Chapter 7
Summary
In an attempt to scientifically validate the anti HIV drug potentials that are claimed in Indian medicinal plants indexed in traditional medicine literatures available in Tamil Nadu, an interdisciplinary study was conducted to identify the potential medicinal plants that could have anti HIV drug potentials and to scientifically analyse them by adopting internationally acceptable study designs. This study was conducted at the Department Of Microbiology, Dr.ALM Post Graduate Institute of Basic Medical Sciences, University of Madras during the years 1998 – 2003.

7.1 SELECTION OF POTENTIAL INDIAN MEDICINAL PLANTS FOR ANTI HIV STUDIES

7.1.1. The Indian medicinal plants having blood purifying, antimicrobial and / or immunomodulatory properties were selected after careful search of literature available on Indian systems of medicine and systematic compilations like Glossary of Indian Medicinal Plants by Kirthikar et al., (1975) and Indian Medicinal Plants by Varier et al.,(1994).

7.1.2. The 33 Indian medicinal plants so selected for the anti HIV studies are:

<table>
<thead>
<tr>
<th>Botanical Name</th>
<th>Family</th>
</tr>
</thead>
<tbody>
<tr>
<td>Amaranthus spinosus Linn</td>
<td>Amaranthaceae</td>
</tr>
<tr>
<td>Aporosa lindleyana</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>Argyreia nervosa</td>
<td>Convolvulaceae</td>
</tr>
<tr>
<td>Asparagus racemosus Willd.</td>
<td>Liliaceae</td>
</tr>
<tr>
<td>Baliospermum montanum</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>Boerhaavia diffusa</td>
<td>Nyctaginaceae</td>
</tr>
<tr>
<td>Eclipta prostrata</td>
<td>Asteraceae</td>
</tr>
<tr>
<td>Euphorbia ligularia</td>
<td>Euphorbiaceae</td>
</tr>
<tr>
<td>Ficus microcarpa</td>
<td>Moraceae</td>
</tr>
<tr>
<td>Gmelina arborea</td>
<td>Verbenaceae</td>
</tr>
<tr>
<td>Gymnema sylvestre</td>
<td>Asclepiadaceae</td>
</tr>
<tr>
<td>Hemidesmus indicus</td>
<td>Asclepiadaceae</td>
</tr>
<tr>
<td>Hedvotis corymbosa</td>
<td>Rubiaceae</td>
</tr>
<tr>
<td>Ipomoea mauritiana</td>
<td>Convolvulaceae</td>
</tr>
</tbody>
</table>
7.2. PREPARATION OF EXTRACTS

The shade dried plants were powdered and extracted using seven polar to non-polar solvents namely Dichloromethane: 2 propanol, Hexane, Petroleum ether, Chloroform, Butanol, Methanol and Water. The extracts were divided into two parts. The first part was retained in the individual solvent for follow up studies and the second part was subjected for antiviral and immunomodulatory assays using either PBS or DMSO as the diluent depending upon the compatibility to the assay system used.

7.3. STANDARDISATION OF TEST SYSTEMS FOR ANTI HIV AND IMMUNOMODULATORY PROPERTIES:

7.3.1. RT Inhibition assays: The following RT inhibition assays were standardised in the study.
7.3.1.1. *MMLV Reverse Transcriptase inhibition assay*

The isotopic reverse transcriptase inhibition assay devised by Ono *et al.*, (1989) using Moloney murine leukaemia virus reverse transcriptase as substrate was standardised with few modifications. AZT at a concentration of 1 μg/ml, which showed 75% inhibition, was used as standard positive control for the assay.

7.3.1.2. *HIV-1 Reverse transcriptase inhibition assay*

In order to compare the specificity of reverse transcriptase inhibition of the selected medicinal plants, the isotopic reverse transcriptase assay of Sahar *et al.*, (1995) with modifications was standardised using HIV-1 RT as substrate in the place of MMLV RT

7.3.2. *Protease Inhibition assays*: Both general protease inhibition assay and HIV-1 specific assay for protease inhibition were standardised in the study. They are:

7.3.2.1. *General protease inhibition assays:*

    a) *a-Chymotrypsin inhibition assay*

    Microtitre plate based colorimetric assay of Cannell *et al.*, (1988) with modifications was standardised to detect the a-Chymotrypsin inhibitory properties of medicinal plants selected for the study. a-Chymotrypsin ex-porcine pancreas and N succinyl phenylalanine 4 nitroanilide were used as enzyme-substrate pairs for the test system.
b) *Leucine Aminopeptidase inhibition assay:*

The colorimetric assay of Cannell *et al.*, (1988) with modifications was standardised. Leucine Aminopeptidase ex-porcine kidney and L-Leucine 4 nitroanilide were used as enzyme substrate pair for this assay.

c) *Papain inhibition assay:*

The colorimetric method of Cannell *et al.*, (1988) was standardised incorporating certain modifications. Papain ex-papaya latex and Benzoyl DL-Arginine 4 nitroanilide were used as enzyme substrate pair.

7.3.2.2. *HIV-1 protease inhibition assay:*

In order to test the specificity of extracts showing general protease inhibition, the extracts were tested for HIV-1 protease inhibition using HPLC. The method of Matsuse *et al.*, (1999) with modifications was adopted. Acetyl Pepstatin having 50% inhibition at 29 μg/ml was used as positive control standard.

7.3.3. *Assay for Immunomodulatory Properties:*

7.3.3.1. *T cell proliferation assay:*

The MTT based assay devised by Mossman *et al.*, (1983) was standardised with modifications, to test the T cell proliferative properties of the 33 Indian medicinal plants used in the study.
7.3.3.2. **RT PCR based IFN γ and IL4 detection assay:**

An in house RT PCR based assay was devised to detect IFN γ and IL4 induction potential of the plants showing T cell proliferation. 50μ g/ml of PHA was used as the standard inductor of IFN γ and IL4.

7.3.3.3. **EASIA based immunomodulatory assay for cytokine Quantitation using commercial kit.**

The level of cytokine produced by cells stimulated by different plant extracts which showed T cell proliferation was also assayed using commercially available Enzyme amplified sensitivity immunoassay kit by BIOSOURCE EUROPA, Belgium.

7.4. **ANTI HIV AND IMMUNOMODULATORY POTENTIALS EXHIBITED BY THE INDIAN MEDICINAL PLANTS TESTED:**

7.4.1. Out of the 33 Indian medicinal plants 221 extracts have been prepared using seven polar to non-polar solvents. All these extracts were tested by both MMLV RT inhibition assay and HIV RT inhibition assay.

7.4.2. The methanolic and aqueous extracts of *Ocimum kilimandscharicum* and methanolic extract of *Pterocarpus marsupium* have exhibited MMLV RT inhibition at the MIC of 400 μg/ml. When the same extracts were retested for the specific HIV RT inhibition, the methanolic extract of *Ocimum kilimandscharicum* revealed HIV RT inhibition at MIC of 25μ g/ml and aqueous extract of *Ocimum kilimandscharicum* at 50 μg/ml. The methanolic extract of *Pterocarpus marsupium* had HIV RT inhibiting property at MIC of 100 μg/ml. In addition, the HIV RT inhibitory assay detected three more plants namely *Rubia cordifolia* (aqueous extract), *Gmelina arborea* (aqueous extract) and *Pueraria tuberosa* (aqueous extract) with HIV RT inhibition at the MIC range of 50-100 μg/ml.
7.4.3. These observations elaborated in table 5.5(a) confirm that HIV specific RT inhibition assay is to be considered as a better sensitive and specific assay than other non-specific RT inhibition assays.

7.4.4. While the extracts of Aporosa lindleyana, Baliospermum montanum, Hedyotis corymbosa and Plectranthus amboinicus showed general protease inhibition at 200-400 μg/ml, only the aqueous extract of Plectranthus amboinicus had significant HIV-1 protease inhibition at 100 μg/ml concentration. Hence this extract was subjected to locate the active constituent.

7.4.5. T cell proliferation assay and IFN γ / IL4 induction study revealed Ocimum kilimandscharicum, Rubia cordifolia and Plectranthus amboinicus as potential immunomodulatory medicinal plants.

7.4.6. In depth analysis revealed that while all the three plants induced T cell proliferation at 100 μg/ml, IFN γ induction was exhibited at 50μg/ml level by Plectranthus amboinicus and Rubia cordifolia and the same was induced by Ocimum kilimandscharicum at 100 μg/ml concentration.

7.4.7. Interestingly only Plectranthus amboinicus in addition to inducing IFN γ was also able to induce IL4 , demonstrating its ability to modulate both TH1 (IFN γ ) and TH 2 (IL4) response.

7.4.8. Quantitation of IL4 and IFN γ using EASIA methodology has shown that the aqueous and methanolic extracts of Rubia cordifolia induced IFN γ at 3 IU and 5 IU levels respectively. The aqueous and methanolic extract of Ocimum kilimandscharicum induced IFN γ at 2 IU and 2.5 IU level respectively. Only the aqueous extract of Plectranthus amboinicus was able to induce both IFN γ and IL 4 at 4.8 IU level and 150 pg/ml respectively.
7.5. BIOLOGY GUIDED FRACTIONATION OF *PECTRANTHUS AMBOINICUS*

7.5.1. The aqueous extract of *Plectranthus amboinicus*, which had both anti-HIV protease inhibitory potential and immunomodulatory properties, was subjected to fractionation using silica gel column. The 15 fractions that were collected by this technique were subjected to HIV-1 specific protease inhibition assay and cytokine detection assay using EASIA. Fraction 9 was found to have both HIV-1 protease inhibitory and immunomodulatory properties.

7.5.2. Column chromatography Fraction 9 of aqueous extract of *Plectranthus amboinicus* was further fractionated using HPLC. Six major peaks were obtained by elution at the retention time of 2.2, 3.4, 4.0, 8, 12.6 and 13.4 minutes using methanol: water gradient system at a flow rate of 1.5 ml/minute. Bioactivity testing of these fractions confirmed that only fraction II reproduced 56% HIV-1 protease inhibitory property.

7.5.3. When the same six fractions were subjected to IFN-γ and IL4 detection assay, fraction II and III were shown to induce both IFN-γ (4 and 2.5 IU respectively) and IL4 to significant levels (190pg/ml and 80pg/ml respectively).

7.6. PHYTOCHEMICAL IDENTIFICATION OF BIOACTIVE FRACIONS

7.6.1. The bioactive extracts of 16 plants were tested for phytochemical analysis by using the test for alkaloids, triterpenoids, flavanoids, phenols, saponin, sugar, anthroquinone, amino acid and sterols.

7.6.2. This analysis has shown that the bioactive extract of *Plectranthus amboinicus* was positive for both alkaloids and flavanoids.

7.6.3. Since column fractionation was attempted on *Plectranthus amboinicus* only in view of its significant anti HIV and immunomodulatory potential, the bioactive fractions
obtained from the plant was reanalysed for phytochemical constituents. The fraction 9 obtained by column chromatography has shown strong positivity for flavanoids.

7.6.4. Based on the above methodology it is reasonably surmised that flavanoids are probably the seat of anti HIV and immunomodulatory potential of *Plectranthus amboinicus*.

7.7. **IN-VITRO CYTOTOXICITY TESTING**

7.7.1. A total of 16 extracts showing protease inhibitory, reverse transcriptase inhibitory and/or immunomodulatory properties were tested for cytotoxicity using VERO cell lines.

7.7.2. The extracts of *Plectranthus amboinicus*, *Rubia cordifolia* and *Baliospermum montanum* were found to be non-toxic above 2mg/ml concentration whereas extracts of *Hedyotis corymbosa*, *Gmelina arborea* were found to be toxic even at a concentration of 10 μg/ml.