Chapter 1

Collection, Extraction, Antibacterial activity and detection of small molecules present in different parts of *Andrographis paniculata*

1.1 Introduction

*Andrographis paniculata* (Acanthaceae) plant is native to China, India and Taiwan. It is a medicinal herb with an extremely bitter taste used to treat liver disorder, bowel complaints of children, colic pain, common cold and upper respiratory tract infections. The ariel part of *Andrographis paniculata* is commonly used in Chinese medicine. According to Chinese medicine theory, *Andrographis paniculata* cools and relieves internal heat, inflammation and pain and hence used for detoxication.

The herb contains diterpenoids, flavonoids and polyphenols as the major bioactive components. In comparison with other Chinese medicinal herbs, *Andrographis paniculata* showed a wide variety of health benefits, due to the presence of bioactive compounds. A few derivatives have been semi-synthesized to enhance their bioactivity than original compounds, suggesting its potential for drug development (Matsuda *et al.*, 1994).

1.1.1 Taxonomic classification

Kingdom: Plantae

Unranked: Angiosperms

Unranked: Eudicots

Unranked: Asteroids
Order: Lamiales
Family: Acanthaceae
Genus: Andrographis
Species: Andrographis paniculata

Binomial name: Andrographis paniculata (Burmf) Wall Ex Nees.

1.1.2 Etymology

Andrographis paniculata is an erect annual herb extremely bitter in taste in each and every part of the plant body. The plant is known in north eastern India as maha-tita literally “king of bitters”, and known by various vernacular names. It is also known as bhui-neem, since the plant is smaller in size and has a similar appearance as that of Neem (Azadirachta indica). In Tamil it is called “sirunangai” or “Siriyanangai”. The genus Andrographis consists of 28 species of small annual shrubs essentially distributed in tropical Asia. Only a few species are medicinal of which Andrographis paniculata is the most popular.

1.1.3 List of vernacular names

Hindi: Kirayat
English: Green chirayta, creat, king of bitters, Andrographis
Sanskrit: Kalmegha, Bhunimba
Tamil: Nilavembu, Siriyanangai
Kannada: Nelaberu
Telugu: Nelavemaa
Malayalam: Nilavembu, kiriyyattu.
Figure 2: *Andrographis paniculata* showing the different plant parts including stem, leaf, flowers, buds and seeds. *Andrographis paniculata* is an erect annual herb, grows to a height of 30 to 110 cm in moist shady places. It has glabrous leaves, about 8 cm long and 25 cm broad, lanceolate and pinnate. The flowers are small whitish or pale pink with brown or purple spots, spreading auxillary and terminal panicles. Capsules are linear or oblong containing numerous seeds which are sub quadracte and yellowish brown in colour.
1.1.4 Antibacterial activity

The wide spread and indiscriminate use of antibiotics especially the broad spectrum antibiotics has confounded the problems of their efficacy as a result of microbial resistance and adverse effects on the host. The global antibacterial resistance is becoming an increasing public health problem. Bacterial resistance to almost all available antibiotics have been recorded. The pharmaceutical industries and fledgling biotechnology companies are intensifying efforts to discover novel antibiotics in attempts to overcome bacterial resistance. Further the newer broad spectrum antibiotics are cost prohibitive and are not within the reach of poor.

Considering these lacunae, suitable experiments were designed to extract and to evaluate the antibiotic potentials of Andrographis paniculata after their collections from the natural habitat.
1.2 Materials and Methods

1.2.1 Collection of plant materials

The whole plant materials of *Andrographis paniculata* were collected from Pottalpudur village in Tirunelveli district and confirmed by the plant taxonomist, Govt Siddha Medical College, Tirunelveli. A herbarium was prepared and maintained in the SPKCES of M.S. University, Tirunelveli. The plants were thoroughly washed with distilled water and separated into desired different parts like leaves, stem, root, branches, seed, and buds (Fig. 3).

1.2.2 Test Bacterial Strains

The Pathogenic bacteria were isolated from clinical samples and collected from M/s Vivek laboratories, Nagercoil. They were identified by different biochemical tests. All the bacterial strains used were maintained as pure cultures in slants and stored in refrigerated conditions. The list is given below:

1. *Bacillus subtilis* CMST, (MSU)
2. *Staphylococcus aureus* Vivek Scientific Laboratory, Nagercoil
3. *Enterobacter faecalis* Vivek Scientific Laboratory, Nagercoil
4. *Escherichia coli* Vivek Scientific Laboratory, Nagercoil
5. *Salmonella typhi* Vivek Scientific Laboratory, Nagercoil
6. *Pseudomonas aeruginosa* CMST, (MSU)

1.2.3 Extract preparation

The extraction procedure given by Chaudhri, (1999) was followed and the methodology is given below:
Figure 3: Different Parts of the plant *Andrographis paniculata*: A. leaves B. branches C. stem D. morphology of whole plant E. root F. seed G. buds
**Comminution/Pulverisation**

Comminution is which the plants were fragmented into small particles by mechanical forces. The plant materials were shredded by sterile surgical blade to the desired size and the particles were used for extraction.

**Preparation of fresh extract**

About 50g of each plant part (i.e. root, stem, leaf, branch, seed and buds) were taken and macerated with mortar and pestle. Then they were extracted with 50ml of methanol and water respectively in sterilized air tight amber coloured container and were stored in refrigerator at 4°C for further analysis.

**Preparation of dry extract**

The pulverised plant materials were dried in shade for a week. After drying, the desired plant parts were weighed and mixed with water and methanol in 1:2 ratio respectively in air tight amber coloured sterilized container. The containers were placed on shaker for a week at room temperature. After one week, the supernatant was transferred to the next sterilized airtight container after filtered by using cheese cloth and was stored in refrigerator at 4°C for further analysis.

**1.2.4 Concentration of crude extract**

The concentration has been done by distilling the solvent from the filterate. For aqueous extract the concentration was done in jacketed open pans, and for concentrating the methanol extract the samples were placed in evaporating dishes.
1.2.5 Recovery/Yield

The extracts were completely dried in evaporating dishes and concentrated. The yield ranges from 0.02 to 0.07% as given below:

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Material</th>
<th>Quantity in gm/ml</th>
<th>Yield in %</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Plant leaf</td>
<td>50</td>
<td>0.06</td>
</tr>
<tr>
<td>2</td>
<td>Branches</td>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td>3</td>
<td>Stem</td>
<td>50</td>
<td>0.07</td>
</tr>
<tr>
<td>4</td>
<td>Root</td>
<td>50</td>
<td>0.05</td>
</tr>
<tr>
<td>5</td>
<td>Seeds</td>
<td>50</td>
<td>0.03</td>
</tr>
<tr>
<td>6</td>
<td>Buds</td>
<td>50</td>
<td>0.02</td>
</tr>
</tbody>
</table>

1.2.6 Inoculum preparation

The nutrient broth was prepared and distributed in different conical flasks. The flasks were sterilized in auto clave at 121°C for 15 minutes. One loopful of culture of different bacterial strains was inoculated in 50 ml broth under aseptic condition. The flasks were labelled and incubated at 37°C for 24 hours. The turbidity was compared with MC Farland Nephalmeter standard and adjusted the turbidity by adding saline or increasing the inoculum concentration (density).

Inoculum standard (Mc Farland Nephalmeter) preparation

To make the turbidity of the inoculam standard, 0.05ml of 1.175% barium chloride solution was mixed with 9.95ml of 1% H₂SO₄.
1.2.7 Medium preparation

**Nutrient Broth**

Autoclaved nutrient broth containing NaCl 5g, Peptone 5g and Beef extract 1.50g (compositions all in g/lit) was used for growing of overnight bacterial culture used in the experiments.

**Muller-Hinton Agar (MHA) medium**

MHA was used as the specialized medium for the antimicrobial assay. (Beef 300g, infusion from casein acid hydrolysate 17.50g and starch 1.50g) at pH 7.3 ± 0.2 was used for antimicrobial well diffusion assay.

1.2.8 Inoculation

The sterilized cotton swab was dipped into the bacterial culture, squeezed on the sides of the tubes to remove excess of inoculum and spread uniformly over the surface of the agar plates by moving back and forth in all directions.

**Well plate method**

The molten MHA medium at 50 °C was poured into sterile petridishes, the plates were allowed to set. The organisms were uniformly swabbed on the plates and wells were then bored into the agar medium using a sterile 6mm cork bores. The wells were later filled up with the extract at a quantity of 20µl. The plates were allowed to stand on for 1 hour to allow proper diffusion of the extract and to prevent spillage onto the surface of the agar medium and then incubated at 37 °C for 24 hours after which they were observed for zone of inhibition. The same procedure was followed
for all the bacterial strains. The zone of inhibition was calculated with standard deviation.

1.2.9 Preparation of control

Positive control

- Leaves extract 20µl
- Kanamycin 20µl
- Ampicillin 20µl

Negative control

- 20µl methanol

1.2.10 Influence of different factors on the bioactivity of *Andrographis paniculata*

1.2.10.1 Effect of heat

The crude extract was autoclaved at 15 lb pressure for 15 minutes. The MHA medium was prepared, poured in petriplates and after solidification and swabbing with test bacterial strains, 6mm well was punched. The autoclaved extract and the normal extracts were loaded in the well (20µl). The loaded petridishes were incubated at 37 °C for 24 hours. After incubation the diameter of zone of inhibition was measured in mm. Replicated plates were maintained and the zone of inhibition was calculated with standard deviation. The zone of inhibition before and after autoclave was observed and compared.

1.2.10.2 Effect of sunlight

The extract powder was exposed in sunlight for 2, 4 and 5 h respectively. The MHA plates were prepared as indicated above and the extract exposed in sunlight was loaded in the respective wells. The extract which was not exposed to the sunlight
served as positive control. Methanol was used as negative control. The zone of inhibition was observed and compared after 24 hours of inhibition at 37°C.

1.2.10.3 Effect on incubation time

The MHA plates were prepared and the wells were loaded with the extract. The plates were incubated at 37°C for 48 hours. After 24 hours the plates were observed for the inhibition zone. The zone of inhibition was observed and the values were tabulated. Then the plates were incubated for another 24 hours, after that the zone of inhibition was observed and compared with the readings of 24 hours.

1.2.10.4 Comparison with antibiotics

The MHA plates were prepared and loaded with common antibiotics like ampicillin and kanamycin in the concentration of 0.2µg. The crude extract was loaded in the concentration of 0.8µg and the zone of inhibition was observed and compared.

1.2.11 Screening of small molecules

Thin Layer Chromatography technique was used for detecting the small molecular compounds present in the different parts of the four different extracts of the plant *Andrographis paniculata*. The tests were performed on a sheet of glass coated with a thin layer of silica gel. Samples were applied on the plate.

1.2.11.1 Preparation of plates

The slurry was prepared by mixing silica gel with water in the ratio 3:2. A few drops of ammonia were added into the slurry to separate the nitrate compounds in the sample. The slurry was coated on the glass plate at a thickness of about 0.25mm and then the plates were allowed to dry at room temperature for 15 to 20 minutes. Then
the plates were kept in hot air oven at 100-120°C for 1 to 2 hours to remove the moisture and to activate the absorbent on the plate. The samples were loaded on the plate about 1.5 to 2 cm from the bottom, the spots were allowed to dry and spotting was carried out repeatedly in order to get a more concentrated spot. For the stationary phase, silica gel was used. For the mobile phase, Chloroform 100% and Acetone 100% were used.

1.2.11.2 Chromatogram development

The solvent chloroform and acetone in the ratio 4:1 was used as the mobile phase. The solvent was poured into the tank and allowed to stand for an hour to ensure that the atmosphere within the tank become saturated with solvent vapours. A sheet of tissue paper was placed inside the chamber for equilibration. After equilibration, the plate was placed vertically in the tank.

1.2.11.3 Identification of compounds

The chromatogram was allowed to dry and the plate was exposed to UV. Some of the compounds were fluoresced in different colours. Then the plates were exposed to iodine vapour.
1.3 Results

The extract of *Andrographis paniculata* exhibited antibacterial activity towards the different bacterial isolates and the salient findings are presented below.

1.3.1 Antibacterial Assay

In order to characterize the antimicrobial activity of the chemicals present in *Andrographis paniculata*, methanol and aqueous extract of the different parts of the plants were prepared both in the fresh and dried form. Antibacterial activities of the different organisms were studied and the results were tabulated. It was found that both the G +ve and G-ve organisms are sensitive to leaves extract. The results are tabulated and presented in Tables 4 to 9 with standard deviation (SD). The trend of inhibition noted in petridishes are given in Fig. 4 to 33. The comparative antibacterial effect of methanol and aqueous extract is given in Table 10.

Table 4: Antibacterial activity of different parts of *Andrographis paniculata* towards *Bacillus subtilis*

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts(20µl)</th>
<th>Zone of inhibition formed by the different parts of plant (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>leaf</td>
</tr>
<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>20±0.7</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>13±0.9</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
<td>18±0.3</td>
</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>8±0.2</td>
</tr>
</tbody>
</table>

R -Resistant
The leaves extract both in the fresh and dried form showed maximum activity towards *Bacillus subtilis*. The extract prepared from buds showed negative result. Among the dried form, only the leaves extract showed significant activity, but in the fresh form except the bud extract all the other extracts showed activity (Table 4).

**Table 5: Antibacterial activity of different parts of *Andrographis paniculata* towards *Staphylococcus aureus***

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts (20µl)</th>
<th>Zone of inhibition formed by the different parts of plant (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>leaf</td>
</tr>
<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>13±0.2</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>12±0.6</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
<td>15±0.5</td>
</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>8±0.2</td>
</tr>
</tbody>
</table>

R-resistant

The aqueous and methanol extract of leaves both in the fresh and dried form showed significant activity against *Staphylococcus aureus*. In the fresh extract, the stem, seed, root and branch showed activity. The dry aqueous extract showed negative result. The extract from buds also showed no activity.
Figure 4: Zone of inhibition against *Bacillus subtilis* in fresh methanol extract

Figure 5: Zone of inhibition against *Bacillus subtilis* in fresh aqueous extract

**Figure 4, 5:** A, B, C, D, E and F was the inhibition zone formed by *Bacillus subtilis* towards the fresh methanol and aqueous extracts of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 6: Zone of inhibition against *Bacillus subtilis* in dry methanol extract

Figure 7: Zone of inhibition against *Bacillus subtilis* in dry aqueous extract

**Figure 6, 7:** A, B, C, D, E, F was the zone of inhibition formed by *Bacillus subtilis* towards dry methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 8: Zone of inhibition against *Staphylococcus aureus* in fresh methanol extract

Figure 9: Zone of inhibition against *Staphylococcus aureus* in fresh aqueous extract

**Figure 8, 9:** A, B, C, D, E, F was the inhibition zone formed by *Staphylococcus aureus* towards fresh methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 10: Zone of inhibition against *Staphylococcus aureus* in dry methanol extract

Figure 11: Zone of inhibition against *Staphylococcus aureus* in dry water extract

Figure 10, 11: A, B, C, D, E, F was the zone of inhibition formed by *Staphylococcus aureus* towards dry methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 12: The antibacterial activity of different parts of *Andrographis paniculata* on *Bacillus subtilis* (with standard deviation).

Figure 13: Antibacterial activity of different parts of *Andrographis paniculata* on *Staphylococcus aureus*.
Table 6: Assay for the different parts of *Andrographis paniculata* towards *Klebsiella pneumoniae*

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts</th>
<th>Zone of inhibition formed by the different plant parts (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>leaf</td>
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<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>12±0.4</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>12±0.3</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
<td>10±0.4</td>
</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>8±0.2</td>
</tr>
</tbody>
</table>

R-Resistant

High activity was shown by the fresh extracts of *Andrographis paniculata* on *Klebsiella pneumoniae*. Except that of the bud extract all the others showed significant activity. In the dried form, negative result was noted except for the leaves extract.

Table 7: Antibacterial activity of different parts of *Andrographis paniculata* towards *Salmonella typhi*

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts</th>
<th>Zone of inhibition formed by the different plant parts (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>leaf</td>
</tr>
<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>12±0.3</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>10±0.5</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
<td>10±0.3</td>
</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>9±0.4</td>
</tr>
</tbody>
</table>

R-Resistant
The leaves extract of *Andrographis paniculata* both in the fresh and dried form exhibit significant activity towards *Salmonella typhi*. Except the aqueous extract prepared from dried parts all the other extracts exhibited broad range of activity. The stem and branch extract of dry methanol showed moderate range of activity.

Table 8: Antibacterial activity of different parts of *Andrographis paniculata* towards *Escherichia coli*

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts</th>
<th>Zone of inhibition formed by the different plant parts (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>leaf</td>
</tr>
<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>10±0.2</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>12±0.4</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
<td>8±0.3</td>
</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>R</td>
</tr>
</tbody>
</table>

R-resistant

The leaves, stem and branch extracts showed activity against *Escherichia coli* both in the fresh and dried form. The different parts of the dried aqueous extract showed negative result. The fresh methanol and fresh aqueous extract of the different parts of the plant *Andrographis paniculata* showed significant activity against the Gram + ve as well as G – ve organisms. The compounds present in the plants are not water soluble. This was confirmed by the zone of inhibition formed by the dry aqueous extract.
Table 9: Antibacterial activity of different parts of *Andrographis paniculata* towards *Enterobacter faecalis*

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts</th>
<th>Zone of inhibition formed by the different plant parts (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>leaf</td>
</tr>
<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>12±0.3</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>10±0.2</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
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</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>R</td>
</tr>
</tbody>
</table>

R-resistant

1.3.2 Comparative antibacterial effect of the methanol and aqueous extract

The leaves extract showed broad range of antibacterial activity and so the dried leaves extract was taken for further experiments. The antibacterial activity of the methanol and aqueous extract was compared and the results were presented in Table 10. According to the result *Staphylococcus aureus, Klebsiella pneumoniae* and *Salmonella typhi* were more sensitive to the extracts (Fig. 34 and Fig. 36).

1.3.3 Influence of different factors on the bioactivity of *Andrographis paniculata*

The effects of different factors which are influencing the antibacterial activity were tested and the results were tabulated (Table 11, 12, 13).
Figure 14: Zone of inhibition against *Klebsiella pneumoniae* in fresh methanol extract

Figure 15: Zone of inhibition against *Klebsiella pneumoniae* in fresh aqueous extract

Figure 14, 15: A, B, C, D, E, F was the zone of inhibition formed by *Klebsiella pneumoniae* towards fresh methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 16: Zone of inhibition against *Klebsiella pneumoniae* in dry methanol extract

Figure 17: Zone of inhibition against *Klebsiella pneumoniae* in dry aqueous extract

**Figure 16, 17:** A, B, C, D, E, F was the zone of inhibition formed by *Klebsiella pneumoniae* towards dry methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 18: Zone of inhibition against Salmonella typhi in fresh methanol extract

Figure 19: Zone of inhibition against Salmonella typhi in fresh aqueous extract

Figure 18, 19: A, B, C, D, E, F was the zone of inhibition formed by Salmonella typhi towards fresh methanol and aqueous extract of the different parts of Andrographis paniculata. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds.

1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 20: Zone of inhibition against *Salmonella typhi* in dry methanol extract

Figure 21: Zone of inhibition against *Salmonella typhi* in dry aqueous extract

**Figure 20, 21:** A, B, C, D, E, F was the zone of inhibition formed by *Salmonella typhi* towards dry methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 22: Antibacterial activity of different parts of *Andrographis paniculata* on *Klebsiella pneumoniae*

Figure 23: Antibacterial activity of different parts of *Andrographis paniculata* against *Salmonella typhi*
Figure 24: Zone of inhibition against *Escherichia coli* in fresh methanol extract

Figure 25: Zone of inhibition against *Escherichia coli* in fresh aqueous extract

**Figure 24, 25:** A, B, C, D, E, F was the zone of inhibition formed by *Escherichia coli* towards fresh methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 26: Zone of inhibition against *Escherichia coli* in dry methanol extract

Figure 27: Zone of inhibition against *Escherichia coli* in dry aqueous extract

**Figure 26, 27:** A, B, C, D, E, F was the zone of inhibition formed by *Escherichia coli* towards dry methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 28: Zone of inhibition against Enterobacter faecalis in fresh methanol extract

Figure 29: Zone of inhibition against Enterobacter faecalis in fresh aqueous extract

Figure 28, 29: A, B, C, D, E, F was the zone of inhibition formed by Enterobacter faecalis towards fresh methanol and aqueous extract of the different parts of Andrographis paniculata. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
Figure 30: Zone of inhibition against *Enterobacter faecalis* in dry methanol extract

Figure 31: Zone of inhibition against *Enterobacter faecalis* in dry aqueous extract

Figure 30, 31: A, B, C, D, E, F was the zone of inhibition formed by *Enterobacter faecalis* towards dry methanol and aqueous extract of the different parts of *Andrographis paniculata*. A. leaves, B. stem, C. seed, D. root, E. branch and F. buds. 1 – Extracts of different parts. NC – Negative control (20 µl methanol).
**Figure 3.2:** Antibacterial activity of different parts of *Andrographis paniculata* on *Escherichia coli*.

**Figure 3.3:** Antibacterial activity of different parts of *Andrographis paniculata* on *Enterobacter faecalis*. 
Table 10: Comparative antibacterial effect of methanol and aqueous extract of dry leaves of *Andrographis paniculata*

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Zone of inhibition formed by the methanol and water extracts (mm)</th>
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<tr>
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<td>Methanol extract</td>
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<td></td>
<td>10µl</td>
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<tr>
<td><em>Bacillus subtilis</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>8</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>12</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>9</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>8</td>
</tr>
</tbody>
</table>

**Effect of heat**

The nature of the compound was not changed when the crude extract was heated to 50°C, whereas autoclaved at 15 lb pressure for 15 minutes denatured the compound present in the crude extract slightly. This was identified by comparing the zone of inhibition before and after autoclave and the results are given in Table 11; Fig. 35 and 37.
Bacillus subtilis

Staphylococcus aureus

Salmonella typhi

Klebsiella pneumoniae

Escherichia coli

Figure 34: Comparative antibacterial effect of dry leaves prepared from methanol and water extract

A, B, C, D, E and F are the results for the comparative antibacterial assay for the methanol and aqueous extracts of the dried leaves tissues. 10M - 10µl of methanol extract, 20M - 20µl methanol extract, 10w - 10µl water extract, 20w - 20µl water extract, 20Mc - 20µl methanol control, 20Wc - 20µl water control
Table 11: Zone of inhibition in response to autoclaving

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Before autoclaving (BA)</th>
<th>After autoclaving (AA)</th>
<th>Reduction in %</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>Bacillus subtilis</em></td>
<td>15</td>
<td>10</td>
<td>33</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>10</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td><em>Klebsiella pneumoniae</em></td>
<td>18</td>
<td>13</td>
<td>28</td>
</tr>
<tr>
<td><em>Salmonella typhi</em></td>
<td>10</td>
<td>8</td>
<td>20</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>12</td>
<td>10</td>
<td>17</td>
</tr>
</tbody>
</table>

In case of *Bacillus subtilis* the zone of inhibition was reduced to 10 mm compared to the value of 15 mm before autoclaving.

**Effect on Sunlight**

The sunlight exposure did not affect the antibacterial activity of the crude extract. The extract was continuously exposed in sunlight for 5 hours. Also assay was carried out at equal intervals of 2 hours and 4 hours. The zone of inhibition was comparatively same as the extract without exposure. The data is furnished in Table 12 and in Fig. 38 and 39.

Table 12: Zone of inhibition in response to sunlight

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Exposed duration in sunlight (mm)</th>
<th>Extract</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>2 hrs</td>
<td>4 hrs</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>15</td>
<td>16</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>15</td>
<td>14</td>
</tr>
</tbody>
</table>
**Figure 35: Effect of Heat**

A, B, C, D and E was the inhibition zone formed by the different organisms against the tissues which are before and after autoclaved. Nc- Negative control Aa- autoclaved extract Ba- extract before autoclaving.
Figure 36: Graph representing the comparative antibacterial effect of extracts of dry leaves of *Andrographis paniculata*.

Figure 37: Graph representing the effect of heat on the bioactivity of the extract of *Andrographis paniculata*. 
**Incubation time**

The plates were kept in incubator for 48 hours and no growth was seen in the zones. This data suggests that the antibiotic substance present in the crude extract was stable and the zones were also clear after 48 hours incubation (Table 13 and Fig. 40, 41).

**Table 13: Zone of inhibition in response to incubation time**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Zone of inhibition (mm)</th>
<th>24 hours</th>
<th>48 hours</th>
<th>24 hours</th>
<th>48 hours</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>10µl</td>
<td>20µl</td>
<td>10µl</td>
<td>20µl</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>R</td>
<td>10</td>
<td>8</td>
<td>13</td>
<td></td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>7</td>
<td>10</td>
<td>9</td>
<td>13</td>
<td></td>
</tr>
</tbody>
</table>

R- Resistant

**Comparison with kanamycin and ampicillin**

The level of 0.8µg of extract was noted to be almost equal to 0.2µg kanamycin and ampicillin. These details revealed that the crude extract of *Andrographis paniculata* had broad range of antibacterial activity towards the tested bacteria (Table 14, 15). The pattern is depicted in Fig. 42 to 45.

**Table 14: Comparison with Ampicilin**

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Dry Extract (0.8µg)</td>
</tr>
<tr>
<td></td>
<td>Ampicillin (0.2µg)</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>15</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>20</td>
</tr>
</tbody>
</table>
Table 15: Comparison with Kanamycin

<table>
<thead>
<tr>
<th>Organisms</th>
<th>Zone of inhibition (mm)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Extract(0.8 µg)</td>
</tr>
<tr>
<td>Bacillus subtilis</td>
<td>18</td>
</tr>
<tr>
<td>Staphylococcus aureus</td>
<td>13</td>
</tr>
<tr>
<td>Klebsiella pneumoniae</td>
<td>15</td>
</tr>
<tr>
<td>Salmonella typhi</td>
<td>15</td>
</tr>
<tr>
<td>Escherichia coli</td>
<td>12</td>
</tr>
<tr>
<td>Enterobacter faecalis</td>
<td>15</td>
</tr>
</tbody>
</table>

1.3.4 Screening of small molecules

The Thin Layer Chromatography technique was carried out for detecting the small molecular compounds present in the different parts of *Andrographis paniculata*. The different parts of the plant both in fresh and dried form were subjected to isolation of antimicrobials by thin layers chromatographic method. Different solvents were used as mobile solvent. Of these, the chloroform and Acetone (4:1) combination gave better results. Eleven compounds were identified from fresh methanol extract. Ten compounds were noted from the fresh water extract and eleven compounds were identified from the dry methanol extract. Totally 32 small molecular compounds were screened by using thin layer chromatography (Table 16 and Fig. 46 to 48). These compounds showed no affinity towards water because the dry water extract did not show antibacterial activity and no significant spots were also seen in TLC.
Figure 38: Zone of inhibition in response to sunlight

A and B was the inhibition zone formed for the leaves extract which was kept under direct sunlight for upto 5 hours. Pc – positive control (extract without exposure), Nc- negative control (methanol).

Figure 39: Graph representing the effect of sunlight on the bioactivity of the extract of *Andrographis paniculata*. 
**Figure 40:** Zone of inhibition in response to incubation time

A and C was the inhibition zone formed by the organisms *Staphylococcus aureus* and *Escherichia coli* after 24 hours incubation. B and D was the zone formed after 48 hours. 10µl and 20µl was the leaves extract of *Andrographis paniculata*, Nc – negative control (methanol).

**Figure 41:** Graph representing the effect of incubation time on the crude extract of *Andrographis paniculata*. 
A, B, C, D, E and F were the comparative antibacterial effect of 0.8µg extract with 0.2µg of Kanamycin. A–*Bacillus subtilis*, B – *Staphylococcus aureus*, C – *Klebsiella pneumoniae*, D – *Salmonella typhi*, E – *Escherichia coli*, F – *Enterobacter faecalis*. 1 – extract and Nc – negative control.
Figure 43: Graph representing the comparative antibacterial effect of the extract of *Andrographis paniculata* and commonly used antibiotics.

Table 16: Small molecules screened from *Andrographis paniculata*.

<table>
<thead>
<tr>
<th>Sl.No</th>
<th>Extracts</th>
<th>Spots formed by different plant parts</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Stem</td>
</tr>
<tr>
<td>1</td>
<td>Fresh methanol extract</td>
<td>2</td>
</tr>
<tr>
<td>2</td>
<td>Fresh water extract</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>Dry methanol extract</td>
<td>3</td>
</tr>
<tr>
<td>4</td>
<td>Dry water extract</td>
<td>-</td>
</tr>
</tbody>
</table>
Figure 44: Comparison with antibiotics – Ampicillin

A and B was the comparative antibacterial effect of 0.8 µg leaves extract with 0.2µg ampicillin.1 – extract, Nc – negative control (methanol)

Figure 45: Graph representing the comparative antibacterial effect of the crude methanol dry extract and ampicillin.
Figure 46 (A to L): Phytochemical components present in the fresh methanol extract of different parts were screened. a, b, c, d, e, f, g, h, i, j, k were the compounds identified, the solvent front is 7 cm and the Rf values were 4/7 =0.57, 5/7 = 0.71, 6.5/7=0.92, 7/7=1, 5/7 = 0.71, 6/7=0.85, 3/7 = 0.42, 2/7=0.28, 4/7=0.57, 5/7 = 0.71 and 3/7=0.42. 1-Negative Control (Methanol), 2-Loaded spot.
Phytochemicals present in the fresh methanol extract of different parts

The presence of different phytochemicals present in the fresh methanol extract of different parts is shown in Fig. 46. The data clearly suggest that the fresh methanol leaves extract contained four compounds. Two compounds were visualized in the stem extract. Root extract showed only one compound. There was no active phytochemical identified in the extract from seed. Three spots were seen in the branch extract and one spot was visualized in the flower extract.

Phytochemicals present in the fresh water extract of different parts

Ten compounds were noted in the fresh water extract of different parts of Andrographis paniculata. Three compounds were noted from the leaves extract three compounds from the stem extract and one compound were visualized in the root, seed, branch and flower extracts (Fig. 47).

Phytochemicals present in the dry methanol extract of different parts

In dry methanol extract, 11 compounds were visualized. Four compounds were screened from leaves and stem one each from root, seed, branch and flower (Fig. 48).
Figure 47: Phytochemicals present in the fresh water extract of different parts of *Andrographis paniculata*

A – before staining with iodine and B – after stained with iodine. Six compounds were visualised by iodine stain. Their Rf values were: $5/20 = 0.25$, $14/20 = 0.7$, $7/20 = 0.35$, $4/20 = 0.2$, $6/20 = 0.3$ and $18/20 = 0.9$.

1 to 6 was the loaded spots of different parts, and 7 was the negative control (methanol).
Figure 48: Phytochemicals present in the dry methanol extract of different parts of *Andrographis paniculata*

The Rf values of the six compounds visualized by TLC were follows. The solvent front was 20 cm and the distance travelled by the compounds was $10/20 = 0.5$, $15/20 = 0.75$, $4/20 = 0.2$, $8/20 = 0.4$, $12/20 = 0.6$, $18/20 = 0.9$.

1 to 6 was the loaded spots of different parts, and 7 was the negative control (methanol).
1.4 Discussion

There are more than 100 drugs of known structure that are extracted from higher plants and used in allopathic medicine (Fransworth, 1990). Results available from a few medicinal plants indicated about presence of promising phytochemicals which can be developed to combat health problems (Gupta, 1994). Herbal drugs have gained importance in recent years due to their efficacy and cost effectiveness. These drugs are invariably single plant extracts or fractions thereof or mixtures of fractions/extracts from different plants which have been carefully standardised for their safety and efficacy. Many single plants extracts are available as prescription drugs in Europe. Some examples of these include: Echinacea spp. (extract of root/aerial part) to stimulate immune system, Panax gin_seng (root extract) to combat fatigue and feelings of lassitude, Allium sativum (bulb extract) as antihyperlipidemic agent, Ginkgo biloba (leaf extract) to treat cerebral and peripheral circulatory disturbances and Serenoa repens (fruit extract) to treat non-malignant prostrate diseases (Suckdev, 1997). Quinine from Cinchona ledgeriana well known about its use in treating malaria (Vieira, 2001). Nimbin from Azadiracta indica has antiviral activity (Singh et al., 1996). The results of screening for chemicals present in Andrographis paniculata and their biological activities are discussed.

1.4.1. Antibacterial activity

The presence of antimicrobial substances in different parts of many plants was documented in literature (Srinivasan et al., 2001). In the present study, the phytochemical screening and antibacterial activity was performed with methanol and aqueous extracts of the leaves, stem, root, branches, seed and buds of Andrographis paniculata using clinical isolates of pathogenic organisms such as G + ve
Staphylococcus aureus and Bacillus subtilis and G – ve Escherichia coli, Salmonella typhi, Klebsiella pneumoniae and Enterobacter faecalis. As could be noted from the results section the extracts prepared from dried (different) parts showed higher inhibitory effect towards pathogenic organisms.

Among the methanol and aqueous extract, methanol extract showed greater activity than the aqueous extract. The fresh parts showed greater activity than the extracts prepared from dried parts. The antibacterial activity of the whole plant Andrographis paniculata suggests that every extract contains the effective active phytochemicals responsible for the elimination of microorganisms responsible for bacterial infections. Among the different extracts, the aqueous extract of bud had no activity. The G + ve as well as the G – ve organisms were resistant to the water extract. The aqueous extract prepared from dried parts had no activity at all, while the aqueous extract of fresh parts of this plant showed moderate activity. In addition, the leaves, stem and branches exhibited greater activity than root, seed and bud. Considering the results, the extract from leaves was investigated in detail.

In the present study Gram positive organisms are more sensitive than Gram negative organisms. The greater susceptibility of G + ve bacteria to the plant extracts has been previously reported in South America (Paz et al., 1995), African (Kudi et al., 1999; Vlietinck et al., 1995) and Australian (Pallombo and Semple, 2001) medicinal plant extracts. Susceptibility differences between G + ve and G – ve bacteria could be connected to the cell wall structural differences between these classes of bacteria. The Gram negative bacterial cell wall outer membrane act as a barrier to different substances including antibiotics (Tortora et al., 2001). The significant results obtained in the present study confirm the antibacterial potential of the plant investigated, and its usefulness in the treatment of bacterial infections.
The *in vitro* study corroborates the antibacterial activity of *Andrographis paniculata* used in folkloric medicine to treat skin infections (Jain, 1991; Ahmad *et al.*, 1998). Inhibitory activity was noted against most of the pathogenic bacteria causing chronic bacterial infections and hence *Andrographis paniculata* could be viewed as a potential source for new antibacterial agent in treating of infections which are associated with these bacteria. The inhibitory effect of these extracts thus justified the medicinal use of *Andrographis paniculata* in the treatment of bacterial infections by traditional practitioners and also warrants further research work to find out the active principles of medicinal value.

Successive isolation of phytochemical compounds from plant material largely depends on the type of solvent used in the extraction procedure. The traditional healers used primarily water as the solvent (Parekh and Chanda, 2007), but in the present study, the methanol extracts of both fresh and in dried form provided consistent antibacterial activity towards G + ve and G – ve organisms.

*Andrographis paniculata* has been valued for centuries by herbalists as a treatment for upper respiratory infections, fever, sore throat and herpes. Other reported applications include its use in cases of malaria, dysentery and even snake bites. At least 26 different Ayurvedic formulations used to treat liver disorders (Kapil *et al.*, 1993). In the *in vitro* study, in the present thesis with *Staphylococcus aureus* confirms the antibiotic capability of the leaves extract. The juice of fresh leaves is a domestic remedy in the treatment of colic pain, loss of appetite, irregular stools and diarrhoea (Saxena *et al.*, 1998). Andrographis extracts are showing promise in relieving diarrhoea associated with *Escherichia coli* bacterial infections (Gupta *et al.*, 1990).
Methanolic extract of kalmegh was reported to exhibit antimalarial activity against *Plasmodium berghei*, one of the parasites that transmit malaria (Misra *et al.*, 1992). Multiplication of the parasites was inhibited by the extract. The extract was also effective in killing filarial worms that obstruct lymph channels in the body leading to gross swelling in elephantiasis. Bacteria such as *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* were highly sensitive to leaf, stem, root and branch extract. *Klebsiella pneumoniae* was sensitive to dry methanol branch extract. *Staphylococcus aureus*, *Salmonella typhi* and *Escherichia coli* were sensitive to fresh extracts prepared from branches. The different parts of the dried aqueous extract, the leaves extract only showed broad range of antibacterial activity.

1.4.2. Influence of different factors on the bioactivity

**Effect of Sunlight and Heat**

(Mishra *et al.*, 2007) dried the aerial part of *Andrographis paniculata* under shade followed by drying in hot air oven for 30 minutes. Then it was powdered in a mechanical grinder. The dried powder was used for extraction with appropriate solvents. These mechanical forces did not change the nature of the compounds. In the present study, the crude extract when exposed to the direct sunlight for 2, 4 and 5 hours respectively did not change the activity of the compounds. Suggesting their stability, similarly when the leaves extract was continuously exposed in sunlight for five hours, no change in the activity was detected. These results also clearly indicated the stability of the compound towards light.

Screening of extracts from several other medicinal plants showed activity against *Staphylococcus aureus*. It was inferred from the results that the *Andrographis paniculata* could be used for treating skin infections conditions produced by gram
positive strain *Staphylococcus aureus*. Some of the bacteria which is used in this study are the common microbes causing various infective ailments. *Klebsiella pneumoniae* is a commonest pathogen for respiratory infections, urinary tract infections and wound infection *(Schneiders et al., 2003)*.

In the present study the autoclaved and non autoclaved crude extract was taken for comparative assay. And it was inferred from the result that the heat did not change the nature of the compound.

**Comparison with Antibiotics**

In the present study, kanamycin and ampicillin were used at 0.2µg level and the results indicated that 0.8µg of the extract showed almost equivalent activity. These details indicated the possibility of the presence of highly active bioactive compounds in the crude extract of *Andrographis paniculata*. Ciprofloxacin was used as a standard compound for comparing the results obtained with two sets of two dilutions (100 and 200 µg/ml) each of *Andrographis paniculata* aerial parts extract. The corresponding zone diameters were compared accordingly. From the zone of inhibition values and their comparison to that of the standard Ciprofloxin, it was evident that the ethanol extract was active against G+ ve and G- ve bacteria as also suggested by Mazumder *et al.*, (2004).

**Screening of small molecules**

Active compounds extracted with ethanol or methanol from the whole plant, leaf and stem included over 20 diterpenoids and more than ten flavonoids. The aerial part of this plant was reported to contain several diterpenoids and diterpene glycosides. The main constituent andrographolide, a diterpene lactone, was
considered responsible for the bitter taste of the plant and its extracts. Besides diterpene lactone, flavones derivatives such as oroxylin and wogonin were also isolated from leaves. From the rhizomes several flavonoids and their glycosides (andrographidine A-F) were found (Kumar et al., 2004).

Saxena et al., (2000) have described a HPTLC method using benzene as one of the mobile phase solvents. Chromatographic techniques like TLC, HPTLC were used to obtain chemical constituents from plants and through the technique of chemoprofiling (Vieira et al., 2001). A total of 32 smaller molecular compounds were screened using Thin Layer Chromatography. The fresh methanol leaves extract showed eleven compounds and in the fresh water extract ten compounds were identified. The dried leaves extract showed eleven compounds.

Although a significant number of studies have been used to obtain purified phytochemicals, very few screening programmes have been initiated on crude plant materials. It has also been widely observed and accepted that the medicinal value of plants lies in the bioactive phytocomponents present in the plants (Veeramuthu et al., 2006).