CHAPTER VI

ANTI-MICROBIAL ACTIVITY
6.1. Introduction:

The development of drug resistance in human pathogens against commonly used antibiotics has necessitated a search for new antimicrobial substances from other sources including plants. Plants are known to produce a variety of compounds to protect themselves against a variety of pathogens and therefore considered as potential source for different classes of antimicrobial substances (Alamagboul A. Z. et al., 1985; Alkofani A.S. et al., 1990; Grayer R.J. et al., 1994; Olukoya D.K. et al., 1993).

Plants used in traditional medicine contain a wide range of substances that can be used to treat chronic as well as acute infectious diseases. The substances that can either inhibit the growth of micro-organisms or kill them are considered as candidates for developing new drugs for treatment of various infectious diseases (Haila F.N. et al., 1999). The plants used in traditional system of medicine are well known as remedies for diseases in the rural areas of developing countries (Catalano S et al., 1998; Martinez M.J. et al., 1996; Sunder R.K. 1996; Taylor R.S.L. et al., 1996). Herbal medicines have been used in developing countries as an alternative to allopathic medicines.

Our extensive review of literature has revealed a variety of medicinal plants possessing anti-inflammatory activity which also exhibited good antimicrobial properties (Valcheva K.S. et al., 2005; Sarker S.D.et al., 2004; Sandler C. et al.,2005 ; Hauser B. et al.,2005; Vuorela S. et al ., 2005). On the basis of these reports the plant extracts were subjected for antimicrobial activity against various bacteria and fungal strains.
6.2. Materials and methods:

The term microbiological assay is a biological assay performed with microorganisms like bacteria, yeast, moulds, etc. This involves the measurement of the relative potency or activity of compounds by determining the amount of test material required for producing stipulated effect on suitable organism under standard conditions.

The procedures employed in microbial assay were,

a. Cylinder plate method or cup plate method

b. Turbidimetric or tube assay method (two fold serial dilution method).

In the present study, antimicrobial screening was carried out by both cup plate and tube assay (two fold serial dilution) method.

In cup plate method, the antimicrobial substance diffuses from the cup through a solidified agar layer in a petridish or a plate to an extent so that the growth of added micro-organism is inhibited entirely in a circular area or zone around the cavity containing the solution of a known quantity of antimicrobial substance. The antimicrobial activity is expressed as the zone of inhibition in millimeters, which is measured with a zone reader.

The leucopyrosinol and crude extract of ethyl acetate, leaves of Fluggea leucopyrus was screened for antimicrobial activity against a wide spectrum of microorganisms and the activity was compared with appropriate reference standards (chloramphenicol for both gram-positive and gram-negative micro-organisms and nystatin for fungal strains). Micro-organisms were grown in nutrient agar medium. Dimethyl sulphoxide (DMSO) and distilled water were used as control and the drug vehicles for the plant extract and reference standards respectively.
6.3. Test Organisms:

The microorganisms used for the experiment were procured from MTCC, IMTECH, Chandigarh (The microbiology department of IISC, Bangalore).

6.3.1. Gram-positive organisms:

Streptococcus pneumoniae
Staphylococcus aureus
Bacillus cereus
Bacillus pumilis

6.3.2. Gram-negative organisms:

Escherichia coli
Enterobacter aerogenes
Pseudomonas aeruginosa
Streptomyces marienensis

6.3.3. Fungal strains:

Aspergillus niger
Rhizopus stolonifer
Saccharomyces cervisiae

6.4. Standardization of micro-organisms:

One loop-full of micro-organisms were inoculated into 100 ml of sterile medium and incubated for 24 hrs at 37°C for bacterial culture and for 48 hrs at 27°C for fungal culture.
After 24 h/48 h of incubation, 1 ml of broth containing the micro-organisms was added to 9 ml of peptone water.

10 fold serial dilutions were made in the range of 10^-1 to 10^-10.

100 μl of the dilutions ranging from 10^-5 to 10^-8 were spread over the sterile nutrient agar (SNA) plates and kept at 37°C and 27°C for 24 / 48 hours respectively.

The number of colony forming units (CFU) was counted and number of micro-organisms per 1 ml of stock culture was calculated.

**6.5. Preparation of test and standard solutions:**

The stock solution of test compounds was prepared by dissolving the dried extracts at a concentration of 5 and 10mg/ml in DMSO respectively. The stock solution of reference standards (chloramphenicol and nystatin) was prepared at a concentration of 0.6 mg/ml in sterile water. Antimicrobial activity was screened by adding 0.05 ml stock solution to each cup by using micropipette.

**6.6. Culture medium:**

The following media were used for antimicrobial studies.

**6.6.1. Nutrient broth for bacteria:**

<table>
<thead>
<tr>
<th>Ingredient</th>
<th>Concentration</th>
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<tbody>
<tr>
<td>Beef extract</td>
<td>0.35%</td>
</tr>
<tr>
<td>Sodium chloride</td>
<td>0.5%</td>
</tr>
<tr>
<td>Peptone</td>
<td>0.5%</td>
</tr>
</tbody>
</table>
The above ingredients weighing 37 g were dissolved in distilled water (1000 ml). pH was adjusted to 7.2-7.4 and sterilized by autoclaving at 15 lbs/inch² for 20 min.

6.6.2. Sabouraud’s dextrose agar medium for fungi (SDA):

Dextrose - 4.0%
Peptone - 1.0%
Agar - 2.5%

The above constituents were dissolved in distilled water and pH was adjusted to 5.6 ± 0.2 and then sterilized by autoclaving at 15 lbs/inch² for 20 min.

6.7. Sterilization:

Sterilization of the media, water, etc., was carried out by autoclaving at 15 lbs/inch² for 20 minutes. The glassware like syringes, petridishes, pipettes, empty test-tubes were sterilized by dry heat in an oven at a temperature of 160°C for one hour.

6.7.1. Nutrient agar for bacteria:

Beef extract - 0.3%
Sodium chloride - 0.5%
Peptone - 0.5%
Agar - 2.0%
P pH - 7.2-7.4

The sterilized medium was cooled to 40°C and poured into the petridishes to contain 6 mm thickness. The media was allowed to solidify at room temperature.


The cylinder plate assay of drug potency is based on measurement of the diameter of zone of inhibition of microbial growth surrounding cylinders (cups), containing various dilutions of test compounds.

A sterile borer was used to prepare four cups of 6 mm diameter in the agar medium spread with the micro-organisms and 0.1 ml of inoculum was spread on the agar plate by spread plate technique. Accurately measured (0.05 ml) solution of each concentration and reference standards were added to the cups with a micropipette.

All the plates were kept in a refrigerator at 2 to 8°C for a period of 2 hours for effective diffusion of test compounds and standards. Later, they were incubated at 37°C for 24 hours. The presence of definite zone of inhibition of any size around the cup indicated antibacterial activity. The solvent control was run simultaneously to assess the activity of dimethyl sulphoxide and water which were used as drug vehicles. The experiments were performed three times. The diameter of the zone of inhibition was measured and recorded.

6.8.2. Determination of minimum inhibitory concentration (MIC) in liquid medium (Indian Pharmacopoeia, 1996; Micheal J, 1998):

The turbidimetric assay to detect the drug potency is based on inhibition of microbial growth as indicated by measurement of the turbidity (transmittance) of a suspension of suitable micro-organisms in a fluid medium to which have been added, graded amounts of the test compounds and known concentration of reference material.
6.8.2.1. Requirements:

Mueller-Hinton broth / Sabouraud’s dextrose broth
Assay tubes
Test organisms (as per IP 1996, A-108)
Escherichia coli ATCC 8739
Staphylococcus aureus ATCC 6538
Pseudomonas aeruginosa ATCC 9027
Aspergillus niger ATCC 16404
Candida albicans ATCC 10231
Micropipettes
Vortex mixer
Test drug

6.8.2.2. Method:

1. Series of dilutions were prepared containing the same volume of medium inoculated with the test organism (the inoculums may vary from $10^3$ to $10^6$ cells / ml).

2. Drug solutions were prepared by serial two-fold dilution technique, (i.e. if the concentration of drug in the first tube is 1000 µg/ml, in the second tube it will be 500 µg/ml and in the third 250 µg/ml and so on) one tube was left without drug, to serve as positive control.
Dilutions = 1000 µg  500 µg  250 µg  125 µg  62.5 µg  31.25 µg

Broth

Containing

Test organism

Test compound

Incubated the tubes at 24 / 48 h at 37°C / 27°C

Serial two fold dilutions of the test compounds were prepared from a high concentration of 1000 µg/ml. The same procedure was followed for positive control. The lowest concentration of the fraction which caused apparently a complete inhibition of growth of organisms was taken as MIC.

1. The cultures were incubated at a temperature optimal for the test organism and for a period of time sufficient for the growth of at least 10 -15 generations (usually 24 hours for bacteria at 37°C and 48 hours for fungi at 27°C).

2. The tubes were inspected visually to determine the growth, as indicated by turbidity (in fact, turbidity of the culture medium is indicative of the presence of a large number of cells) and the tubes in which the antibiotic is present in concentration sufficient to inhibit bacterial growth remain clear.

3. In experimental terms, the minimum inhibitory concentration (MIC) is the minimum concentration at which the drug or test compound inhibits the growth of the micro-organisms.
### Results and discussion:

**Table 1: Antibacterial activity of leucopyrosinol and ethyl acetate (crude) extract of Fluggea leucopyrus**

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Dose (µg/cup)</th>
<th>Zone of inhibition&quot; (diameter in mm)</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>gram (+)ve</td>
<td>gram (−)ve</td>
</tr>
<tr>
<td></td>
<td></td>
<td>S.p</td>
<td>B.c</td>
</tr>
<tr>
<td>Fluggea leucopyrus:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethyl acetate</td>
<td>100</td>
<td>12</td>
<td>9</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>13</td>
<td>13</td>
</tr>
<tr>
<td>Leucopyrosinol</td>
<td>100</td>
<td>14</td>
<td>24</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>19</td>
<td>29</td>
</tr>
<tr>
<td>Standard:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Chloramphenicol</td>
<td>30</td>
<td>19</td>
<td>18</td>
</tr>
<tr>
<td>Vehicle:</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td></td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

S.p=Streptococcus pneumoniae; B.c=Bacillus cereus; B.p=Bacillus pumilis; S.a=Staphylococcus aureus; E.c=Escherichia coli; E.a=Enterobacter aerogens; P.a=Pseudomonas aeruginosa; S.m=Streptomyces marienensis; -=No activity

#Values are the average of triplicate; Includes the cup diameter (6mm)
Table 2: Antifungal activity of lecopyrosinol and ethylacetate (crude) extract of *Fluggea leucopyrus*

<table>
<thead>
<tr>
<th>Plant material</th>
<th>Dose (μg/cup)</th>
<th>Zone of inhibition* (diameter in mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td><em>A.n</em></td>
</tr>
<tr>
<td><em>Fluggea leucopyrus:</em></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Ethylacetate</td>
<td>100</td>
<td>17</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>19</td>
</tr>
<tr>
<td>Leucopyrosinol</td>
<td>100</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>150</td>
<td>25</td>
</tr>
<tr>
<td>Standard:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Nystatin</td>
<td>30</td>
<td>18</td>
</tr>
<tr>
<td>Vehicle:</td>
<td></td>
<td></td>
</tr>
<tr>
<td>DMSO</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Water</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

A.n = Aspergillus Niger; R.s = Rhizopus stolonifer; S.c = Sacharomyces cerevisiae; - = No activity

*Values are the average of triplicate; Includes the cup diameter (6mm).
Table 3: Determination of Minimum Inhibitory Concentration (MIC)

<table>
<thead>
<tr>
<th>Organism</th>
<th>Antimicrobial concentration (µg/ml)</th>
<th>Growth Control</th>
<th>Sterility Control</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1000</td>
<td>500</td>
<td>250</td>
</tr>
<tr>
<td><em>E. coli</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>S. aureus</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>P. aeruginosa</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>A. niger</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. albicans</em></td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

Note: (+) Growth; (-) No growth

The leucopyrosinol at a concentration of 100 µg, 150 µg where as ethyl acetate at 100 µg and 150 µg exhibited antibacterial and antifungal activities against one or the other organisms in dose dependent manner. The plant extracts have exhibited considerable activity on the tested fungi. Leucopyrosinol had produced good antibacterial activity against gram +ve and gram –ve bacteria and fungal strains when compared to ethyl acetate crude. The plant did not produced inhibition against *Staphylococcus aureaus*, *Pseudomonas aeruginosa* and *Streptomyces marienensis* at a dose of 100 µg/cup.

Table 3. Shows that test tubes containing 31.25, 62.5 & 125 µg/ml respectively of antimicrobial drug exhibited growth evident in the form of turbidity when compared against the sterility control (blank), both in the case of bacteria and fungi. Whereas test tubes containing 250, 500 & 1000 µg/ml respectively of antimicrobial drug did not show any growth. Hence, 250 µg/ml is the minimum inhibitory concentration of the antimicrobial drug both in the case of bacteria and fungi. The above results clearly demonstrated that the extracts had significant and considerable anti-microbial activity against variety of pathogens.
6.10. References:


Indian Pharmacopoeia Vol-II, the Controller of Publications, New Deihi, 1996.


Valcheva Kuzmanove S, Marazova K, Krasnaliew I, Galunska B, Bosrisova P, Belcheva. An Effect of Aronia meanocarpa fruit juice on indomethacine