>17.0gm</td>
</tr>
<tr>
<td>pH</td>
<td>7.3</td>
</tr>
<tr>
<td>Distilled water</td>
<td>1000ml</td>
</tr>
</tbody>
</table>
6.3. Results

The effect of crude extract from morphologically different 5 actinomycetes strains were tested for minimum inhibitory concentration (MIC) against 8 chosen human bacterial pathogens and the results are represented in figures. It clearly reveals that, 5 strains of actinomycetes from mangrove ecosystem showed varied MIC values range from 10-40µg.ml⁻¹. The minimum MIC value (10 µg.ml⁻¹) was recorded against the *Escherichia coli* and *Proteus vulgaris* and maximum MIC value was (40 µg.ml⁻¹) was recorded against *Salmonella paratyphii* (A), *Salmonella paratyphii* (B), *Klebsiella pneumoniae*, *Staphylococcus aureus*, *Pseudomonas aeruginosa* and *Clostridium sp.* (Fig. 49).

The antibacterial activity of well- diffusion method was also carried out by the present study and shows that, the maximum zone of inhibition (43mm dia.) was noticed by the actinomycetes strain ACT-1 against the human bacterial pathogens *Escherichia coli* (Fig. 50).

The present study also made an attempt to find out the antibacterial sensitivity of crude extracts from Actinomycetes against 8 human bacterial pathogens by conventional streaking method. The results are categorized from a minimum inhibition (3⁺) to maximum inhibition (5⁺). It is very interesting to notice that, the Actinomycetes strain ACT-1 showed maximum antibacterial sensitivity (5⁺) against all the 8 human pathogens followed by ACT-2. However, the ACT-3, ACT-4, ACT-5 showed minimum antibacterial sensitivity when compared with ACT-1 and AC2 (Fig. 51).
Fig. 49. Minimum inhibitory concentration of crude extract from 5 actinomycete strains against human pathogens

![Graph showing MIC values for different pathogens against various extracts.]

**MIC µg.mL⁻¹**
- Salmonella paratyphi A
- Salmonella paratyphi B
- Klebsiella pneumoniae
- Pseudomonas aeruginosa
- Staphylococcus aureus
- Escherichia coli
- Proteus vulgaris
- Clostridium Sp.

Human pathogens

Fig. 50. Antibacterial sensitivity of crude extracts from 5 actinomycetes strains against human pathogens by well diffusion method

![Graph showing zone of inhibition for different pathogens against various extracts.]

**Zone of inhibition (mm dia)**
- Salmonella paratyphi A
- Salmonella paratyphi B
- Klebsiella pneumoniae
- Pseudomonas aeruginosa
- Staphylococcus aureus
- Escherichia coli
- Proteus vulgaris
- Clostridium Sp.

Human pathogens

Fig. 51. Antibacterial sensitivity of 5 actinomycetes strains against human pathogens by conventional streaking method

![Graph showing frequency of inhibition for different pathogens against various extracts.]

**Frequency of inhibition**
- Salmonella paratyphi A
- Salmonella paratyphi B
- Klebsiella pneumoniae
- Pseudomonas aeruginosa
- Staphylococcus aureus
- Escherichia coli
- Proteus vulgaris
- Clostridium Sp.
Picture showing antagonistic activity of actinomycetes against 8 human pathogens - Cross Streak Assay
Microbial activity of actinomycetes against 8 human pathogens - Well Diffusion method

Contd........
Microbial activity of actinomycetes against 8 human pathogens - Well Diffusion method

Contd........
Microbial activity of actinomycetes against 8 human pathogens - Well Diffusion method