CHAPTER 5:
ANTIMICROBIAL ACTIVITY
OF CRUDE LEAF EXTRACTS
OF Lasianthus lucidus
Blume

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5.1 INTRODUCTION

Antimicrobials are those groups of substances which inhibits the growth of micro organisms (bacteria, fungi or protozoan) by adopting various mechanisms of actions. Antimicrobial substances either kill microbes or prevent the growth of microbes. A great variety of chemicals and compounds of plant origin are used as antimicrobials commercially. Various ethnic groups traditionally used various plants to cure infectious diseases. Some of these plants have been investigated in laboratory for their possible antimicrobial mechanism of actions due to which now a day’s large numbers of antimicrobial agents of plant sources are available in market. A number of these antimicrobial agents appear to have structures and modes of action that are distinct from those of the antibiotics in current use, suggesting that cross-resistance with agents already in use may be minimal. Within the recent years, people are much dependent on antimicrobials of plant origin because antibiotic resistance have become an ever increasing therapeutic problem (Austin et al., 1999).

Field survey reveals that local peoples traditionally use the leaf paste of Lasianthus lucidus Blume for curing various infectious ailments. This ethnomedicinal importance of the plant has repelled to test the antimicrobial activity of the leaf extracts of the plant. Further more the presence of various secondary metabolites like flavonoids, alkaloids, etc signifies the probable presence of active antimicrobial agents in different leaf extracts of L. lucidus.

From Rubiaceae approximately 53 genera have been recorded on which antibacterial studies have been carried out (table- 1.3). The method employed by most of the workers for antibacterial investigations is Filter Paper Disc Diffusion Method (Vincent and Vincent, 1944). Values of Minimum Inhibitory Concentration also have been reported by some workers by using several methods like Serial Dilution Technique (Petersdorf and Piorde, 1963). After studying research articles on antibacterial activity of various plants belonging to Rubiaceae five most common pathogenic bacterial strains were selected (Staphylococcus aureus, Bacillus subtilis, Escherichia
coli, Pseudomonas aeruginosa and Klebsiella pneumoniae) for investigating antibacterial properties of L. lucidus. These selected strains of bacteria are causing serious threats to human health. Pathogenic strains of E. coli cause gastroenteritis, urinary tract infections and neonatal meningitis (Eckburg et al., 2005). B. subtilis is responsible for causing food poisoning (Nakano and Zuber, 1998); K. pneumoniae is a common hospital acquired pathogen which can cause nosocomial pneumonia, urinary tract infections and infections in gastrointestinal tract (Podschun and Ullman, 1998). P. aeruginosa generally infects lungs and urinary tracts and develops wounds, burns and blood infections. It is a common pathogen available in hospitals and clinics causing cross infections (Fine et al., 1996). S. aureus typically causes various skin infections, pneumonia, meningitis, osteomyelitis, sepsis, etc (Mathews et al., 1997). As now-a-days these microbial strains have developed resistance to many antibiotics, the situation forced scientists to find out new antimicrobial agents from other sources. With a view to sort out these clinical problems, plant products are developing with potential activity and less side effects. Keeping the cross resistance of these bacterial strains against some antibiotics in mind, these strains were selected to determine the antimicrobial activity of different leaf extracts of my study material.

5.2 PRELIMINARY ANTIMICROBIAL SCREENING OF Lasianthus lucidus LEAF EXTRACTS

5.2.1 Material and methods

5.2.1.1 Plant Material

The procedure of preparation of PE, EA, AC and ME leaf extracts were discussed in section 2.2 of the dissertation. These extracts were decanted and transferred into the rotary evaporator. Here the solvents were evaporated under reduced pressure. This extraction procedure was repeated three times for
each plant sample. The dark residues obtained were used for the screening programme.

5.2.1.2 Test Organisms

Antibacterial activity and MIC were determined against two Gram-positive bacteria (*Bacillus subtilis, Staphylococcus aureus*) and three Gram-negative bacteria (*Escherichia coli, Klebsiella pneumoniae and Pseudomonas aeruginosa*). The pure cultures of these bacteria were collected from the Microbiological Laboratory of the Department of Microbiology, Medical College, Silchar, Assam.

5.2.1.3 Media

Standard Nutrient agar (Himedia Laboratories Pvt. Ltd., Mumbai, India) pH 7.2, Standard Nutrient Broth No. 1 (Himedia Laboratories Pvt. Ltd., Mumbai, India) pH 6.8, were used for antibacterial screening. 10 mcg antibiotic disc (kanamycin, chloramphenicol, ciprofloxacin, amikacin, penicillin, streptomycin, gentamycin, erythromycin, ampicillin) having 6 mm diameter manufactured from Thermo Fisher Scientific India Pvt. Ltd, Mumbai, India is used as positive control.

5.2.1.4 Antimicrobial Assay

The disc diffusion method (Bauer *et al.*, 1966; Carson *et al.*, 1995; Dash *et al.*, 1995) was used to test antimicrobial activity against five pathogenic bacterial strains. Solutions of known concentration (10 mg/ml) of the test samples were prepared by dissolving measured amount of the samples (leaf extracts) in calculated volume of solvents. Whatman Filter paper discs of 6 mm diameter were prepared and then these discs were dried, sterilized and kept under UV light for 5-10 minutes. Now the discs were impregnated with test materials (plant extracts) of known amounts by using micropipette and evaporated to dryness. 20 ml quantities of nutrient agar medium were plated in petriplates, then 0.1 ml of a $10^{-2}$ dilution of 18 hours old bacterial cultures were uniformly spread on those petriplates and then the filter paper discs were...
placed and marked. Nine different types of standard antibiotic discs (Kanamycin, chloramphenicol, ciprofloxacin, amikacin, penicillin, streptomycin, gentamycin, erythromycin, ampicillin; 10mcg/disc) and blank discs (impregnated with solvents) were used as a positive and negative control respectively. These plates were then kept at low temperature (4°C) for 24 hours to allow maximum diffusion. A gradual change was observed in concentration in the media surrounding discs. The petridishes were then incubated for 24 hours at 37°C to allow maximum growth of the organisms. The tested extracts which have antimicrobial property were successful in inhibiting the growth of the bacterial strains and a distinct, clear zone of inhibition was detected surrounding the agar medium. The antibacterial activity of the test agent was determined by measuring the diameter of zone of inhibition expressed in mm. Each sample was used in triplicate for the determination of antibacterial activity.

5.2.1.5 Statistical Analysis

All data presented are means of three determinations along with standard deviation (SD). Data were analysed by one-way ANOVA in Microsoft Excel software.

5.2.2 Results and Discussion

The results of antibacterial activity of PE, EA, AC and ME leaf extracts are presented below:
<table>
<thead>
<tr>
<th>Leaf extracts (10mg/disc)</th>
<th>Test Organisms [Diameter of Zone of Inhibitions (mm)]</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Bacillus subtilis</td>
</tr>
<tr>
<td>Petroleum ether (PE)</td>
<td>7.66±0.33</td>
</tr>
<tr>
<td>Ethyl acetate (EA)</td>
<td>8.33±0.33</td>
</tr>
<tr>
<td>Acetone (AC)</td>
<td>10.6±0.27</td>
</tr>
<tr>
<td>Methanol (ME)</td>
<td>16±0.57</td>
</tr>
</tbody>
</table>

**Standard Antibiotics (10mcg/disc)**

<table>
<thead>
<tr>
<th>Antibiotic</th>
<th>Bacillus subtilis</th>
<th>Staphylococcus aureus</th>
<th>Pseudomonas aeruginosa</th>
<th>Escherichia coli</th>
<th>Klebsiella pneumoniae</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ciprofloxacin</td>
<td>25.6±0.38</td>
<td>19±9</td>
<td>32.6±0.27</td>
<td>28±0</td>
<td>32±0.1</td>
</tr>
<tr>
<td>Amikacin</td>
<td>22±0</td>
<td>22.3±32</td>
<td>21±0</td>
<td>24±0</td>
<td>22±0.34</td>
</tr>
<tr>
<td>Penicillin</td>
<td>28.3±0.32</td>
<td>22.6±0.34</td>
<td>23±0</td>
<td>25±0</td>
<td>Nil</td>
</tr>
<tr>
<td>Kanamycin</td>
<td>13.6±0.38</td>
<td>17.3±0.32</td>
<td>17.6±0.2</td>
<td>17.6±0.2</td>
<td>14.6±0.43</td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>17.45±0.32</td>
<td>18.33±0.32</td>
<td>19±0</td>
<td>15.6±0.2</td>
<td>18±0.2</td>
</tr>
<tr>
<td>Streptomycin</td>
<td>9.6±0.38</td>
<td>25±0</td>
<td>20±0</td>
<td>18±0</td>
<td>18±0.2</td>
</tr>
<tr>
<td>Gentamycin</td>
<td>9.6±0.38</td>
<td>26.6±0.38</td>
<td>17.6±0.27</td>
<td>17.6±0.27</td>
<td>16.7±0.34</td>
</tr>
<tr>
<td>Erythromycin</td>
<td>31.6±0.38</td>
<td>15.6±0.38</td>
<td>Nil</td>
<td>17.3±0.32</td>
<td>12.4±0.32</td>
</tr>
<tr>
<td>Ampicillin</td>
<td>10±0</td>
<td>28.6±0.38</td>
<td>Nil</td>
<td>Nil</td>
<td>Nil</td>
</tr>
</tbody>
</table>
PE extract has shown zone of inhibition in case of *B. subtilis* and *E. coli*, in case of other three pathogens no inhibition was observed. EA extract was active against all the four pathogenic strains except *K. pneumoniae*, but the zone diameters were not significant as compared to the standards. AC and ME extracts exhibited inhibition against all the strains. Zone diameters in case of both the extracts against *K. pneumoniae* were negligible. Among the standard antibiotics used, ciprofloxacin showed best results against *P. aeruginosa* (32.6 mm) and *K. pneumoniae* (32 mm), amikacin against *E. coli* (24 mm), chloramphenicol against *E. coli* (19 mm) and *S. aureus* (18.33 mm), streptomycin and gentamycin against *S. aureus* (25 mm and 26.6 mm respectively), erythromycin against *B. subtilis* (31.6 mm) and ampicillin against *S. aureus* (28.6 mm). Kanamycin and chloramphenicol showed nearly similar diameter of inhibition zones against all test organisms, hence these two antibiotics were selected as standards for further studies on antimicrobial activity of the plant.

Results suggest that AC and ME extracts exhibited wide inhibition zone diameters nearly similar to that exhibited by some of the standards (kanamycin, chloramphenicol). In case of both AC and ME extracts maximum zone of inhibition was observed against *Pseudomonas aeruginosa* and least inhibition zone diameter was observed in case of *Klebsiella pneumoniae*. PE and EA extracts were less effective as compared with the activities shown by AC and ME extracts against all these microorganisms selected for investigation. The inhibitory activity was found to be maximum in case of ME extract followed by AC, EA and PE extract on the bacterial strains. Depending on the results obtained after performing preliminary antimicrobial screening, AC an ME extract were selected for antimicrobial activity studies in detail and minimum inhibitory zone determination processes.
Figure 5.1: Photograph showing inhibition zone diameters of various leaf extracts of *L. lucidus* and standard antibiotics (Ciprofloxacin and gentamycin; 10mcg/disc) against *Pseudomonas aeruginosa*. [A = PE Extract; B = EA extract; C = AC extract; D = ME extract].
5.3 ANTIMICROBIAL ACTIVITY AND MIC DETERMINATION OF ACETONE AND METHANOLIC LEAF EXTRACTS OF L. lucidus

5.3.1 Materials and methods

The procedure is carried out in a similar way to the previous one (5.1), only change is that here four concentrations (10 mg/ml, 1 mg/ml, 500 mcg/ml and 250 mcg/ml) of each of the plant extracts were made by dissolving measured amount of the samples (leaf residues) in calculated volume of solvents to prepare 4 sets of sample discs (10 mg/disc, 1 mg/disc, 500 mcg/disc, 250 mcg/disc) of each leaf extracts. 500 mcg/ml and 250 mcg/ml concentrations were prepared by serial dilution of 1 mg/ml test sample. Here, standard antibiotic discs (Kanamycin & Chloramphenicol; 10 mcg/ disc) and blank discs (impregnated with solvents) were used as a positive and negative control respectively.

5.3.2 Minimum inhibitory concentration (MIC) determination

Serial tube dilution technique (Rahman et al., 2000; Mosaddik et al., 2003) was used to determine MIC of the crude extracts against these bacterial strains. Crude extracts (1.024 mg) dissolved in 2 ml distilled water to obtain stock solution having concentration of 512 µg/ ml. To facilitate dissolution a few drops of Tween 80 was added. In serial dilution technique, 1 ml prepared stock solution was added to test tube containing 1 ml nutrient broth medium to prepare concentration 256 µg/ml. From this solution 1 ml was transferred to another test tube containing 1 ml nutrient broth medium to give concentration 128 µg/ml. This procedure was repeated to prepare concentration up to 2 µg/ml. An 18 hours old culture of bacterial strains was diluted with a sterile physiological saline solution [PS; 0.85% (w/v) sodium chloride] with reference to the 0.5 McFarland standards to get an inoculum size of approximately 10^7 colony forming unit per millilitre. Then a drop of suspension (0.02 ml) was added to each broth dilution. After 24 hours
incubation at $37^0$ C, the tubes were examined for the growth of microbes. The MIC of crude extracts was considered as the lowest concentration that showed no growth. Growth observed in those test tubes where the broth appeared turbid or cloudy and the spectral readings were noted down. Distilled water with few drops of Tween 80 was considered as negative control and kanamycin was used as positive control.

**5.3.3 Statistical Analysis**

All data presented are means of three determinations along with standard deviation (SD). Data were analysed by one-way ANOVA in Microsoft Excel software.

**5.4 RESULTS AND DISCUSSION**

Preliminary antimicrobial screening assay of the *L. lucidus* leaf extracts is shown in both methanolic and acetone extracts gave relatively wide inhibition zones against the test strains compared with the positive control. The relatively wider spectrum of activity of the methanolic extracts over the positive controls (chloramphenicol and kanamycin) is significant ($P<0.05$) from the disk diffusion assay. The negative control methanol was devoid of any antimicrobial activity. Except *Klebsiella pneumoniae*, all other test organism’s growth was inhibited by both acetone and methanol extracts. Acetone has shown range of inhibition diameter from 8 to 16.33 mm, whereas methanol has shown inhibition range of 7.6–18 mm. *Pseudomonas aeruginosa* is more sensitive and *Bacillus subtilis* is least sensitive to both the extracts. After the preliminary antimicrobial screening assay, the extracts that showed significant antimicrobial property were selected for the determination of MIC. The methanolic and acetone extracts MICs for the tested bacterial strains were from 8 µg/ ml - 128 µg/ ml respectively. Disc diameter of Chloramphenicol and Kanamycin ranged from 13.6-25 mm at a concentration of 10 mcg/ disc. All data corresponding to test organisms are tabulated in Table 5.2, 5.3 and 5.4. In this study both methanolic and acetone extracts demonstrated
antibacterial activity which may explain anonymous claim on the topical use of *Lasianthus lucidus* leaves for infected wound.

**Table 5.2:- Antimicrobial activity of different concentrations of methanolic extract (ME) of *Lasianthus lucidus***

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Diameter of Zone of Inhibitions (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>250 mcg/disc</td>
</tr>
<tr>
<td><strong>Staphylococcus aureus</strong></td>
<td>9.3 ± 3</td>
</tr>
<tr>
<td><strong>Bacillus subtilis</strong></td>
<td>7.6 ± 0.3</td>
</tr>
<tr>
<td><strong>Pseudomonas aeruginosa</strong></td>
<td>9.3 ± 0.3</td>
</tr>
<tr>
<td><strong>Escherichia coli</strong></td>
<td>9.6 ± 0.3</td>
</tr>
<tr>
<td>Test Organisms</td>
<td>250 mcg/disc</td>
</tr>
<tr>
<td>---------------</td>
<td>--------------</td>
</tr>
<tr>
<td><em>Klebsiella pneumonia</em></td>
<td>-</td>
</tr>
</tbody>
</table>

* Zone are mean ± SD for n =3

- No zone of inhibition

Table 5.3: Antimicrobial activity of different concentrations of acetone extract (AC) of *Lasianthus lucidus*

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>Diameter of Zone of Inhibitions (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>9.66±0.38</td>
</tr>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>9±0.57</td>
</tr>
<tr>
<td><em>Pseudomonas aeruginosa</em></td>
<td>9.6±0.3</td>
</tr>
</tbody>
</table>
Table 5.4:- Minimum inhibitory concentration of acetone and methanolic extracts of *Lasianthus lucidus*

<table>
<thead>
<tr>
<th>Test Organisms</th>
<th>MIC values of AC extract (µg/ml)</th>
<th>MIC values of ME extract (µg/ml)</th>
<th>MIC values of Kanamycin (µg/ml)</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Escherichia coli</em></td>
<td>9.66 ± 0.38</td>
<td>13 ± 0.32</td>
<td>20 ± 0</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>14.2 ± 0.32</td>
<td>14.8 ± 0.54</td>
<td>-</td>
</tr>
</tbody>
</table>

* Zone are mean ± SD for n = 3
- No zone of inhibition

Results of antimicrobial activity of AC and ME extracts were also represented graphically by bar diagrams showing error bars (figure 5.2). In cases of all the test organisms, bars showed a descending trend from higher concentration to lower concentration. These graphical representations clearly signify that the potentiality of microbial inhibition is more in ME extract followed by AC extract.
Staphylococcus aureus

![Graph showing disc diameter (mm) against concentration (mg/disc) for Staphylococcus aureus. The graph compares the effects of AC and ME at concentrations of 10, 1, 0.5, and 0.25 mg/disc.]

Escherichia coli

![Graph showing disc diameter (mm) against concentration (mg/disc) for Escherichia coli. The graph compares the effects of AC and ME at concentrations of 10, 1, 0.5, and 0.25 mg/disc.]

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Figure 5.2:- Antibacterial activity of methanolic extract of *Lasianthus lucidus* leaf representing A= *Staphylococcus aureus*; B= *Escherichia coli*; C= *Pseudomonas aeruginosa*; D= *Bacillus subtilis*.
From Rubiaceae approximately 53 genera have been recorded on which antibacterial studies have been carried out (table-1.3). Leaves of some of the plants belonging to Rubiaceae with reported antibacterial activity are *Borreria latifolia*, *Canthium coromandelicum*, *Ixora coccinea*, *Morinda citrifolia*, *Morinda tinctora*, *Mitragyna speciosa*, *Mussaenda frondosa*, *Psychotria microblasta*, *Saprosma foetans*, *Tarea asiatica*, etc (Ali et al., 1995; Jayasinghe, 2001; Annapurna et al., 2003; Usha et al., 2010; Parthasarathy, et al., 2009; Khan et al., 2001).

Amongst these plants maximum zone of inhibition have been reported in case of methanolic extract of *Mitragyna speciosa* (30 mm) against *Bacillus subtilis* indicating the presence of potential antimicrobial agent in this plant (Parthasarathy et al., 2009). In case of *L. lucidus* maximum activity was represented by methanolic leaf extract showing disc diameter 18 mm against *Pseudomonas aeruginosa* which reflects moderate antimicrobial activity of the plant. The plants of Rubiaceae have been found to be more or less active against the pathogenic strains of *E. coli*, *P. aeruginosa*, *B. subtilis* and *S. aureus*. Against *K. pneumoniae* only two plants have been reported to take preventive action, these are methanolic and ethanolic extracts of *Borreria hispida* and methanolic extract of *Oldenlandia umbellata* (Muthu et al., 2010; Arun et al., 2010). Antibacterial studies on *L. lucidus* did not show any preventive action against *K. pneumoniae* but found to be protective against all the other test organisms. Though various types of plant extracts have been found to be effective against large number of pathogenic bacterial strains, in maximum number of cases it was represented by methanolic extracts of different plants in this family, suggesting the presence of potential phytochemicals in these extracts (Jayasinghe, 2001; Annapurna et al., 2003; Usha et al., 2010; Parthasarathy, et al., 2009; Khan et al., 2001). In this study of anti-microbial activity of *L. lucidus* also antimicrobial activity have been greatly focused by methanolic extract reflecting the presence of valuable phyto-chemical(s), which if synthesized can be utilised as a potential phyto-medicine against various pathogenic diseases.
Figure 5.3:- Photograph showing antimicrobial activity of acetone (AC) and methanolic (ME) leaf extracts of *Lasianthus lucidus*. [A= ME extract against *Bacillus subtilis*; B= AC extract against *Bacillus subtilis*; C= ME extract against *Staphylococcus aureus*; D= AC extract against *Staphylococcus aureus*; E= ME extract against *Escherichia coli*; F= AC extract against *Escherichia coli*.]
5.5 CONCLUSION

It may be concluded from this study that crude leaf extracts of *L. lucidus* is active against the tested pathogenic microorganisms except *Klebsiella pneumoniae*. In addition, the results confirm the use of the plant in traditional medicine. The results of the investigation do not reveal that which chemical compound is responsible for aforementioned activity. The future prospect of this work is to explore the lead compound liable for aforementioned activity from this plant.

REFERENCES


