Chapter 6
Discussion
Any antiretroviral agent during its evolutionary stage has to be screened first for its effectiveness, *in vitro* and *in vivo* efficacy and later for its toxicity in animal and cell culture systems. As newer antiviral agents are getting developed either through synthetic pathway or through natural products, the pressure for developing suitable and practicable methods to evaluate these agents has increased. The need for simpler and reliable procedures along with guidelines for evaluating candidate antiviral agents has arisen. In reviewing the literature it could be noted that studies on assessment of antiviral agents varied greatly from one lab to the other. (Hu et al., 1989). This variation and difficulty in comparability between the results of different laboratories has effected the momentum of success in the area of antiviral agent development.

The fields of antiretroviral therapy has witnessed remarkable progress during the past 15 years both in terms of developing newer therapeutic agents and in designing rapid and reliable antiviral screening procedures. Since the advent of Azidothymidine (AZT), the therapy of HIV has been dominated by agents that target two essential enzymes of the virus, the reverse transcriptase and the protease. There are now sixteen approved therapeutic agents for infection with HIV, a pathogen that once caused nearly uniform fatal illness. The era of potent antiretroviral therapy also termed Highly active antiretroviral therapy (HAART) began in 1996 which resulted in dramatic decline in morbidity and mortality due to HIV disease in the developed world.

Antiretroviral management brings a complex series of choices about when to initiate therapy, what regimen to use, which drugs within each class of drugs to use, when to change therapy and which alternative drug to use. (Hammer, 2002). The three most commonly prescribed initial antiretroviral regimens are a protease inhibitors+2 nucleoside analogues, a non nucleoside RT+2 nucleoside analogues, and 3 nucleoside analogues. Antiviral potency is the key to the initial success of drug regimen, as well as to the durability of their success, the restoration of immune function, prevention of emergence of resistance and prevention of disease.
progression. The key is to link potency with the other desirable aspects of therapeutic regimen, low pill burden, excellent tolerability, absence of major drug interactions, absence of long term toxic effects, absence of cross resistance to other agents (Hammer., 2002).

These advances in HIV therapy have not been without a cost in terms of drug resistance and side effects, particularly metabolic abnormalities such as lipodystrophy. Concern about these negative effects has lead to a more conservative approach to the timing of the initiation of therapy to clinical trials of intermittent therapy in an attempt to decrease the total exposure to drugs over time.

It is disturbing to note that in the absence of any dramatic turn of events in the form of a therapeutic vaccine against HIV effective in all communities of the world a stream of global access to at least one or two anti HIV drugs, almost all the 40 million HIV infected people in the world will mostly go untreated and die of this disease in the next 5-10 years. The fact that nearly 90% of the worlds 40 million HIV positive people are present in sub Saharan Africa and developing countries of Asia, and these countries are those which can least afford the cost of the presently available anti HIV drugs.

It is in this context cost effective, alternate modes of treatment regimen for HIV diseases are being continuously explored.

6.1 REVERSE TRANSCRIPTASE INHIBITORS OF NATURAL ORIGIN

Plants, marine and microbial organisms have been explored for possible anti HIV leads for over a decade. Twanya et al., (1997) in their extensive study on plants, marine invertebrates, fungi , micro and macro algae, cyanobacteria and other microbes for anti HIV and cytotoxic leads have found Stellatta species., a marine sponge ; two tropical plants of the genera Erythrina ; fungi in the genera Fusarium and Alternaria to possess in vivo anti HIV activity.

Mathee et al., (1999) have also screened an array of natural compounds belonging to a wide range of structural classes like coumarines, flavanoids,
tannins, alkaloids, lignans, terpenes, naphtho and anthraquinones and polysaccharides for their activity as reverse transcriptase inhibitors. Out of the many tested, Calanolide A, isolated from the terrestrial plant *Calophyllum lanigerum* emerged as the most interesting natural reverse transcriptase inhibitors. Algae like *Nostoc sp*, *Phormidium sp*, *Ocillatoria sp*, *Liagora boergeseni*, *Champia parvula*, *Sargassum*, *Asparagopsis armata* showed HIV RT inhibition. (Knuble *et al.*, 1990, Matthee *et al.*, 1999, Hastin *et al.*, 2000)


Takayama *et al.*, (1991) demonstrated that PC6, a natural product extracted from cones of *Pinus parviflora* Sieb et Zucc inhibited HIV-1 reverse and forward transcription in CEM cells. Eberhardt *et al.*, (1996) isolated a phenylpropanoid (lignin) component from *Pinus nigra* Arnold (Pinaceae) seed cones which may be the component having the anti-HIV-1 activity.

Ayisi *et al.*, (2003) showed that extracts of *Ocimum gratissimum*, *Ficus polita*, *Clausena anisata*, *Alchornea cordifolia*, *Elaeophorbia drupifera* extracts inhibited HIV-1 reverse transcriptase (RT) activity at EC<sub>50</sub> values of 0.01–0.03 mg/ml.

In the present study 33 Indian medicinal plants as listed in table 5.1 were analysed for their anti HIV properties. The reverse transcriptase inhibition studies have shown the following plants either in their aqueous and/or methanolic extracts to have HIV RT inhibition properties *Ocimum kilimandscharicum*, *Gmelina arborea* Roxb. *Pureria tuberosa*, *Pterocarpus marsupium* and *Rubia cordifolia*. 
With the available literature no other study is available to show the anti-HIV properties of the above plants except in *Ocimum gratissimum*. However the species analysed in the study is different from the above being *Ocimum kilimandscharicum*.

6.2. ASSAYS FOR REVERSE TRANSCRIPTASE ACTIVITY AND ITS INHIBITION.

Assays for reverse transcriptase activity have become accepted techniques for the detection and quantification of retroviruses in cell culture. Reverse transcriptase testing has been used to detect residual infectivity after virus inactivation and to screen inhibitors (Eberle, 1992). A number of methods like detection of reverse transcriptase in cell culture, ELISA, both isotopic and non-isotopic methods have been devised for this purpose.


While Premanathan *et al.*, (1999) used cell culture studies to report a new polysaccharide from *Rhizophora apicuata* to inhibit cell to cell transmission of HIV and also transmission of free infectious virions to the cells. Lau *et al.*, (1993) conducted a comparative study between AMV RT inhibition and HIV RT inhibition with compounds of natural origin and showed that the extracts could inhibit HIV RT to a higher level than AMV RT.

In our present study MMLV RT inhibition was compared with HIV RT inhibition by isotopic methods. While the MMLV RT inhibition study had shown only two out of the 33 medicinal plants analysed to have RT inhibitory potentials,
the HIV RT inhibition assay has revealed five plants to possess HIV RT inhibitory properties.

6.3. HIV – PROTEASE INHIBITORS

HIV Protease inhibitors represent a potent new class of antiretroviral agent with short-term monotherapy activity in vivo superior to that reported for established nucleoside analogues. In contrast to reverse transcriptase inhibitors, which are effective only against acute HIV-1 infections, protease inhibitors are also effective against chronic HIV-1 infection because of their ability to block a late stage of viral maturation. Protease inhibitors containing regimen are considered by many to be the standard of care for the treatment of HIV infection. Protease inhibitors are currently the most effective agents available and used, and may continue to yield promising results for patients infected with HIV [Kakuda et al., (1998)].

Various natural as well as synthetic substances have been used to test their protease inhibitory properties. The agents containing Statine or hydroxy isostere mimic and rennin substrate analogues being the first to be postulated as potential candidates for protease inhibition. The FDA has approved the following HIV-1 protease inhibitors namely Saquinavir mesylate (Invirase), Ritonavir (Norvir), Indinavir sulfate (Crixivan) and Nelfinavir.

Synthetic peptide analogue UK-88,947 [Baboonian et al., (1991)], a compound from Abbot laboratories called A-77003 [Robins et al., (1993)], Aminodiol inhibitors (Bechtold et al.,1995), Oxim derivatives containing halogenomethyl ketone and phenyl moeties [Komai et al., (1997)], have all been shown to inhibit HIV-1 protease.

L-735, 524,a synthetic compound developed by Dorsey et al., (1994) inhibited the spread of HIV-1 IIIB -infected MT4 lymphoid cells at a concentration of 50nM. This compound also did not have any activity against human rennin, cathepsin D, pepsin and bovine chymosin at concentration exceeding 10μM.
A number of companies have developed different protease inhibitors
some of which have been discontinued after Phase I trial. Others have gone to phase
II and III. Some of these which are undergoing trials are ABT 538(Abbott), AG
1284,AG 1343(Agouron), BILA 1906 BS,BILA 218 5BS(Boehringer –Ingelheim),
BMS 182193,BMS 186318 (Bristol-Myers Sqibb),CGP5820 (Ciba - Geigy).Other
compounds undergoing Phase I and II clinical trials are AG 1343 (Agouron),CGP
3437(Ciba - Geigy), DMP 450 (DuPont Merck),U96988 (Upjohn) VX 478 (Vertex).

6.4 PROTEASE INHIBITORS OF NATURAL ORIGIN

While studies on synthetic compound and their protease inhibitory properties
are plenty, similar approaches on natural products are limited in literature. Plants
like Rosa damascena [Mahmood et al., (1996)], Garcinia mangostana [Chen et
al.,(1996)], were some of the initial reports on naturally occurring protease
inhibitors. Matsuse et al.,(1999) in their study on 39 Panamanian medicinal plants
for HIV RT inhibition and HIV protease inhibition have shown protease inhibitory
properties in Erythroxylum citrifolium ,Waltheria indica and Xylopia frutescens at
the IC₅₀ concentration range 43 to 48 µ g/ml.

In our present analysis four out of 33 plants exhibited either general protease
and/or HIV protease inhibitory potentials. They were Aporosa lindleyana,
Baliospermum montanum, Hedyotis corymbosa, and Plectranthus amboinicus.

6.5. ASSAYS FOR ACTIVITY OF PROTEASE AND ITS INHIBITION

Assay systems for evaluating protease inhibitory potentials have been
periodically designed to identify potential molecules that can emerge as HIV
protease inhibitors. Richards et al., (1989) tested the activity of Pepstatin ,a
compound which could inhibit aspartic proteinases ,using different buffering
conditions and found that at a pH 4.7 using sodium acetate buffer acetyl Pepstatin
could inhibit HIV protease and this provided strong support for the theory that HIV
protease is an aspartyl protease. Thyagi et al., (1992) devised a flourometric assay to
test the activity of protease inhibitors. Baboonian et al.,(1991) used recombinant
HIV protease and radiolabelled substrate to detect protease activity. Bechtold et al.,(1995) used two cell systems namely 8ES cell line and CEM-SS cells. 8ES cell line contains a single integrated copy of HIV-1 LAV and provides an excellent model to ascertain the effects of inhibitors which can act on post integration stages.

Komai et al.,(1997) used recombinant p55 Gag precursor polypeptide which in presence of protease would cleave to give p17 and p24 polypeptides that could be monitored using western blot analysis with anti p17 monoclonal antibodies. They reported the inhibition of HIV-1 protease by oxim derivatives which did not show any significant inhibition of serine protease namely Chymotrypsin and Trypsin. The activity of oxim derivatives was shown to be very rapid. The compound could bring about inhibition of viral maturation even before any cytotoxic effect could develop. Matsuse et al., (1999) tested HIV-1 protease inhibition by natural products using HPLC analysis whereby the substrate and enzyme are added in the presence of inhibitors at a pH 5.0, incubated and subject to HPLC and the product obtained is determined. Martinez et al., (2000) have devised bacteriophage λ based genetic screen for characterisation of the activity and phenotype of HIV-1 protease using different phages coding for HIV-1 protease which could be inhibited by various protease inhibitors in a dose dependent manner. Lindstner et al., (2001) have devised a fluorometric assay for testing new protease inhibitors. In the assay devised by Lindstner et al.,(2001) an artificial precursor protein is used which harbours green fluorescent protein (GFP-PR). This precursor is activated in vivo by auto catalytic cleavage resulting in elimination of GFP. In the presence of protease inhibitors this precursor does not undergo cleavage resulting in accumulation of GFP, which can be quantified using flow cytometric assay.

In the background of all the above information on the possible test systems for protease inhibition we developed test systems for general protease inhibition like α Chymotrypsin inhibition assay, Leucine Aminopeptidase inhibition assay and papain inhibition assay besides HIV specific protease inhibition assay by HPLC.
The results have shown that out of the four plants that had demonstrable inhibitory potentials for general proteases, only *Plectranthus amboinicus* possessed HIV-1 specific protease inhibition properties at the MIC of 100 μg/ml. It is also noteworthy to mention that it is the first report in literature, to report HIV-1 specific protease inhibitory property with *Plectranthus amboinicus*.

### 6.6 IMMUNOMODULATORS AND HIV

Recent advances in HIV-1 pathogenesis, and in defining virological and immunological responses to highly active antiretroviral therapy (HAART), along with the identification of the numerous drawbacks of HAART, have clearly demonstrated that the eradication of the virus is not a feasible therapeutic goal and that there is an urgent need to develop other approaches to fight HIV-1 infection. Novel therapeutic approaches of immune modulation have been recently been evaluated in pilot clinical trials. First, treating primary HIV-1 infection with Cyclosporin A (CsA) coupled with HAART to target massive immune activation extends the benefits achieved with HAART during primary HIV-1 infection and might contribute to the establishment of a more favourable immunological set point affecting the ultimate pattern and rate of disease progression. Second, treating chronic HIV-1 infection in patients with long term suppression of virus replication induced by HAART, with the addition of mycophenolate mofetil (MMF) reduces the pool of activated CD4+ T lymphocytes able to support productive HIV-1 infection, and might have an indirect impact on the pool of resting, latently infected CD4+ T cells, contributing to its depletion *in vivo*. The important question is clearly whether these results will have an impact on the clinical management of patients with HIV-1 infection, determining the precise therapeutic function of drugs like CsA and MMF, thus investigating the effects of these drugs on residual viral replication and the decay of the latent reservoir, on long term immunological benefit and ultimately on clinical benefit (Rizzardi *et al.*, 2002).
In addition to the above, earlier work has focused on IL2 in combination with reverse transcriptase or protease inhibitors, researchers have continued to explore the potential role of IFN α as a component of combination therapy for HIV diseases. Combination of didanosine (ddI) + interferon at appropriate doses has been shown to be administered safely to HIV infected people over a prolonged period of time. (Folkers, 1996)

Eventhough the presently adopted HAART therapy for HIV diseases with human immunodeficiency virus (HIV), the lengthening of life and decrease in HIV related complications associated with these triple cocktail HAART therapy works out to be of an exorbitant price more especially for people in developing and underdeveloped countries. The other dimension of the problem is that HIV rapidly develops resistance to these agents unless they are taken precisely on schedule and they can cause a variety of both minor and serious side effects. These situations have made the development of effective adjuncts and alternatives to antiretroviral drugs as a priority.

6.7. IMMUNOMODULATORS OF NATURAL ORIGIN IN RELATION TO HIV DISEASES

6.7.1. HERBAL IMMUNOMODULATORS IN TRADITIONAL MEDICINE

In clinical perspective, an immunomodulator is defined as a substance, biological or synthetic, which can stimulate, suppress or modulate any of the components of immune system including both innate and adaptive arms of the immune response. These immunomodulators can be either immunoadjuvants which are used for enhancing efficacy of vaccines or Immunostimulants which are envisaged to enhance bodies resistance against infections or Immunosupressents which are agents that could be used for control of pathological immune response in
autoimmune diseases, graft rejection, graft-versus host diseases, hypersensitivity immune reaction and immunopathology associated with infections.

The traditional systems of medicine which firmly believe on the holistic approach of treating the body as a whole in any particular disease, has often included herbal plants known to have immunomodulatory potentials in their formulations. However, systematic studies to evaluate such properties by modern parameters are of recent origin only. From 1980, attempts have been made to document and investigate several medicinal plants in the Indian system of literature for immunomodulatory properties (Sharma, 1981, Chopra et al., 1996, Dev 1997, Agarwall and Singh, 1999).

Several investigators by using in vitro and in vivo methods, have shown the following plants to possess immunomodulatory properties:


Andrographis paniculata stimulated macrophage migration, phagocytosis of $^{14}$C leucine labelled E.coli, proliferation of splenic lymphocytes, inhibited NO production. (Puri et al., 1993, Chiou et al. 1998).

Asparagus racemosus induced lag in tumour development, prevented leucopenia, produced by cyclophosphamid, inhibited ochratoxin A induced suppression of IL1, TNF $\alpha$ and macrophage chemotaxis. (Seena et al., 1993, Thatte et al., 1988, Dhuley, 1997).

Azadirachta indica stimulated IL1, IF $\gamma$, TNF $\alpha$ production, enhanced proliferative response of spleen cells to Con A and tetanus toxoid. (Upadhyay et al., 1992, Ray et al., 1996, Katiyar et al., 1997, Sai Ram et al., 1997).

Curcuma longa increased mitogenic response of lymphocytes and inhibited NO (Gatak et al., 1972, Mukhopadyay et al., 1982, Deodhar et al., 1980, Srimal, 1997, Kuttan et al., 1985, Brouet et al., 1995, Rao et al., 1995).

Gann (1984) showed that an antitumor cyclic hexapeptide RA-V obtained from the roots of Rubia cordifolia had significant effects against human nasopharynx carcinoma (KB), P388 lymphocytic leukemia and MM2 mammary carcinoma cells.

Nyctanthes arbor tristis stimulated macrophage migration, protected against candida albicans (Puri et al., 1994, Khan et al., 1995), Paul et al., (1997) showed that the water soluble fraction of the ethanol extract of Nyctanthes arbor-tristis (NAT) reduced plasma interferon-gamma level. Ocimum sanctum inhibited tumour development in mice, increases colony forming units in spleen and protects mice after irradiation. (Das et al., 1983, Senna et al., 1993, Devi and Ganasoundari, 1995, Ganasoundari et al., 1998).
Panax ginseng protects mice against viral encephalitis and enhances protective effect of interferon inducer, stimulates T cell proliferation, augments NK cells, production of IL1, IL2, TNF α, GM-CSF (Singh et al., 1983, Mizuno et al., 1994, Lee et al., 1997, Kim et al., 1998, Xiaoguang et al., 1998).

Panax pseudoginseng stimulated macrophage migration (Dua et al., 1989), Phyllanthus emblica inhibited PMN activity induced by leucotriene B4 and FMLP (Suresh and Vasudevan, 1994, Ihantola-Vormisto 1997), Picrorrhiza Kurroa enhanced phagocytosis, stimulated PHA, Con A and LPS induced lymphocyte proliferation (Atal et al., 1986, Dhuley et al., 1997, Sharma et al., 1994, Puri et al., 1992, Fatma et al., 1994, Rege et al., 1999), Tinospora cordifolia is cytotoxic to D11 and EAC tumour cells, mitogenic to splenocytes and lymph nodes, inhibits ochratoxin A induced suppression of IL1, TNF α and macrophage chemotaxis, enhances IgG antibody and macrophage activation. (Thatte et al., 1992, Nagarkatti et al., 1994, Sainis et al., 1997, Dhuley, 1997). Withania somnifera enhanced radio sensitisation for V97 Chinese hamster cells, inhibits tumour development (Ziauddin et al., 1996, Sharad et al., 1996). Mungantiwar et al., 1998 showed that alkaloid extract of Boerhaavia diffusa had some in vivo immunostimulatory activity whereas no such activity was seen in vitro. 6-MFA, an interferon inducer has been isolated from Aspergillus ochraceus. Syringin (TC-4) and Cordiol (TC-7) obtained from Tinospora cordifolia have been found to possess immunomodulatory activities (Aggarwal et al., 1999).

6.7.2. IMMUNOMODULATORS OF HERBAL ORIGIN IN HIV

As a part of research on alternative and complementary therapies during the last couple of decades, immunomodulating substances from herbal sources of either terrestrial or marine origin have been explored as adjuncts and cost effective supplements for antiretroviral therapy.
Echinacea purpurea (purple coneflower or echinacea), E. angustifolia (narrow-leaf coneflower), and E. pallida (pale coneflower) are popular herbs that were used originally by native Americans and are known to have immune-enhancing effects in humans. In the test tube, Echinacea flower and leaf extracts, particularly their polysaccharides, increased production of tumour necrosis factor (TNF), which has been shown to contribute to the pathogenesis of HIV-related disease. The German Commission E monograph on various approved echinacea preparations recommended that the herb not be given to people infected with HIV. However, most studies have used polysaccharides derived from echinacea grown in tissue culture, which yields different plant compounds than are found in the actual plant, and preliminary results from a double-blind trial in progress show that 12 patients given one gram three times a day of E. angusifolia extract have dramatic improvements in immune function.

Viscum album (European mistletoe) leaf has been used since ancient times for a variety of ailments. Similar to echinacea, in vitro studies originally found that European mistletoe increases TNF production by leukocytes.

There is a theoretical contraindication for European mistletoe for people infected with HIV. However, an uncontrolled preliminary trial showed that regular subcutaneous injection of European mistletoe extracts had no negative effects in people infected with HIV. In a follow-up, dose-escalation study in 40 HIV positive patients there were strong signs of immune enhancement, including a 20 percent or greater increase in CD4+ lymphocytes levels. While equivalency is thought to be unlikely, it isn’t known whether oral European mistletoe would have the same effects as injections.

Results with most other immunomodulating herbs are more preliminary than for echinacea and European mistletoe. Eleuthero (Eleuthero-coccus senticosus) and other Araliaceae family immunomodulators (particularly Panax ginseng [Asian
ginseng]) have also been used in patients who are HIV-positive. *Ganoderma lucidum* (reishi) mushroom immunomodulators have been used in complex treatment protocols for patients who are infected with HIV and seen "good results."

Two of the most thoroughly studied botanical immunomodulators in the treatment of HIV are the European plant *Glycyrrhiza glabra* (licorice) and its close Asian cousin *G. uralensis* also known by its Chinese name *gan cao*. Licorice, particularly its main active glycoside, known as glycyrrhizin, appears to act both as an immunomodulating agent and an antiviral, an ideal combination to use in the treatment of HIV infection. At least three long-term studies from Japan have shown that oral administration of glycyrrhizin (150 to 225 milligrams per day) is effective for maintaining immune function and suppressing HIV replication in patients who are infected with the virus. Deglycyrrhizinated licorice, an adjusted licorice extract designed to reduce adverse side effects, is not likely to be beneficial for HIV.

Licorice, echinacea, European mistletoe, eleuthero, reishi, and other immunomodulating natural products alone, combined with one another, and combined with antiretroviral drugs should all be studied further, as they may provide quite promising therapeutic options. (Webb, 2000).

It is under this context that the present search for a herbal preparation which possesses both HIV specific antiviral activity and immunomodulatory activity gains significance. Of the 33 medicinal plants that were screened for HIV RT and HIV-1 protease inhibition and immunomodulatory activities only one plant, *Plectranthus amboinicus* had significant HIV-1 protease inhibitory property and immunomodulatory activity as proved by T cell proliferation property at 100 µ g/ml and cytokine induction of IFN-γ and IL4 at 50 µ g/ml and 100 µ g/ml concentration.
There were three other plants which had shown HIV RT inhibition at 25-50 μ g/ml concentration and immunomodulatory activity of T cell proliferation at 100 μ g/ml and only IFN γ induction at 50-100μ g/ml concentration. In view of the usefulness of a herbal compound which could demonstrate both HIV-1 protease inhibition and profound immunomodulatory properties, *Plectranthus amboinicus* was chosen for in depth study. With the available search mechanisms we could not find any other report on a medicinal plant possessing both HIV-1 protease inhibition and immunomodulatory properties.

**6.8. CHEMICAL CHARACTERISATION OF BIOACTIVE NATURAL PRODUCTS**

The present day traditional medicinal research against HIV in the western world has concentrated on a) Chemical class of bioactive compounds that can be identified from the potential plants. b) their ethnomedicine background c) the mechanism of their anti HIV potentials and d) the level of research on the compounds derived or the extracts prepared. Under this concept tens of thousands of crude natural extracts have so far been screened for anti HIV activity Of plants and herbs screened, 70 compounds and 76 crude extracts from 123 species ranging from common food and drink (e.g. soy) to tropical rainforest specimens (e.g. *Calophyllum lanigerum*) reportedly exhibited HIV inhibitory activity in vitro. Amongst the bioactive materials, there were 29 terpenes (15 diterpenes, 14 triterpenes), 29 flavanoids, 15 polysaccharides, 8 coumarins, 6 tannins, 4 lectins, 4 quinolones, 2 peptides, and 7 other alkaloids. Mechanism of action elucidated included competitive and non-competitive reverse transcriptase inhibition, protease inhibition, and interference of infection at the viral cell entry level. Of bioactive species, 63 are found in the Chinese materia medica and at least another 8 are derived from other ethnomedicines. However, only a handful of such active extracts and compounds (e.g. ganoderma, momordica, viscum album, curcumin, acemannan, glycyrrhizin,
Lentinan, hypericin, GLQ233, PCK-4,) have been formally assessed in clinical studies of HIV patients, and most trials were observational and uncontrolled. (Chang et al., 1997).

The following table narrates some of the reports on the identified natural compounds from herbal and plant sources that have shown anti HIV potentials at different levels.

<table>
<thead>
<tr>
<th>Name of compound</th>
<th>Plant name and source</th>
<th>Activity</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>MAP 30</td>
<td>Momordica charantia</td>
<td>HIV-1 RT inhibition</td>
<td>Huang et al., 1990</td>
</tr>
<tr>
<td>Guttiferone A</td>
<td>Garcinia livingstonii</td>
<td>Inhibits cytopathic effects of HIV</td>
<td>Gustafson et al., 1992</td>
</tr>
<tr>
<td>Guttiferone E</td>
<td>Garcinia ovalifoliaoliv</td>
<td>Inhibits cytopathic effects of HIV</td>
<td>Gustafson et al., 1992</td>
</tr>
<tr>
<td>Gemin D</td>
<td>Geum japonicum</td>
<td>HIV induced cytopathic effect</td>
<td>Nakashima et al., 1992</td>
</tr>
<tr>
<td>O-Demethylbuchenavianine</td>
<td>Buchenavia capitata</td>
<td>Anti HIV</td>
<td>Beutler et al., 1992</td>
</tr>
<tr>
<td>Bershacolone (diterpene)</td>
<td>Maprounea africana Muell.-Arg</td>
<td>Anti HIV</td>
<td>Bernart et al., 1993</td>
</tr>
<tr>
<td>Circulins A and B(macrocylic peptides)</td>
<td>Chssalia parvifolia Schumann</td>
<td>Anti HIV</td>
<td>Gustafson et al., 1994</td>
</tr>
<tr>
<td>Ent-4-o-methylgallocatechin</td>
<td>Panda oleosa Pierre</td>
<td>HIV inhibition</td>
<td>Bokesch et al., 1994</td>
</tr>
<tr>
<td>Michellamine B</td>
<td>Ancistrocladus korupensis</td>
<td>Anti HIV-1 and HIV-2 activity</td>
<td>Boyd et al., 1994</td>
</tr>
<tr>
<td>Koumbalones A and B</td>
<td>Maprounea africana Muell.-Arg</td>
<td>Anti HIV activity</td>
<td>Kashman et al., 1994</td>
</tr>
<tr>
<td>Nitidine</td>
<td>Toddalia</td>
<td>Cytoprotec</td>
<td>Rashid</td>
</tr>
</tbody>
</table>
The approach of deducing the chemical structure of a bioactive compound from the medicinal plant, even though it may run the risk of loss of the specific bioactivity, has stood as the standard methodology for obtaining a reproducible bioactive drug molecule. However, the traditional medicine experts belonging to various systems of medicine like Chinese system of traditional medicine, African system of traditional medicine, Tibetan system of traditional medicine, Indian system of traditional medicine, Srilankan system of traditional medicine etc have vociferously been arguing against the methodology of targeting at isolating chemically defined molecules. Their argument of bio synergism in the whole plant and loss of the same when they are broken has also been proved on several occasions.

In the present study we also attempted to fractionate the plant *Plectranthus amboinicus* by adopting a biology guided fractionation methodology. After partial fractionation using column chromatography, HPLC was adopted to localise the bioactive fractions. At every stage of fractionation the originally expressed property of HIV-1protease inhibition and Immunomodulatory properties of IFNγ and IL4 inductions were assessed to check for reproducibility of these properties at every
level of fractionation. Interestingly fraction 9 of the fifteen collections obtained through column chromatography possessed both HIV protease inhibition and immunomodulatory properties.

Further separation of fraction 9 obtained from the column through HPLC yielded six major fractions. Fraction II had specifically demonstrated 56% HIV protease inhibition and Fraction II and III showed significant immunomodulatory potential by induction of IFNγ and IL4.

In view of loss of activity on further fractionation attempts to identify a chemical molecule were not continued. This might be another experimental proof to show the existence of bio synergism in medicinal plants and the loss of biological property on extreme fractionation.

6.9 TOXICOLOGICAL STUDIES ON NATURAL PRODUCTS

There is a prevailing notion that all traditional medicine drugs in general and herbal products in particular are harmless without any side effect. Over the years, studies have shown that this popular view that herbal drugs are natural and hence harmless is always not true. Shaw et al.,(1997) in their five year toxicological study report on traditional remedies and food supplements have shown toxic effects of some of the traditional remedies. Yoshida et al.,(1996) while evaluating Chinese herbal medicines in fulminant hepatic and liver transplantation also reported the occurrence of toxicity. Hepatotoxicity of herbal remedies has been reported by Larrey and Pageaux (1995) and Kaplowidz (1997). A survey by the national poison information service in Europe for the years 1991-1995 documented 785 cases of possible or confirmed adverse reactions due to herbal drugs among which hepatotoxicity was the most frequent (Shaw et al., 1997). Hence safety studies have formed an integral component in drug development and to obtain regulatory clearance for a candidate drug.

Under these circumstances we selected Plectranthus amboinicus, which turned out and to be the most promising Indian medicinal plant among the 33
evaluated in the study, for toxicological studies. This has become pertinent since
the plant has specifically shown to possess HIV-1 protease inhibition property and
specific immunomodulatory properties. Conventionally animal systems like rats,
mice, rabbit etc are subjected for acute, subacute and chronic toxicological studies.
(Dwivedi et al., 1991). More recently transgenic animals and transgenic cell lines
are also used to evaluate the tissue specific safety of medicinal plant preparations.
(Lee et al.,1996  Otto et al.,1997)

Presently continuous cell lines like VERO, Hep 2 and He La are also used to
assess the cytotoxic and cytotoxic effects of any possible Indian medicinal plant
preparations. (Jayaram et al.,1987)

In the present study 16 extracts of ten medicinal plants, which showed either
antiviral and/or immunomodulatory potentials were assessed for their tissue toxicity
using VERO, cell line. The tissue culture toxicity dose was calculated keeping cell
death in 50 % of the cells of the culture plate as cut off level. The results have shown
that the TCTD 50 dose varied from 10µ g/ml upto 2000µ g/ml. Three plants namely
Baliospermum montanum, Rubia cordifolia and Plectranthus amboinicus have
shown significant in vitro safety on VERO cell line even after addition of 2000 µ
g/ml of either aqueous or methanolic extracts of these bioactive plants. These
observations are to be considered preliminary. The identified bioactive fractions
shall have to be subjected for the full range of toxicological evaluation before this
protease inhibitor cum immunomodulator from Plectranthus amboinicus is projected
as a candidate drug for HIV.