CHAPTER 3
Scope and Plan
3. **SCOPE AND PLAN**

Scientific exploration based on evidence based medicine ideology has emerged as the international perspective in drug development from natural products of both terrestrial and marine origin. The consensus approach has been to:

a) Identify the so called, "powerful plants / organisms" from the innumerable naturally occurring living organisms on the basis of traditional medicine literature / medicines, folk medicine practices etc.

b) Explore whether the so identified natural sources possess the anticipated lead potentials / properties by various internationally acceptable laboratory test systems / screens.

c) Confirm the tentatively identified lead properties and decipher the mechanism of action of the natural products developed.

d) Conduct pre-clinical toxicology studies as per the regulatory requirements to document the level of safety profile of these natural products which are predicted for human use.

e) Organise and conduct phase I and II randomised controlled clinical trials (RCT) to document clinical efficacy, safety and dosage regulation.

Only after these stages, the"lead natural product(s)" shall emerge as" Candidate drug(s)" for technology transfer and commercialisation along with multi-centric Phase III/IV clinical trials.

With this plan, the present pilot study was conceived and conducted with the following objectives so as to provide the future scope for development of a possible natural product based anti-HIV drug.
3.1 To short list and select a tentative list of Indian Medicinal Plants which are accessible in Tamil Nadu and Kerala for analysing their anti-HIV and Immunomodulating properties.

Akin to the international approach, the basis for analysing anti-HIV and immunomodulating properties of medicinal plants was conceived as the documentations already available in traditional medicine literatures, folk medicine practices and formulatory reviews published during the 20th century. Accordingly the first part of the study after in depth literature search attempted to short list a list of medicinal plants that are commonly available in Tamil Nadu and Kerala for analysing their anti-HIV and immunomodulatory properties.

3.2 To standardise the following in vitro screening tests for RT-inhibition, Protease inhibition and Immunomodulatory potentials and analyse the identified medicinal plants for these properties.

3.2.1 MMLV-RT Inhibition assay.

Reverse transcriptase is the hallmark of retroviruses. With the advent of HIV/AIDS, a lot of research work is being carried out to study the mechanism of action of candidate drugs using this enzyme as the model. The plants selected in our study were screened initially for their RT inhibition potentials using Moloney Murine Leukaemia Virus (MMLV).

3.2.2 Assays for Protease Inhibition: Initially, a set of three general protease inhibition assays were standardised as screening tests to identify the medicinal plants possessing protease inhibition property. They were:
3.2.2.1. **α-Chymotrypsin Inhibition Assay:**

α-Chymotrypsin is a serine protease and an essential enzyme in the metabolic pathway. In an attempt to study the property of the protease inhibition, the plants identified were screened for activity against α-Chymotrypsin.

3.2.2.2. **Leucine Aminopeptidase Inhibition Assay:**

Leucine aminopeptidase is also a serine protease and an essential enzyme in the metabolic pathway. To confirm this parameter, a second enzyme Leucine aminopeptidase was used to test the identified plants.

3.2.2.3. **Papain Inhibition Assay:**

Papain is another serine protease and is also an essential enzyme in the metabolic pathway. To check the reproducibility and specificity of protease inhibition, the identified plants were screened for their activity against Papain.

3.2.3 **ASSAY FOR IMMUNOMODULATION**

3.2.3.1 **T-Cell proliferation assay**

The proliferation or suppression of lymphocytes indicate the status of the immune system and its ability to defend the body from foreign elements. As HIV/AIDS is an immunodeficiency disease, the current antiretroviral regimen has included immunomodulators along with the anti HIV drugs. Hence the immunomodulatory potentials of the selected plants were studied using T cell proliferation as a screening marker of immunostimulation.
3.3 To standardise the following confirmatory tests to authenticate the lead potentials revealed by the identified medicinal plants.

The third part of the study concentrated on standardising certain specific assay systems to confirm the properties of reverse transcriptase inhibition, protease inhibition and Immunomodulation shown by certain medicinal plants.

3.3.1 **ISOTOPIC HIV-RT INHIBITION ASSAY**

Since HIV-RT also is to be specifically inhibited by the medicinal plants that have shown MMLV RT inhibition in order to be looked upon as an anti HIV drug, the lead extracts were further evaluated for HIV specific RT inhibition by this assay.

3.3.2 **HPLC BASED HIV-1 PROTEASE INHIBITION ASSAY**

The plants initially screened for general protease inhibition need to be further analysed for HIV specific protease inhibitory potentials since HIV protease is an aspartic protease. The plants, which had inhibited general protease, were taken up for this study. HIV–1 specific protease inhibition was standardised by the highly sensitive methodology using HPLC. A lower initial concentration of the extract was used in order to eliminate those plants, which could inhibit only the general protease. The end points of activity of bioactive extracts were worked out.

3.3.3 **RT-PCR BASED CYTOKINE INDUCTION ASSAYS FOR INTERFERON GAMMA (IFNγ) AND INTERLEUKIN-4 (IL-4).**

Cytokines are induced in the body as a defence mechanism by the immune system. A shift from production of TH1 type of cytokines to TH2 type of cytokines has been documented as a mark of disease progression in AIDS cases. With the successful antiretroviral therapy a shift from TH2 type to TH1 has also
been reported. In this part of the study an attempt has been made to study the cytokine induction potential of plants which initially showed T cell proliferating activity. The IFN γ and IL 4 induction were analysed to represent the potentials of T_{H1} and T_{H2} induction.

3.3.4. **ENZYME AMPLIFIED SENSITIVITY IMMUNOASSAY (EASIA) BASED CYTOKINE DETECTION ASSAY.**

The amount of cytokines induced can be recorded using various techniques like ELISA and real time PCR. In this part of the study an attempt has been made to quantitated the amount of representative cytokines induced by the plants using the technique of enzyme amplified sensitivity assay.

3.4. To adopt the biology guided fractionation strategy to locate the fraction(s) that possess specific anti-HIV potentials.

The time-tested methodology in drug development has been to identify chemical compounds of predictable biological properties and subsequently validating them by various test systems to confirm the specific biological properties predicted. In recent times, this methodology has seldom helped in identifying bioactive compounds, more especially in the case of phytochemical research. A novel approach developed has been to design a biology guided fractionation methodology so that the demonstrated property was reproduced at every level of fractionation, so that the final compound can be obtained without loosing the earlier property identified. This methodology was adopted in this study to fractionate the medicinal plant, *Plectranthus amboinicus* that had shown anti HIV and Immunomodulating properties.
3.5. To attempt to elucidate the chemical structure of the bioactive molecule(s) possessing HIV-1 specific protease and RT Inhibition and/or Immunomodulatory properties.

The final answer in phytochemical research and drug development has always been identifying a bioactive chemical compound with fully characterised chemical structure. This end result facilitates potent drug development in the most successful way taking care of intellectual property rights and the production of drugs under the synthetic pathway instead of resorting to the original plant source repeatedly from which a bioactive compound is derived. With this objective, an attempt was made in this study to see whether a chemically characterised bioactive molecule can be identified and the bioactive fractions obtained.

3.6. To conduct in vitro toxicological studies using tissue culture models on the extracts of the medicinal plants with reproducible properties of HIV-protease inhibition, HIV-RT inhibition and/or Immunomodulation.

Although herbal products are believed to be free of side affects, there are several reports on plant product induced cytotoxicity. In addition mandatory regulatory norms warrant the conduct of toxicological studies on any potential drug molecule. Hence, as a preliminary analysis, the bioactive plants were analysed for cytotoxicity using VERO cells.