CHAPTER - V

ANTIBACTERIAL ACTIVITY OF DERIVED COMPOUNDS FROM GLYPTOPETALUM CALOCARPUM
ABSTRACT

Background: In preliminary studies, the crude extract of Glyptopetalum calocarpum (an endemic plant of Andaman & Nicobar Islands) showed significant antibacterial activity. Six triterpene compounds were extracted, purified and characterized. In the present investigation, pharmacologically active compounds were isolated from this plant, and their antimicrobial efficacy was evaluated against the human pathogenic bacterial strains studied earlier (Chapter-2).

Materials and Methods: The antimicrobial activity was estimated using disc diffusion method. The minimum inhibitory concentrations and minimum bactericidal concentrations were estimated by using the standard protocols.

Results and Discussion: The compounds Lupenone, Stigmasterol and β-amyrin acetate displayed antimicrobial activity against the pathogens studied. The compound, Lupenone showed activity against P. aeruginosa, S. aureus and B. cereus, with MIC and MBC ranging from 62.5 – 125 and 125 – 500 respectively. The compound, Stigmasterol showed activity against P. aeruginosa, S. aureus, S. flexeneri and B. cereus, with MIC and MBC ranging from 31.25 – 250 and 25 – >500 respectively; whereas β-amyrin acetate showed activity against S. aureus and B. cereus.

Conclusions: Three of the six compounds isolated from Glyptopetalum calocarpum displayed antibacterial activity. Further in vivo studies are required to confirm these compounds as antibacterial agents.

INTRODUCTION

The antimicrobial activity of medicinal plants has been evaluated previously, using agar well diffusion method. In this study disc diffusion method was employed. The dilution assays were those, which require a homogeneous dispersion of the sample in solvent (Rio et al., 1988). These methods were mainly used to determine the Minimum Inhibitory Concentration (MIC) values of an extract or pure compound.

The MIC (Minimal Inhibitory Concentration) of a bacterium to a certain antimicrobial agent provides a quantitative estimate of their susceptibility. MIC is defined as the lowest concentration of antimicrobial agent required to inhibit the growth of the organism. The principle involved is: Agar plates, tubes or microtitre trays with two-fold dilutions of antibiotics are inoculated with a standardised inoculum of the bacteria,
and incubated under standardised conditions following NCCLS guidelines (NCCLS, 2000; NCCLS, 1998). On the subsequent day, the MIC is recorded as the lowest concentration of antimicrobial agent with no visible growth.

The MIC provides the degree of resistance, and might reveal significant information regarding the resistance mechanism, and the resistant genes involved. MIC-determination by agar dilution method is regarded as the gold standard for susceptibility testing.

Agar diffusion tests are often used as a qualitative method to determine whether a bacterium is resistant, intermediate resistant or susceptible. However, the agar diffusion method can be used to determine the MIC values, provided the necessary reference curves for conversion of inhibition zones into MIC values are available. After an agar plate is inoculated with the bacteria, a tablet, disk or paper strip with the antimicrobial agent is placed on the surface. During incubation, the antimicrobial agent diffuses into the agar and inhibits the growth of the bacteria, if susceptible. Diffusion tests are cheap, compared to other MIC-determination methods.

Standardised methods are essential for all kinds of susceptibility testing, since the methods are highly sensitive to variations in several factors, such as size of inoculum, contents and acidity of the growth medium, time and temperature of incubation. The agar diffusion methods are also strongly influenced by factors, such as agar depth, diffusion rate of the antimicrobial agent and growth rate of the specific bacteria.

The MIC-determination and disk diffusion methods described in this protocol are in accordance with the international recommendations given by the National Committee for Clinical Laboratory Standards (NCCLS). The NCCLS describes how to perform the tests, and sets international guidelines for interpretation of the results. It should be noted that the WHO does not prescribe any specific method for performance and interpretation of susceptibility tests.

**MATERIALS AND METHODS**

**Disc Diffusion Method**

*Preparation of extract and drug impregnated discs*

The sterile discs of 6mm diameter were procured from Himedia (India). 1mg methanolic extract and compounds were suspended in sterile 100μl of dimethyl
sulfoxide, as the extracts were not fully soluble in water, and subsequently diluted in sterile distilled water to make the required stock solutions. For each extract, three stock solutions were prepared. The stock solutions of the control antibiotics were prepared by dissolving the required amount of gentamicin in 10ml of sterile distilled water separately. All the stock solutions were kept at 4°C, and used within three months.

**Bacterial strains**

Authenticated cultures of Gram positive bacteria, such as *Escherichia coli* (MTCC 443), *Staphylococcus aureus* (MTCC 737), *Pseudomonas aeruginosa* (MTCC 1688), *Vibrio cholera* (MTCC 3906), *Bacillus cereus* (MTCC 1272), *Staphylococcus epidermidis* (MTCC 3615), *Proteus mirabilis* (MTCC 425), *Shigella flexneri* (MTCC 1457), *Salmonella enterica typhi* (MTCC 733), *Klebsiella pneumonia* (MTCC 129), which were procured from Microbial Type Culture Collection, Chandigarh, India, were used for the test.

**Preparation of inoculums**

Bacterial inoculums were prepared from 18 hours old pure culture, grown on nutrient agar. Bacterial colonies were pre-cultured in nutrient broth medium and kept overnight, which were later centrifuged at 10,000 rpm for 5 minutes. Pellet was suspended in sterilized distilled water, and the cell turbidity was assessed spectroscopically, and compared with of 0.5 McFarland standards (approximately 1.5 x 10^8 CFU/ml). These inoculums were used for the antibacterial assays (Mahesh and Satish, 2008).

**Antibacterial assay**

Antibacterial activity of the derived compounds of *G. calocarpum* was determined using disc diffusion method (Kavitha and Satish, 2013). Briefly, 100μl of the test bacteria was spread onto the nutrient agar. The different test compounds were loaded onto sterile 6mm discs, allowed to dry and later, the impregnated discs with 100μl (1mg/ml concentration of test compounds) onto the inoculated plates. The plates were allowed to stand at 4°C for 2 hours, before incubation with the test microbial agents. Plates were incubated at 37°C for 24 hours. The diameters of the inhibition zones were measured (in millimetre). All the assays were carried out in triplicate, and the results were provided as mean ± SD. Standard antibiotic, streptomycin served as positive control.
**Determination of MIC of crude extract and derived compounds by Micro broth dilution method**

The minimum inhibitory concentration was determined by using the micro dilution method in 96 well micro titre plates (NCCLS, 1999). The concentration tested ranged from 500μg/ml to 7.81μg/ml for crude extract and derived compounds. In sterile flat bottom 96-well plates, two fold dilutions of each extract or compound was made in Muller Hinton Broth. The starting inoculum was 5x10^5 CFU/ml of log phase culture. Final volume of broth achieved in each well was 100μl. Wells containing no extract, but inoculated with test strains were considered as positive control. Negative control wells consisted of serial dilution of extracts or compounds only. Plates were incubated at 37°C aerobically, for 18hrs, which were examined later with Elisa reader (TECAN) at 620nm. The lowest concentration at which the extract showed nil growth, was considered as its minimum inhibitory concentration (MIC). All the samples were tested in triplicate to confirm the activity, and the values were noted.

**Determination of Minimum Bactericidal Concentration (MBC) of crude extract and plant derived compounds**

After determination of MICs by micro broth method, plant crude extract and derived compounds were processed to check for their bactericidal activity. Briefly, 100μl of MHB from the wells containing compounds of the plant extract was taken and washed with PBS to remove any residual plant material in it. Sediment was resupended in PBS, and was inoculated on the extract or antibiotic free MH plates. The plates were later incubated for 18hrs at 37°C aerobically. The MBC was considered as the lowest concentration of each drug that resulted in nil bacterial growth, following the removal of the drug.

**RESULTS AND DISCUSSION**

Antibacterial activity of the six compounds derived from *G. calocarpum* was studied using the disc diffusion method. The compounds Lupenone, Stigmasterol and β-amyrin acetate displayed antimicrobial activity against the pathogens studied. Zones of bacterial growth inhibition, for the different compounds are provided in Table 5.1. Minimum inhibitory concentration (MIC) and minimum bactericidal activity (MBC) of the compounds are provided in Table 5.2.
Table 5.1: Antimicrobial activity of derived compounds from *G. calocarpum*

| Inhibition zone diameter (mm) | Lupe
tone | Stigmasterol | α- Lupene | Lupeol | β-amyrin | β-amyrin acetate | Crude | Streptomycin |
<table>
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<tr>
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<th></th>
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<th></th>
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<th></th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>E.coli</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>12.33±0.58</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>10.33±0.58</td>
<td>10.67±0.58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.33±0.58</td>
<td>13.67±0.58</td>
<td>12.67±0.58</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>13.00±1.00</td>
<td>11.67±1.15</td>
<td>-</td>
<td>-</td>
<td>10.33±0.58</td>
<td>11.33±1.53</td>
<td>12.33±0.58</td>
<td></td>
</tr>
<tr>
<td><strong>B. cereus</strong></td>
<td>15.33±0.58</td>
<td>18.67±0.58</td>
<td>-</td>
<td>-</td>
<td>11.00±0.00</td>
<td>12.67±1.15</td>
<td>21.67±1.15</td>
<td></td>
</tr>
<tr>
<td><strong>K. pneumonia</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.33±0.58</td>
</tr>
<tr>
<td><strong>S. typhi</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.00±1.00</td>
</tr>
<tr>
<td><strong>P. mirabilis</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>11.33±1.53</td>
</tr>
<tr>
<td><strong>S. flexeneri</strong></td>
<td>-</td>
<td>10.33±0.58</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>14.33±0.58</td>
</tr>
<tr>
<td><strong>V. cholerae</strong></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>10.67±0.58</td>
</tr>
</tbody>
</table>

*'-'- indicates no activity.

Table 5.2: MIC and MBC of derived compounds from *G. calocarpum*

<table>
<thead>
<tr>
<th>Lupenone</th>
<th>Stigmasterol</th>
<th>β-amyrin acetate</th>
<th>Crude</th>
</tr>
</thead>
<tbody>
<tr>
<td>MIC</td>
<td>MBC</td>
<td>MIC</td>
<td>MBC</td>
</tr>
<tr>
<td><strong>P. aeruginosa</strong></td>
<td>125</td>
<td>500</td>
<td>125</td>
</tr>
<tr>
<td><strong>S. aureus</strong></td>
<td>125</td>
<td>250</td>
<td>125</td>
</tr>
<tr>
<td><strong>B. cereus</strong></td>
<td>62.5</td>
<td>125</td>
<td>31.25</td>
</tr>
<tr>
<td><strong>S. flexeneri</strong></td>
<td>ND</td>
<td>ND</td>
<td>250</td>
</tr>
</tbody>
</table>

ND= Not Done
The compound Lupenone showed activity against *P. aeruginosa*, *S. aureus* and *B. cereus*, with MIC and MBC range of 62.5–125μg/ml and 125–500μg/ml respectively. The compound Stigmasterol showed activity against *P. aeruginosa*, *S. aureus*, *S. flexeneri* and *B. cereus* with MIC and MBC range of 31.25μg/ml – 250μg/ml and 25μg/ml – >500μg/ml respectively whereas β-amyрин acetate showed activity against *S. aureus* and *B. cereus*. Crude extract showed antibacterial activity against *P. aeruginosa*, *S. aureus* and *B. cereus*, at given concentrations.

Compound Stigmasterol isolated from the stem of *Sida rhombifolia* Linn. and twigs of *Eriosema robustum* were reported to show activity against *E. coli*, *P. aeruginosa*, *S. aureus*, *S. typhimurium* and *E. faecalis* (Woldeyes et al., 2012; Awouafack et al., 2013). In the present study, Stigmasterol showed activity against *P. aeruginosa*, *S. aureus*, while no activity was observed on *E. coli*. Schinor et al., (2007) observed that compounds, Lupeol and β-amyрин acetate, isolated from *Chresta scapigera* showed activity against *S. aureus* at concentration of 500μg/ml. In the present study, β-amyрин acetate showed activity against *S. aureus* and *B. cereus*, but Lupeol had no effect on the pathogens tested.

**CONCLUSIONS**

Three of the six compounds isolated from *Glyptopetalum calocarpum* displayed antibacterial activity, and it is also be possible that some of the active compounds were not isolated.

Lack of biological activity in few compounds does not necessarily indicate their lack of effectiveness of the compounds. They may act in other ways to effect a cure, such as by stimulating the immune system of the patient, or by creating internal conditions unfavourable for the multiplication of bacteria. On the other hand, if more than one plant is used in a mixture, the synergistic effects of the principle components in different plants of the mixture may cause relief from the ailments. During purification, it is also possible that some of the active compounds from the plant were not isolated.