CHAPTER - VI

ANTI-LEPTOSPIRAL ACTIVITY OF DERIVED COMPOUNDS FROM GLYPTOPETALUM CALOCARPUM
ABSTRACT

Background: Many plant species proved to be effective against various pathogens in vitro, however, data regarding the efficacy of Leptospira are very scanty. Six derived compounds of Glyptopetalum calocarpum (an endemic plant of Andaman & Nicobar Islands), were studied for its antimicrobial activity, and three compounds showed activity against the human pathogenic bacteria. In the present investigation, the antimicrobial efficacy of pharmacologically active compounds isolated from this plant was evaluated against the Leptospiral strains.

Materials and Methods: Microdilution was performed with 96-well, round-bottom microtiter plates using Alamar Blue dye. Each plate included positive control (bacteria without any antimicrobial compound), negative control (medium only), and serial two fold dilutions of each of the six antimicrobials, all carried out in EMJH medium. The MICs were observed visually, and the lowest concentration of antimicrobials were those, which did not show any colour change, from blue to pink/purple. Macrodilution was performed in glass test tubes. Isolated compounds and the antibiotic-containing tubes were prepared to contain serial two fold dilutions of antimicrobials in EMJH medium, to obtain final concentrations of 400 to 0.01 µg/ml.

Results: Out of six, two compounds, viz., Lupenone and Stigmasterol, showed anti-leptospiral activity. The MICs of the two compounds tested against pathogenic leptospiral strains belonging to 10 serovars, were in the range of 100-200µg/ml. The range of MBCs was 400-800µg/ml.

Conclusions: Lupenone and Stigmasterol showed promising antileptospiral activity against the pathogenic serovars studied. Further in vivo studies are needed to investigate the pharmacological and toxicological properties of Glyptopetalum calocarpum, before it can be considered as a new anti-leptospiral agent.

INTRODUCTION

Leptospirosis is an infectious disease that occurs in humans and a wide range of animals worldwide, and is caused by serovars of Leptospira interrogans. Close association with water and soil contaminated animal urine, leads to the risk of being infected with this pathogen (Faine et al., 1999). Symptoms and signs of leptospirosis are variable, and laboratory support is a prerequisite for its confirmation. In animals, the infection results
in fever, abortion, icterus and uveitis, and lead to partial or total blindness. The infection in humans is associated with multi-organ injury with case fatality ratios ranging from 3% to 70% (Gouveia et al., 2008). Definitive diagnosis relies either on culture, which has a low yield, and requires incubation for 4 to 6 weeks, or on serological testing of acute and convalescent phase samples. Therefore, initial therapy of leptospirosis is often empirical, based upon a broad differential diagnosis that includes other etiologies of acute febrile illness in the community.

As many rural communities lack modern medical facilities, they utilize the local plants available in their surroundings to treat various illnesses in their health care system (Tabuti et al., 2003). In recent years, development of drug resistance in human pathogens against commonly used antibiotics necessitated the search for new antimicrobial substances from other sources, including plants (Erdogrul, 2002).

Knowledge of herbal medicine has been acquired for treating different illness in areas where the use of plants is still of great importance (Diallo et al., 1999). Traditional medicines can provide a valuable lead in the discovery of new and more efficacious drugs. Till date, very few of these are utilized in drug and pharmaceutical industries (Mehrotra and Mehrotra, 2005, Verma et al., 2007).

Many plant species proved to be effective against various pathogens in vitro, however data regarding the efficacy of antibiotics or natural pharma against Leptospira are very scanty. In vivo testing of the antimicrobial activity on Leptospira sp. is limited to few agents, mainly observed during epidemic situations (Murray and Hospenthal, 2004; Vedhagiri et al., 2009; Azevedo et al., 2011). For randomized trials in humans, data is available only for a limited number of drugs (Azevedo et al., 2011; Sehgal et al., 2000). A simple and effective method in testing various antimicrobial agents against Leptospira sp. would result in an effective alternative for leptospirosis.

MATERIALS AND METHODS

Leptospiral strains

In the present study, a total of 10 leptospiral reference strains were used (obtained from the Leptospira Reference Laboratory, Port Blair). All the strains were maintained in Ellinghausen-McCullough-Johnson-Harris (EMJH) medium at 30°C (Faine et al., 1999).
**Solvent toxicity assay**

Toxicity of various solvents and their concentrations were tested against leptospiral strain. All solvents used were of analytical grade. Dimethyl sulfoxide (DMSO), Methanol and Ethyl acetate were used to prepare serial two fold dilutions of antimicrobials in EMJH medium in final concentrations of 8-0.1%. To each tube, *Leptospira* spp. at a final concentration of $10^6$ organisms/ml (final volume, 2 ml) was added, and was incubated at 30°C for 7 days. The solvent concentration with the highest leptospiral count was the control (without any solvent), and was considered as a nontoxic concentration for studying antileptospiral activity of plant compounds.

**Susceptibility testing**

Testing of each combination of leptospiral strain, and the plant compound was performed in parallel. Three parallel tests were performed at different times to determine the reproducibility of results. Cumulative efficacy was described as MIC$_{90}$ and MBC$_{90}$, i.e., the concentrations at which 90% of the leptospiral isolates are inhibited and killed, respectively.

**Anti-Leptospiral activity**

**Microdilution method**

The antileptospiral activity of the compounds were studied by Broth microdilution testing method, using Alamar Blue dye as previously described (Murray and Hospenthal, 2004). Briefly, Broth microdilution testing was performed with 96-well, round-bottom microtiter plates. Each plate included positive control (bacteria without any antimicrobial compound), negative control (medium only), and serial two fold dilutions of each of the six antimicrobials, all carried out in EMJH medium. Antimicrobial-containing wells included final concentrations of each compound, ranging from 400 to 0.01 µg/ml. For inoculum preparation, *Leptospira* cultures were grown for 7 days ($2 \times 10^8$ cells/ml) at 30°C. Following the addition of 100-µl inoculum (containing $2 \times 10^6$ leptospiral organisms/ml) to the antimicrobial-containing and positive control wells, the plates were incubated at 30°C. The final volume of each well was 200 µl. After 3 days of incubation, 20 µl of concentrated (10X) Alamar Blue was added to all the wells and incubated for 48 h at 30 °C. On the fifth day of incubation, the colour in each well was noted. The MICs were
observed visually, and the lowest concentration of antimicrobials in the wells were those without any colour change from blue to pink/purple. The plate was also read by spectrophotometer at 570 nm.

*Macrodilution method*

Broth macrodilution, MICs and MBCs were obtained with a modification of previously described technique (Murray and Hospenthal, 2004). Isolated plant compounds and antibiotic-containing tubes were prepared to contain serial two fold dilutions of antimicrobials in EMJH medium, to obtain final concentrations of 400 to 0.01 µg/ml (200 to 0.01 U/ml for penicillin G, and 200 to 0.01 µg/ml for Doxycycline). Leptospiral strains were added to each tube at a concentration of 10^6 organisms/ml (final volume, 2 ml), and were incubated at 30°C for 7 days. The antimicrobial concentration present in the tube without visual growth was observed using Petroff-Hauser counting chamber, under dark field microscope, and was recorded as the MIC. MBC testing was performed by transferring 10 µl of the fluid from each tube without visible growth into 2 ml of fresh EMJH medium. The lowest antimicrobial concentration that did not yield any growth by visual inspection, post three weeks of incubation at 30°C was recorded.

**RESULTS AND DISCUSSION**

*G. Calocarpum* is one of the plants used as a treatment for fever, body ache and joint pain. Preliminary screening of the crude extract of this plant was observed to have antimicrobial activity against human pathogenic bacteria (Chapter 5). Hence, further extraction and purification of compounds from this plant species was attempted, to evaluate its potential to treat microbial infection.

Leptospirosis is a potentially fatal infection that is often treated empirically due to the lack of rapid and accurate testing method (Murray and Hospenthal, 2004). Treatment options are limited, as only few drugs have been evaluated in randomized human trials (Murray and Hospenthal, 2004; Suputtamongkol and Niwattayakul, 2004; Azevedo et al., 2011). Currently, very few reports are available on the natural products used against leptospirosis (Chandan et al., 2012; Prabhu et al., 2008; Vedagiri et al., 2009). Also, there is a lack of standard method to assess plant based natural extract or derived compounds for antileptospiral activity. The most frequently employed method is a cumbersome broth macrodilution method (Murray and Hospenthal, 2004).
Toxicity of solvents may influence microbiological evaluation of poorly water-soluble substances (Matzneller et al., 2011). There is no data available on the working concentration of solvents for Leptospira testing. However, few studies report the use of DMSO, as a solvent for water in soluble substances for Leptospira. In view of this, different solvents were tested against leptospires, and a working concentration was determined. Three commonly used solvents tested were Dimethyl sulfoxide (DMSO), Methanol and Ethyl acetate. Methanol was less toxic at a working concentration of < 0.2% \( (\text{MIC}_{90} = 1.5\% \text{ and } \text{MBC}_{90} = 2.5\%) \), when compared to DMSO at < 0.01% \( (\text{MIC}_{90} = 1\% \text{ and } \text{MBC}_{90} = 2\%) \). Ethyl acetate was found to possess high toxicity.

A simple broth microdilution method was employed to evaluate the susceptibility of Leptospira to plant derived compounds, as described by Murray et al 2004 (Murray and Hospenthal, 2004). This method was comparable to the traditionally employed broth macrodilution technique, but is advantageous of requiring less labour, and consumes less time with better reproducibility. The use of plant derived compounds and solvents did not have any problem for visualizing colour change with Alamarblue dye, as all the compounds tested were colourless. Difficulties may arise in visualizing, if coloured compounds are used. Penicillin and doxycycline were used as standards in the present study, which were well studied drugs, and are administered in the treatment of human leptospirosis (Edwards et al., 1988; Hospenthal and Murray, 2003; Sehgal et al., 2000). Our results were in accordance with the in vitro studies reported on penicillin and doxycycline (Hospenthal and Murray, 2003; Murray and Hospenthal, 2004).

The observed reproducibility of both MICs and MBCs by microdilution and macrodilution method produced results between test runs, which fell within 2 dilutions among the serovars used (Table 6.1). Out of six, only two compounds, viz., Lupenone and Stigmasterol, showed MIC\(_{90}\) to be 100 µg/ml and 200 µg/ml in microdilution and macrodilution respectively. MBC\(_{90}\) was 400 µg/ml in macrodilution. MIC\(_{90}\) for Stigmasterol was as low as 50 µg/ml for Genomospecies/serovars \( L. \ borgpetersenii \)/Poi and \( L. \ interrogans \)/Canicola. Other four compounds, viz, Alpha-Lupene, Lupeol, β-Amyrin and β-Amyrin acetate, did not show any activity within the range of concentrations tested (Data not shown). In comparison to the two antibiotic standards, MIC was higher for the derived compound. However, results were similar to
### Table 6.1: Susceptibility of 10 serovars of *Leptospira* spp. to plant derived compounds and antibiotics by microdilution and macrodilution

<table>
<thead>
<tr>
<th>Genotype/Serovar</th>
<th>Crude MIC</th>
<th>Crude MBC</th>
<th>Lupenone MIC</th>
<th>Lupenone MBC</th>
<th>Stigmasterol MIC</th>
<th>Stigmasterol MBC</th>
<th>Doxycycline MIC</th>
<th>Doxycycline MBC</th>
<th>Penicillin G MIC</th>
<th>Penicillin G MBC</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Micro</td>
<td>Macro</td>
<td>Micro</td>
<td>Macro</td>
<td>Micro</td>
<td>Macro</td>
<td>Micro</td>
<td>Macro</td>
<td>Micro</td>
<td>Macro</td>
</tr>
<tr>
<td><em>L. interrogans/Australis</em></td>
<td>400/200</td>
<td>400/400</td>
<td>800/800</td>
<td>100/100</td>
<td>200/100</td>
<td>400/800</td>
<td>200/100</td>
<td>200/200</td>
<td>800/800</td>
<td>3.1/3.1</td>
</tr>
<tr>
<td><em>L. interrogans/Bankinang</em></td>
<td>400/400</td>
<td>400/400</td>
<td>800/800</td>
<td>200/200</td>
<td>200/200</td>
<td>400/400</td>
<td>200/200</td>
<td>200/200</td>
<td>400/800</td>
<td>3.1/3.1</td>
</tr>
<tr>
<td><em>L. interrogans/Canicola</em></td>
<td>200/200</td>
<td>200/400</td>
<td>800/800</td>
<td>200/200</td>
<td>200/100</td>
<td>400/400</td>
<td>50/50</td>
<td>100/200</td>
<td>400/800</td>
<td>1.6/3.1</td>
</tr>
<tr>
<td><em>L. krisscheri/Cynopteri</em></td>
<td>200/200</td>
<td>400/400</td>
<td>800/800</td>
<td>200/200</td>
<td>200/200</td>
<td>400/400</td>
<td>200/200</td>
<td>200/200</td>
<td>400/800</td>
<td>6.3/6.3</td>
</tr>
<tr>
<td><em>L. interrogans/Grippotyposa</em></td>
<td>200/200</td>
<td>400/400</td>
<td>800/800</td>
<td>200/200</td>
<td>400/200</td>
<td>400/400</td>
<td>200/200</td>
<td>200/200</td>
<td>800/800</td>
<td>3.1/6.3</td>
</tr>
<tr>
<td><em>L. interrogans/Hebdomadis</em></td>
<td>200/100</td>
<td>200/400</td>
<td>800/800</td>
<td>200/200</td>
<td>200/200</td>
<td>400/400</td>
<td>200/200</td>
<td>200/200</td>
<td>400/800</td>
<td>3.1/6.3</td>
</tr>
<tr>
<td><em>L. borgpetersenii/Poi</em></td>
<td>200/200</td>
<td>400/400</td>
<td>800/800</td>
<td>100/200</td>
<td>200/200</td>
<td>400/400</td>
<td>50/100</td>
<td>200/200</td>
<td>400/800</td>
<td>3.1/6.3</td>
</tr>
<tr>
<td><em>L. noguchii/Panama</em></td>
<td>200/200</td>
<td>200/400</td>
<td>800/800</td>
<td>100/200</td>
<td>200/200</td>
<td>400/400</td>
<td>100/200</td>
<td>100/200</td>
<td>400/800</td>
<td>3.1/6.3</td>
</tr>
<tr>
<td><em>L. interrogans/Pomona</em></td>
<td>200/400</td>
<td>400/400</td>
<td>800/800</td>
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<td>400/200</td>
<td>800/400</td>
<td>200/200</td>
<td>200/200</td>
<td>800/800</td>
<td>6.3/6.3</td>
</tr>
<tr>
<td><em>L. interrogans/Hardjo</em></td>
<td>200/200</td>
<td>400/400</td>
<td>800/800</td>
<td>100/200</td>
<td>200/200</td>
<td>400/400</td>
<td>200/200</td>
<td>100/200</td>
<td>800/400</td>
<td>3.1/6.3</td>
</tr>
</tbody>
</table>

MIC or MBC:

- Crude MIC/MBC: 100/200
- Lupenone MIC/MBC: 100/200
- Stigmasterol MIC/MBC: 100/200
- Doxycycline MIC/MBC: 100/200
- Penicillin G MIC/MBC: 3.1/3.1

Note: Broth microdilution (Micro) and macrodilution (Macro) MICs and macrodilution MBCs (run 1/run 2 for each) are given in micrograms per milliliter (units per milliliter for penicillin G).
the other natural pharmacological studies against *Leptospira* elsewhere (Prabhu et al., 2008; Vedhagiri et al., 2009; Chandan et al., 2012).

**CONCLUSIONS**

With the passing of time, indigenous communities have learned to use the locally available plants for the treatment of various infections and disease. Only few of those remedies were tested for their efficacy against Leptospirosis, one of the most dreaded zoonotic infection worldwide, especially in developing countries. This study demonstrates the antileptospiral efficacy of *G. Calocarpum*, a plant commonly used by the Nicobarese as a herbal remedy. The activity of these promising agents needs to be tested in an animal model, because *in-vivo* activity in animal models will allow the selection of agents for human trials.