

**IN VITRO TESTING OF THE ANTI-HIV PROPERTIES OF
MANGROVE AND SOME OTHER MEDICINAL PLANTS**

**THESIS SUBMITTED
UNIVERSITY OF MUMBAI**

**FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY
IN
MICROBIOLOGY**

**SUBMITTED BY
ANUYA A. REGE**

**UNDER THE GUIDANCE OF
DR. (MRS.) R. A. DESHMUKH**

**DEPARTMENT OF VIROLOGY
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MUMBAI – 400 012**

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SEPTEMBER 2008

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STATEMENT BY THE CANDIDATE

As required by the University Ordinance 770, I wish to state that the work embodied in this thesis titled **"In-vitro testing of the Anti-HIV properties of Mangrove and some other Medicinal plants"** forms my own contribution to the research work carried out under the guidance of **Dr. (Mrs.) Ranjana A Deshmukh** at the Haffkine Institute for Training, Research and Testing, Mumbai – 12. This work has not been submitted for any other degree of this or any other University. Whenever references have been made to previous works of others, it has been clearly indicated as such and included in the Bibliography.

Signature of the Candidate


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DEDICATED WITH DEEP
GRATITUDE TO MY GURUS
SHREE RAGHAVENDRA SWAMY,
SHREE ANNA KAKA
&
MY DEAREST AAI

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INTRODUCTION

Acquired Immuno Deficiency Syndrome [AIDS] is a devastating human disease and is caused by Human Immuno Deficiency Virus [HIV]. AIDS is characterized by extensive immunosuppression that predisposes patients to life-threatening opportunistic infections and unusual forms of neoplasm.¹

Epidemiology:

Global Overview:

According to a joint UNAIDS and WHO report, the estimated number of persons living with HIV worldwide in 2007 was 33.2 million [30.6~36.1 million]. As per the report, every day, over 6800 persons become infected with HIV and over 5700 persons die from AIDS, mostly because of inadequate access to HIV prevention and treatment services. Global and regional trends suggests the pandemic has formed 2 broad patterns: generalized epidemics sustained in the general populations of many sub-Saharan African countries, especially in the southern part of the continent and epidemics in the rest of the world that are primarily concentrated among populations most at risk, such as men who have sex with men, injecting drug users, sex workers and their sexual partners.

AIDS remains a leading cause of mortality worldwide and primary cause of death in sub-Saharan Africa. The estimated number of deaths due to AIDS in 2007 was 2.1 million [1.9~2.4 million] worldwide, of which 76% occurred in sub-Saharan Africa. Global HIV incidence likely peaked in the late 1990s, and was estimated to be 2.5 million [1.8~4.1million] new infections in 2007 of which over 68% occurred in sub-Saharan Africa. There are an estimated 11.4 million orphans due to AIDS in this region.

The 92000 adults and children estimated to be newly infected with HIV in East Asia in 2007 represent an increase of almost 20% over the people who acquired HIV in 2001. In sub-Saharan Africa, almost 61% of adults living with HIV in 2007 were women, while in Caribbean that percentage was 43%. The proportions of women living with HIV in Latin America, Asia and Eastern Europe are slowly growing. In East Europe and Central Asia, it is estimated that women accounted for 26% of the adults with HIV in 2007, while in Asia that proportion reached 29% in 2007. Globally the number of children living with HIV was estimated to be 2.5 million in 2007. Whereas the deaths due to AIDS among children were estimated to be 330000 in 2007.

Indian Scenario:

Overall in Asia, an estimated 4.9 million [3.7 million-6.7million] people were living with HIV in 2007, including the 440000 people who became newly infected and approximately 300000 died from AIDS-related illness in 2007. In India approximately 2.5 million [2 million~3.1 million] people are currently living with HIV, with national adult HIV prevalence of 0.36%. The first AIDS case in India was detected in 1986 in Chennai; since then HIV infection has been reported in all states and union territories. Prevalence trends in India vary between states and regions. The highest HIV prevalence rates are found in Maharashtra and Gujarat in the west; Andhra Pradesh, Tamil Nadu and Karnataka in the south; and Manipur and Nagaland in the north-east. Reported adult HIV prevalence in six states included in the recent national population-based survey varied from 0.07% in Utter Pradesh to 0.34% in Tamil Nadu, 0.62% in Maharashtra, 0.69% in Karnataka, 0.97% in Andhra Pradesh, and 1.13% in Manipur.

The prevalence in all other states together was 0.13%. As India is one of the largest and most populated countries in the world, a mere 0.1% increase in HIV prevalence would increase the estimated number of people living with HIV by over half a million. In the southern states, HIV primarily spread through heterosexual contact, while infections are mainly found amongst injecting drug users in Manipur and Nagaland. Women currently account for 39% of HIV infections in India and it is thought that this figure is rising. The 'high-risk groups' in India include sex workers, injecting drug users, truck drivers, migrant workers and men who have sex with men. Another significant trend is that most of the people becoming infected are in the sexually active and economically productive 15 to 44 age group. This means that most of the people living with HIV are in the prime of their working lives and are supporting families. Unfortunately, HIV treatment is severely limited in India, with only 95000 people (less than 15% of those in need) receiving antiretroviral drugs in India, as the vast majority of the people can not afford to buy treatment privately.

Various groups have made predictions about the effect that AIDS will have on India and the rest of Asia in the future, which have suggested that:

- India's adult HIV prevalence will peak at 1.9% in 2019
- The number of AIDS deaths in India will rise to 12.3 million during 2000-15, and to 49.5 million during 2015-50.
- Economic growth in India will slow by almost a percentage point per year as a result of AIDS by 2019.

Thus HIV and AIDS will have a devastating effect on India in the future, and that as much as possible needs to be done to minimize this impact.^{2,3,4,5}

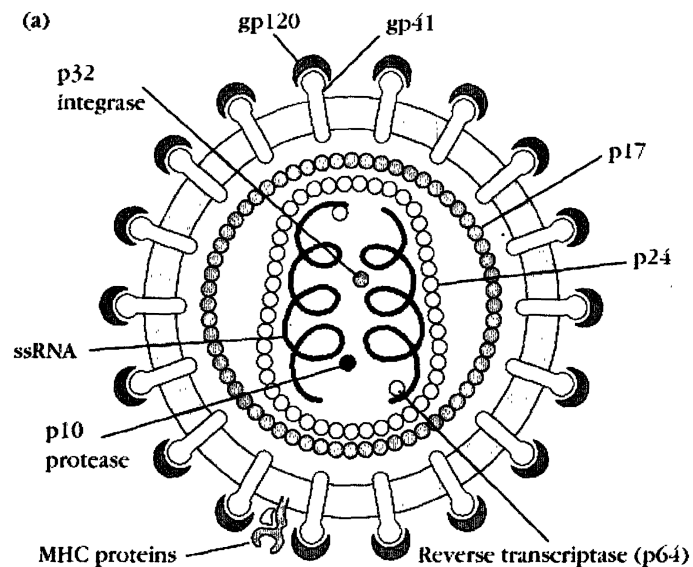
History and Origin:

AIDS was officially recognized for the first time in June, 1981 at Centers for Disease Control, U.S.A. in previously healthy homosexual men dying with two very rare diseases, *Pneumocystis carinii* pneumonia and Kaposi's sarcoma. Since then, AIDS has been reported from all continents. The virus causing AIDS was independently identified by a team of French scientists led by Dr. Luc Montagnier of Pasteur Institute and American scientists lead by Dr. Robert C. Gallo of National Cancer Institute in 1983-1984. The virus has been called by different names LAV i.e. Lymphadenopathy Associated Virus by French and HTLV III i.e. Human T Lymphocytotropic Virus type III by the Americans. The International committee on nomenclature of viruses named it the "Human Immunodeficiency Virus" (HIV) and to date two types, HIV-1 and HIV-2 have been identified.⁶

Structure of HIV:

HIV, the etiological agent of AIDS, belongs to the family **Retroviridae** subgroup **Lentivirus** (from L. *lentus* = slow). HIV is a spherical enveloped virus, about 90-120 nm size surrounded by a lipoprotein membrane. Each viral particle contains 72 glycoprotein complexes which are integrated into this lipid membrane and are each composed of external glycoprotein gp120 and a transmembrane protein gp41. During the process of budding,

the virus may also incorporate different host proteins from the membrane of the host cell into its lipoprotein layer, such as HLA class I and II proteins or adhesion proteins such as ICAM-1 that may facilitate adhesion to other target cells. Matrix protein p17 is anchored to the inside of viral lipoprotein membrane.



Cited: tutor.Iscf.ucsb.edu

The viral core (or capsid) is usually bullet-shaped and is made from the protein p24. Inside the core are three enzymes required for HIV replication called reverse transcriptase, integrase and protease. Also held within the core is HIV's genetic material, which consists of two identical strands of RNA.

The genome of HIV contains 3 structural genes: *gag*, *pol* and *env*. The classical structural scheme of a retroviral genome is: 5' LTR-*gag*-*pol*-*env*-LTR 3'. The LTR (long terminal repeat) regions represent the 2 end parts of the viral genome, that are connected to the cellular DNA of the host cell after integration and do not encode for any viral proteins.

gag (represents: group antigen) gene determines the core and shell of the virus. It is expressed as precursor protein p55, which is cleaved into 3 proteins p15, p17 and p24, which make up the viral core and shell.

env (represents: envelop) determines the synthesis of envelop glycoprotein gp160 which is cleaved into the 2 envelop components, gp120 that forms the surface spike and gp41 which is transmembrane anchoring protein.

pol (represents: polymerase) gene codes for reverse transcriptase and other viral enzymes. In addition, HIV-1 contains six genes (*vif*, *vpu*, *vpr*, *tat*, *rev* and *nef*).

Tat and *rev* are regulatory proteins that accumulate within the nucleus and bind to defined regions of the viral RNA: TAR (transactivation-response elements), found in the LTR; and RRE (rev response elements), found in the *env* gene, respectively. The *tat* protein is a potent transcriptional activator of the LTR promoter region and is essential for viral replication in almost all *in vitro* culture systems. *Tat* and *rev* stimulate the transcription of proviral HIV-1 DNA into RNA, promote RNA elongation, enhance the transportation of HIV RNA from the nucleus to the cytoplasm and are essential for translation. *Rev* is also a nuclear export factor that is important for switching from the early expression of regulatory proteins to the structural proteins that are synthesized later.

Nef has been shown to have a number of functions. *Nef* may induce downregulation of CD4 and HLA class I molecules from the surface of HIV-1-infected cells, which may represent an important escape mechanism for the virus to evade an attack mediated by cytotoxic CD8+ T-cells and to avoid recognition by CD4+ T-cells. *Nef* may also interfere with T-cell

activation by binding to various proteins that are involved in intracellular signal transduction pathways.

Vpr seems to be essential for viral replication in non-dividing cells such as macrophages. *Vpr* may stimulate the HIV-LTR in addition to a variety of cellular and viral promoters. More recently, *vpr* was shown to be important for the transport of the viral pre-integration complex to the nucleus and may arrest cells in the G2 phase of the cell cycle.

Vpu is important for the virus budding process and is also involved when CD4-gp160 complexes are degraded within the endoplasmic reticulum and therefore allows recycling of gp160 for the formation of new virions.^{7,8}

HIV types, groups and subtypes:

There are two types of HIV: HIV-1 and HIV-2. Both types are transmitted by sexual contact, through blood, infected needles and syringes and from mother to child, and appear to cause clinically indistinguishable AIDS. However worldwide, the predominant virus is HIV-1. The relatively uncommon HIV-2 type is concentrated in West Africa and is rarely found elsewhere.

The strains of HIV-1 can be classified into three groups: the "major" group M, the "Outlier" group O and the "new" group N. These three groups may represent three separate introductions of simian immunodeficiency virus into humans. Group O appears to be restricted to west-central Africa and group N – discovered in 1998 in Cameroon- is extremely rare. More than 90% of HIV-1 infections belong to HIV-1 group M. Within group M there are known to be at least nine genetically distinct subtypes (or clades) of

HIV-1. These are subtypes A, B, C, D, F, G, H, J and K. In India subtype C exists.⁶

HIV Tropism:

The term viral tropism refers to which cell types HIV infects. HIV can infect a variety of immune cells such as CD4⁺ T cells, macrophages, and microglial cells. HIV-1 entry to macrophages and CD4⁺ T cells is mediated through interaction of the virion envelope glycoproteins (gp120) with the CD4 molecule on the target cells and also with chemokine coreceptors.

Macrophage (M-tropic) strains of HIV-1 or non-syncytia-inducing strains (NSI) use the β -chemokine receptor CCR5 for entry and are thus able to replicate in macrophages and CD4⁺ T cells. This CCR5 coreceptor is used by almost all primary HIV-1 isolates regardless of viral genetic subtype.

T-tropic isolates or syncytia-inducing (SI) strains replicate in primary CD4⁺ T cells as well as in macrophages and use the α -chemokine receptor, CXCR4, for entry.⁹

SDF-1 (stromal cell-derived factor 1) was identified as the natural ligand of CXCR4 and is able to inhibit the entry of T-tropic HIV-1 isolates into activated CD4⁺ T-cells. RANTES (regulated upon activation T cell expressed and secreted), MIP-1 α (macrophage inhibitory protein) and MIP-1 β represent the natural ligands of CCR5 and are able to inhibit the entry of M-tropic HIV-1 isolates into T cells.⁸

Viral replication:

Although CD4⁺ T cells appear to be HIV's main target, other immune system cells with CD4 molecules on their surfaces such as cells of the

mononuclear phagocyte lineage (principally blood monocytes and tissue macrophages), natural killer (NK) cells, dendritic cells (Langerhans cells and follicular dendritic cells in lymph nodes), hematopoietic stem cells, endothelial cells, microglial cells in the brain and gastrointestinal epithelial cells are infected as well. Long-lived cells called monocytes and macrophages can harbor large quantities of the virus without being killed. Even CD4⁺ T cells serve as important reservoirs of HIV: a small proportion of these cells maintain HIV in a stable, inactive form. Normal immune processes may activate these cells, resulting in the production of new HIV virions.

a) Entry to the cell:

HIV enters macrophages and CD4⁺ T cells by the adsorption of its surface glycoproteins to the receptors on the target cell. Once gp120 is bound with the CD4 protein, the envelope complex undergoes a structural change, exposing the chemokine binding domains of gp120 and allowing them to interact with the target chemokine receptor. This allows for a more stable two-pronged attachment, which allows the N-terminal fusion peptide gp41 to penetrate the cell membrane, allowing fusion of the membranes and subsequent entry of the viral capsid.

b) Replication and Transcription:

Once the viral capsid enters the cell, an enzyme called reverse transcriptase liberates the single-stranded (+)RNA from the attached viral proteins and copies it into a complementary DNA. The reverse transcriptase then makes a complementary DNA strand to form a double-stranded viral DNA intermediate (vDNA). This, vDNA is then transported into the cell nucleus. The integration of the viral DNA into the host cell's

genome is carried out by another viral enzyme called integrase. This integrated viral DNA may then lie dormant, in the latent stage of HIV infection. To actively produce the virus, certain cellular transcription factors need to be present, the most important of which is NF- κ B (NF kappa B), which is upregulated when T cells become activated. In this replication process, the integrated provirus is copied to mRNA which is then spliced into smaller pieces. These small pieces produce the regulatory proteins Tat (which encourages new virus production) and Rev. As Rev accumulates, it gradually starts to inhibit mRNA splicing. At this stage, the structural proteins Gag and Env are produced from the full-length mRNA. The full-length RNA is actually the virus genome; it binds to the Gag protein and is packaged into new virus particles.

c) Assembly and Release:

The final step of the viral cycle, assembly of new HIV-1 virions, begins at the plasma membrane of the host cell. The Env polyprotein (gp160) goes through the endoplasmic reticulum and is transported to the Golgi complex where it is cleaved by protease and processed into the two HIV envelope glycoproteins gp41 and gp120. These are transported to the plasma membrane of the host cell where gp41 anchors the gp120 to the membrane of the infected cell. The Gag (p55) and Gag-Pol (p160) polyproteins also associate with the inner surface of the plasma membrane along with the HIV genomic RNA as the forming virion begins to bud from the host cell. Maturation either occurs in the forming bud or in the immature virion after it buds from the host cell. During maturation, HIV proteases cleave the polyproteins into individual functional HIV proteins and enzymes. The various structural components then assemble

to produce a mature HIV virion. The mature virus is then able to infect another cell.⁹

Stages of HIV infection:

The Center for Disease Control and Prevention (CDC) defined a set of guidelines and recommendations for HIV-infected adolescents and adults on the basis of clinical conditions associated with the HIV infection and CD4+

T lymphocyte counts. The system is based on three ranges of CD4+ T-lymphocyte counts or CD4% and the three clinical categories. These CDC guidelines have been based on studies done mostly in developed countries.

Table-1: CDC classification of system for HIV infection

Clinical Categories	CD4+ T-cell count (cells/μl) (CD4%)
A [Asymptomatic, acute (primary) HIV or PGL]	>500 (28%)
B [Symptomatic conditions]	200-499 (15-28%)
C [AIDS indicator-conditions]	<200 (14%)

Category A: asymptomatic HIV infection, persistent generalized lymphadenopathy (PGL).

Category B: oropharyngeal and vulvovaginal candidiasis, constitutional symptoms such as fever (38.5° C) or diarrhea lasting >1 month, herpes zoster (shingles).

Category C: *Mycobacterium tuberculosis* (pulmonary and disseminated), *Pneumocystis carinii* pneumonia, candidiasis of bronchi; trachea or lungs, extrapulmonary cryptococcosis, CMV, HIV-related encephalopathy, Kaposi's sarcoma, wasting syndrome due to HIV.¹⁰

Besides the CDC classification, the World Health Organization (WHO) has also published a staging system for HIV infection. The WHO classification is an approach for use in resource-limited settings and is widely used in Africa and Asia. This staging system for HIV disease (in adults and adolescents) is based on clinical symptoms. (2006 revision)

Clinical Stage I: Asymptomatic, Persistent generalized lymphadenopathy

Clinical Stage II: Moderate unexplained weight loss (under 10% of presumed or measured body weight), Recurrent respiratory tract infections (sinusitis, tonsillitis, otitis media, pharyngitis), Herpes zoster, Angular chelitis, Recurrent oral ulceration, Papular pruritic eruptions, Seborrhoeic dermatitis, Fungal nail infections

Clinical Stage III: Unexplained severe weight loss (over 10% of presumed or measured body weight), Unexplained chronic diarrhoea for longer than one month, Unexplained persistent fever (intermittent or constant for longer than one month), Persistent oral candidiasis, Oral hairy leukoplakia, Pulmonary tuberculosis, Severe bacterial infections (e.g. pneumonia, empyema, pyomyositis, bone or joint infection, meningitis, bacteraemia), Acute necrotizing ulcerative stomatitis, gingivitis or periodontitis, Unexplained anaemia (below 8 g/dl), neutropenia (below 0.5 billion/l) and/or chronic thrombocytopenia (below 50 billion/l)

Clinical Stage IV: HIV wasting syndrome, Pneumocystis pneumonia, Recurrent severe bacterial pneumonia, Chronic herpes simplex infection (orolabial, genital or anorectal of more than one month's duration or visceral at any site) , Oesophageal candidiasis (or candidiasis of trachea, bronchi or lungs), Extrapulmonary tuberculosis, Kaposi's sarcoma, Cytomegalovirus infection (retinitis or infection of other organs), Central nervous system toxoplasmosis, HIV encephalopathy, Extrapulmonary cryptococcosis including meningitis, Disseminated non-tuberculous mycobacteria infection, Progressive multifocal leukoencephalopathy, Chronic cryptosporidiosis, Chronic isosporiasis, Disseminated mycosis (extrapulmonary histoplasmosis, coccidiomycosis), Recurrent septicaemia (including non-typhoidal Salmonella), Lymphoma (cerebral or B cell non-Hodgkin), Invasive cervical carcinoma, Atypical disseminated leishmaniasis, Symptomatic HIV-associated nephropathy or HIV-associated cardiomyopathy.¹¹

The clinical course of infection:⁷

The natural evolution of HIV infection can be considered in the following stages:

Acute HIV infection:

Within few weeks of infection with HIV, 10-15% of persons experience low grade fever, malaise, headache, lymphadenopathy and rash. Tests for HIV antibodies are usually negative at the onset of the illness but become positive during its course. Hence this syndrome has been called 'seroconversion illness'.

Asymptomatic infection:

All persons infected with HIV pass through a phase of symptom less infection lasting for several months or years. They show positive HIV antibody tests during this phase and are infectious. In some, infection may not progress any further, while in others it may lead to full blown AIDS either directly or through minor opportunistic infections, persistent generalized lymphadenopathy or AIDS related complex.

Persistent generalized lymphadenopathy (PGL):

This has been defined as the presence of enlarged lymph nodes, at least 1 cm in diameter, in two or more noncontiguous extrainguinal sites, that persists for at least 3 months, in absence of any current illness or medication that may cause lymphadenopathy. This may itself is benign but a proportion of cases may progress to ARC or AIDS.

AIDS related complex (ARC):

This group includes patients with considerable immunodeficiency, suffering from various constitutional symptoms or having minor opportunistic infections. The typical constitutional symptoms are fatigue, unexplained fever, persistent diarrhea and marked weight loss of more than 10% of body weight. The common opportunistic infections are oral candidiasis, herpes zoster, hairy cell leukoplakia, salmonellosis or tuberculosis. Generalized lymphadenopathy and splenomegaly are usually present.

AIDS:

This is the end stage of disease representing the irreversible breakdown of immune defense mechanisms, rendering the patient susceptible to opportunistic infections and malignancies.

a) In developing countries, the most important pathogen is *M. tuberculosis*, with many strains being multidrug resistant. Especially India is facing a double epidemic, jointly with HIV and tuberculosis.

b) Gastrointestinal system: The mouth is often involved in AIDS, with thrush gingivitis, hairy leukoplakia or Kaposi's sarcoma. A characteristic intestinal pathogen in AIDS is cryptosporidium. Salmonellae, mycobacteria, cytomegalovirus (CMV) or adenoviruses also frequently cause intestinal infections. Chronic colitis is common in male homosexuals, from which amoeba, giardia have been reported.

c) Central nervous system: The typical CNS opportunistic infections are toxoplasmosis and cryptococcosis. Infections are seen with CMV, herpes simplex, aspergillus and candida. Lymphomas of CNS are common. HIV may cause direct cytopathogenic damage in CNS. It can cross the blood-brain barrier and cause encephalopathy leading to loss of higher functions, progressing to dementia.

d) Malignancies: Kaposi's sarcoma is the characteristic lesion seen in male homosexuals. The other tumors commonly seen are lymphomas, both the Hodgkin and non Hodgkin types.

e) Cutaneous: Herpes lesions, candidiasis, xeroderma, prurigo and impetigo are common cutaneous lesions.

Table-2: Opportunistic infections and malignancies commonly associated with HIV infection¹²

Type of Disease	Diseases
Bacterial	Tuberculosis (TB caused by <i>Mycobacterium tuberculosis</i>), <i>Mycobacterium avium</i> complex disease (MAC), bacterial pneumonia and septicaemia
Protozoal	<i>Pneumocystis carinii</i> pneumonia (PCP), toxoplasmosis, microsporidiosis, cryptosporidiosis and leishmaniasis
Fungal	Candidiasis, cryptococcosis (cryptococcal meningitis) and penicilliosis
Viral	Diseases caused by cytomegalovirus, herpes simplex and herpes zoster virus
Malignancies	Kaposi's sarcoma, lymphoma and squamous sarcoma

f) Pediatric AIDS: About one third to one half the number of babies born to infected mothers are infected with HIV. Many of them may not survive for a year. Children may also acquire infection from blood transfusion. There are many differences between adult and pediatric AIDS. Children develop humoral immunodeficiency early, leading to recurrent bacterial infections. Chronic diarrhea, lymphadenopathy, tuberculosis and opportunistic bacterial infections are common manifestations in pediatric AIDS. Lymphatic interstitial pneumonia is seen exclusively in children, while Kaposi's sarcoma, toxoplasmosis and cryptococcosis are less common than in adults.⁷

Table-3: 2006 WHO Human Immunodeficiency Virus Pediatric Classification System¹³

Classification of HIV associated immunodeficiency	Age related CD4 Values			
	< 11 months (%CD4+)	12-35 months (%CD4+)	36-59 months (%CD4+)	>5 years (absolute Number per mm ³ or %CD4+)
None or not significant	>35	>30	>25	>500
Mild	30-35	25-30	20-25	350-499
Advanced	25-29	20-24	15-19	200-349
Severe	<25	<20	<15	<200 or 15%

Immunopathogenesis:

The principle impact of HIV infection on immune system is destruction of the CD4⁺ T lymphocytes. This ultimately leads to the inability of infected person to deal with opportunistic organisms. The half life of most HIV infected T cells *in vivo* is 12-36 hr.¹⁴ HIV brings about the destruction through multiple mechanisms that are,

❖ Direct cell killing:

Infected CD4⁺ T cells may be killed directly when large amounts of virus are produced and bud off from the cell surface, disrupting the cell membrane.

❖ Syncytia formation:

Infected cells also may fuse with nearby uninfected cells through CD4-mediated fusion, forming balloon-like giant cells called syncytia. This mechanism of cell-to-cell spread of HIV has been associated with the death of uninfected cells.

❖ **Apoptosis:**

Infected CD4⁺ T cells may be killed when cellular regulation is distorted by HIV proteins, probably leading to their suicide by a process known as programmed cell death or apoptosis. Investigators have shown in cell cultures that the HIV envelope alone or bound to antibodies sends an inappropriate signal to CD8⁺ T cells causing them to undergo apoptosis even though not infected by HIV.

❖ **Innocent bystanders:**

Uninfected cells may die in an innocent bystander scenario: HIV particles may bind to the cell surface, giving them the appearance of an infected cell and marking them for destruction by killer T cells.¹⁵

The period between the infection with HIV till progression to AIDS, is a long period of clinical stability that may last for 8 to 10 years. At the one end of the spectrum are the individuals who are able to mount the immune response that is capable of controlling the virus replication successfully and such individuals may remain clinically normal with minimal loss of CD4⁺ cells and very low to undetectable viral load. These are 'Long term Non Progressors' (LTNPs). At the other end of the spectrum are the persons who are unable to control viral multiplication and shows rapid decline in CD4⁺ cell counts and may develop AIDS within 2 to 3 years of acquiring HIV infection.¹⁶ Progression of HIV infection is largely dependent on the interaction between the viral factors and host factors.

The genetic factors that have been studied in the context of HIV disease progression include human leukocyte antigen (HLA) and polymorphism in

HIV co-receptor genes. A number of studies have revealed association between certain HLA alleles with rapid and slow disease progression. The alleles B14, B27, B51, B57 and C8 are associated with a slow disease progression, while, the presence of HLA A23, B37 and B49 are associated with the rapid disease progression.¹⁷

One of the strongest association of genetic polymorphism and HIV susceptibility and disease progression has been reported in case of a mutation of 32 base pair deletion in the CCR5 receptor gene (CCR5Δ32). Individuals homozygous for this mutation have decreased susceptibility to HIV infection, while persons heterozygous for CCR5Δ32 are more likely to become LTNPs. A mutation in stromal derived factor 1 (SDF-1), ligand for CXCR4, may delay progression to AIDS and also increase survival after diagnosis of AIDS.¹⁶

The innate responses may play a major role in initial containment of infection and hence may be crucial in acute primary HIV infection. Two cell populations, plasmacytoid dendritic cells (PDC) and natural killer cells play predominant role in innate cellular immune response. PDCs were first detected in lymph nodes in 1975 and described as T-like plasmacytoid cells, the major producer of type 1 interferons. Loss of these cells, reflected by decreased interferon-alpha (IFN-α) production, is associated with higher HIV RNA levels and progression to AIDS.¹⁸ PDCs not only participate in the innate immune response through interferon production, they may become mature dendritic cells [DCs] and participate in the adaptive immune response. DCs residing with epithelial surfaces are the initial cells infected by HIV after mucosal exposure to virus.¹⁹ DC-SIGN [Dendritic cell-specific ICAM-3 non-integrin], a novel lectin expressed by

DCs, has recently been shown to play a role in transport of HIV to the local lymph nodes. It has been observed that virus bound to DC-SIGN on DCs can remain infectious for several days and virus-pulsed DCs efficiently transmit virus when they come into contact with CD4- and co-receptor-positive cell types.²⁰

NK cells have the natural capacity to kill virus infected cells in the absence of prior sensitization. They can kill target cells either by direct contact or by antibody-dependent cellular cytotoxicity (ADCC). However NK cells display a decreased lytic activity in HIV-1 infected patients as compared to uninfected controls. This effect appears to be associated with very low expression of natural cytotoxicity receptors (NCR) on HLA-DR+NK cells and with a differential content in perforin molecules.¹⁶

The adaptive immune response plays major role in control of viraemia. HIV-specific neutralizing antibodies, CTL responses and T helper response are the major players in HIV-specific adaptive immune response. Neutralizing antibodies may be detected 8 week or more after infection.^{21,22} Appearance of neutralizing antibody response results in complete replacement of neutralization sensitive virus by successive population resistant virus. Escape virus may contain mutation in Env protein that alters conformation of gp120 protein, which diminishes antibody binding. However the role of neutralizing antibodies still remains to be understood completely in disease progression.¹⁶

The decrease in CD4 cell count due to HIV infection could affect the CD4 T cell response. T cell help is required for generation of neutralizing antibody response as well as cytotoxic T cell (CTL) responses. The CD4 T cell produce an array of cytokines that not only activate the cells of

immune system, they also influence the expression of various critical molecules on the cell surface and may upregulate or downregulate HIV multiplication in infected cells. Cytokines such as IL2, IFN- γ , IL12 are proregulatory cytokines (Th1 type) while IL4, IL10, IL15, IL13 are proinflammatory cytokines (Th2 type). It has been observed that HIV disease is associated with overproduction of type 2 cytokines and decreased production of type 1 cytokines, especially IL2 that enhances the growth of other T cells and helps to stimulate other cells' response to invaders. Thus the profile of cytokines produced by the CD4 helper cells may decide the progression of disease.¹⁶

Cytotoxic T lymphocytes (CTL) recognize HIV infected cells through HIV antigens expressed on the surface of the infected cells in association with HLA class I peptides. They induce biochemical pathways in the target cells that ultimately lead to the destruction of cells. By destroying infected cells the CTLs put halt to multiplication of HIV. However CTL responses are unable to prevent HIV-1 infection or prevent viral escape from immune recognition due to several reasons.²³ First, depletion of essential helper CD4+ T cells by HIV may be an important cause for the progressive loss of CTL function. These cells are critical in sustaining optimum virus-specific CTL response. Secondly, HIV-specific CTLs may suffer functional defects such as defects in perforin expression or decline in IFN- γ production have been reported. Thirdly, CTLs are not able to maintain the control of HIV multiplication as a result of viral (mutant) escape from recognition by CTLs.

Thus it is clear that it is not a single component but orchestrated action of different immune mechanisms will decide the outcome of HIV infection.

Treatment:

Since HIV belongs to a group of viruses called retroviruses, treatment to fight HIV infection is known as Anti-Retroviral Therapy (ART). The goals of ART are,

- ❖ To ensure maximal and durable suppression of the virus
- ❖ To reconstitute and preserve immunologic quantity and function
- ❖ To improve quality of life
- ❖ To reduce morbidity and mortality due to HIV infection

Additionally, antiretroviral drugs can be used to reduce transmission of HIV in various situations, for example, transmission of HIV from infected mother-to-child (MTCT), after occupational post-exposure prophylaxis (PEP) and non-occupational exposures.

Since ART is life-long and associated with long-term adverse events, the decision to initiate ART is made after weighing the risk of progression to AIDS and other important determinants such as the incidence of short and long term side effects, commitment to high levels of adherence, development of resistance to antiretrovirals and affordability and patient's readiness to therapy.

ART is indicated for all patients who are symptomatic with an AIDS defining illness, irrespective of CD4 counts or viral load levels. In addition patients with non-AIDS defining illness like recurrent oral candidiasis, oral hairy leukoplakia may have a risk of rapid progression and therapy is indicated in these patients if CD4 count is $<350/\text{mm}^3$. Adequate control of opportunistic infections (OIs) with appropriate antimicrobial therapy and stabilizing patients is crucial before initiating ART. This reduces pill burden and prevents additive toxicities.²⁴

Currently, 25 single or combination preparations (Table-4) from four classes of drugs are licensed. These 4 classes include the Nucleoside Reverse Transcriptase Inhibitors (NRTIs), Non-nucleoside Reverse transcriptase Inhibitors (NNRTIs), Protease Inhibitors (PIs) and Entry Inhibitors.

Table 4: Antiretroviral agents²⁶

Trade name	Abbrev.	Drug	Manufacturer
Nucleoside and Nucleotide Reverse Transcriptase Inhibitors (NRTIs)			
Combivir™	CBV	AZT+3TC	GSK
Emtriva™	FTC	Emtricitabine	Gilead
Epivir™	3TC	Lamivudine	GSK
HIVID™*	ddC	Zalcitabine	Roche
Kivexa/Epzicom™	KVX	3TC+ABC	GSK
Retrovir™	AZT	Zidovudine	GSK
Trizivir™	TZV	AZT+3TC+ABC	GSK
Truvada™	TVD	FTC+TDF	Gilead
Videx™	ddI	Didanosine	BMS
Viread™	TDF	Tenofovir	Gilead
Zerit™	d4T	Stavudine	BMS
Ziagen™	ABC	Abacavir	GSK
Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTIs)			
Rescriptor™	DLV	Delavirdine	Pfizer
Sustiva/Stocrin™	EFV	Efavirenz	BMS/MSD
Viramune™	NVP	Nevirapine	Boehringer Ingelheim
Protease Inhibitors (PIs)			
Aptivus™	TPV	Tipranavir	Boehringer-Ingelheim
Agenerase™	APV	Amprenavir	GSK
Crixivan™	IDV	Indinavir	MSD
Invirase 500™	SQV	Saquinavir	Roche
Kaletra™	LPV	Lopinavir/ritonavir	Abbott
Norvir™	RTV	Ritonavir	Abbott
Reyataz™	ATV	Atazanavir	BMS
Telzir/Lexiva™	FPV	Fosamprenavir	GSK
Viracept™	NFV	Nelfinavir	Roche/Pfizer
Fusion inhibitors			
Fuzeon™	T-20	Enfuvirtide	Roche
Entry inhibitors			
Celsentri/Selzentry™	MVC	Maraviroc	Pfizer

*Distribution ceased

❖ **Nucleoside Reverse Transcriptase Inhibitors (NRTIs):**

They are also referred to as Nucleoside analogs and were the first drugs to be used in HIV treatment. Their target is the HIV enzyme reverse transcriptase. Acting as alternative substrates, they compete with physiological nucleosides, differing from them only by a minor modification in the ribose molecule. The incorporation of nucleoside analogs induces the abortion of DNA synthesis, as phosphodiester bridges can no longer be built to stabilize the double strand. Nucleoside analogs are prodrugs that must first enter the infected cell and then be phosphorylated to the active triphosphate moiety, similar to the natural deoxynucleosides whose functions they mimic. Zidovudine (AZT) and stavudine (d4T) serve as analogs of deoxythymidine (dT). Zalcitabine (ddC) and lamivudine (3TC) are analogs of deoxycytidine (dC). Didanosine (ddI) is analog of deoxyadenosine (dA) and abacavir is guanosine analog.^{25,26}

Zidovudine (Azidothymidine/AZT):

It is a synthetic pyrimidine analog that differs from thymidine in having azido substituent instead of a hydroxyl group at the 3' position of deoxyribose ring. It was initially developed as an anticancer agent and subsequently found to inhibit the reverse transcriptase of Friend leukemia virus.²⁷ Soon after the identification of human retrovirus as the etiologic agent of AIDS, AZT was shown to have anti-HIV activity *in vitro*.²⁸ Studies conducted in 1987 demonstrated that zidovudine, the first antiretroviral agent, at a dose of 1500 mg/day was able to reduce the frequency of HIV-associated diseases and deaths in HIV-positive patients. In 1990, two studies were published showing that a dose of 1200 mg and even 500

mg/day was as effective as higher doses. The same studies tested the efficacy of zidovudine in patients with less advanced disease: AIDS-related complex, asymptomatic HIV infection and moderate cellular immunosuppression. These studies led to the recommendation in the US that minor symptomatic or asymptomatic HIV positive patients with less than 500 CD4+ cells/ μ l should be given zidovudine monotherapy. However results from the Concorde and the Australian-European study, which assessed the efficacy of zidovudine monotherapy in asymptomatic patients with more than 500 CD4 cells, led to the conclusion that zidovudine monotherapy was only beneficial over a limited period of about 1 year.

The oral bioavailability of AZT is 60%. It is metabolized in liver and excreted renally. The penetration in cerebrospinal fluid (CSF) is approximately 50% of the serum concentration and the intracellular half-life is 3 hr.²⁹

Didanosine (ddI):

In 1991, didanosine was the second nucleoside analog to be licensed. After initial studies investigating safety and tolerance of ddI, a comparative study between ddI and AZT was conducted. These studies showed a survival advantage for treatment-naïve patients with AZT+ddI compared to AZT monotherapy. This effect of ddI was less marked in AZT pretreated patients. In more recent studies, ddI is only used seldomly.²⁶

Its bioavailability is 40% and about 50% of drug is excreted renally. Penetration in CSF is 20% of serum concentration. It has long intracellular half-life of 25-40 hr.²⁹

Zalcitabine (ddC):

In 1992, zalcitabine was the third NRTI to reach the market. The weak efficacy as well as problems with the pharmacokinetics and side effects resulted in ddC being taken from the market in 2006 - a first for HIV medicine.²⁶

Lamivudine (3TC):

In 1996, lamivudine was the fifth NRTI to be licensed in Europe. It is a well-tolerated cytidine analog, whose substantial disadvantage is rapid development of resistance. A single point mutation (M184V) is sufficient to cause loss of efficacy and results in a moderate *in vitro* cross resistance to didanocine, abacavir and zalcitabine. On monotherapy, this mutation is likely to lead to resistance after only a few weeks. The full effect of 3TC only emerges in combination with other NRTIs. As a component of Combivir™, Kivexa™ and Trizivir™, 3TC is actually one of the most frequently used antiretroviral agents of all. An important effect of 3TC is also its relatively good efficacy against hepatitis B viruses, although this is again limited by the relatively rapid development of resistance.²⁶

Its bioavailability is 85%. It is eliminated unchanged by way of renal excretion. The concentration in CSF is 6% of the serum concentration and the intracellular half-life is 12 hr.²⁹

Stavudine (d4T):

It was the second thymidine analog to be introduced after AZT. d4T is often initially tolerated better than AZT, is certainly just as effective and used to be one of the most frequently prescribed HIV drugs. It is now beyond doubt that long-term toxicity occurs more frequently with d4T than with other NRTIs. d4T is a risk factor for lactic acidosis and

hyperlactacidemia.^{30,31,32} In cohort studies, the risk of lipoatrophy on d4T doubled in one year³³ and it tripled in two years.³⁴ However, in the so-called developing countries it remains an important combination partner, particularly due to the lack of myelotoxicity.

The bioavailability of stavudine is 85%. About 50% is excreted renally. The concentration in CSF is about 10%-40% of serum concentration and intracellular half-life is 3.5 hr.²⁹

Abacavir (ABC):

It is a guanosine analog, which can lower viral load by approximately 1.4 logs within 4 weeks. In 2004 abacavir was licensed for once-daily therapy. It is also a component of Trizivir™ and Kivexa™. A regimen that is failing virologically can be successfully intensified with abacavir if it is added early enough, and if the viral load is not too high. Abacavir is also used to simplify HAART. Numerous randomized studies have demonstrated that patients on a successful PI- or NNRTI-regimen can switch relatively safely to abacavir plus two NRTIs.^{35,36,37,38} With respect to mitochondrial toxicity, abacavir is more favorable than several other substances. Lipoatrophy improves on changing from d4T to Abacavir.^{39,40,41,42} One drawback to the use of abacavir is the risk of a hypersensitivity reaction (HSR), an allergic reaction that is associated with fever and lethargy. Severe HSR can occur after only a single abacavir tablet⁴³ or even after treatment interruption despite prior tolerability.⁴⁴

Emtricitabine (FTC):

FTC is a cytidine analog, which is biochemically, very similar to 3TC, but has a longer half-life. Like 3TC, it has HBV efficacy, tolerability is good, and it has a narrow interaction potential. In monotherapy studies as well

as in combination with AZT, FTC was at least as effective as 3TC.^{45,46} Subsequent to data from the FTC-301 Study⁴⁷, the drug was licensed in 2003. This randomized, double blind trial showed that FTC was clearly more effective and tolerable than d4T.⁴⁸ The ALIZE study demonstrated the good long-term tolerability and efficacy of a once-daily combination of FTC+ddI+Efavirenz.⁴⁹ Today, FTC is an important combination partner in HAART, particularly in the fixed-dose combination with tenofovir (Truvada™).

Tenofovir (TDF):

It acts as a false building block similar to nucleoside analogs, targeting the enzyme reverse transcriptase. However, in addition to the pentose and nucleic base, it is monophosphorylated and therefore referred to as a nucleotide analog. The accurate description of the substance is tenofovir DF (tenofovir disoproxil fumarate = TDF) and it has been licensed in 2001. In the 934 study, TDF+FTC was significantly better than AZT+3TC, particularly due to the improved tolerability.⁴⁸ Tenofovir can also help to improve d4T-induced lipoatrophy and dyslipidemias.^{50,51} However, the potential risk of nephrotoxicity is a serious problem for tenofovir, which is associated with a mild to moderate disturbance of renal function.^{52,53}

❖ Non-nucleoside reverse transcriptase inhibitors (NNRTIs):

NNRTIs were first described in 1990. As with the nucleoside analogs, the target enzyme is reverse transcriptase. However, NNRTIs bind directly and noncompetitively to the enzyme at a position in close proximity to the substrate binding site for nucleosides. The resulting complex blocks the catalyst activated binding site of the reverse transcriptase. In contrast to

NRTIs, NNRTIs do not require activation within the cell. The three available NNRTIs- nevirapine, delavirdine and efavirenz were introduced between 1996 and 1998.²⁶

Nevirapine:

Nevirapine, a NNRTI, was one of the first medicaments with antiretroviral activity to be tested in humans. However, as with lamivudine, within few weeks, nevirapine led to a rapid development of resistance associated with a loss in clinical efficacy. Even in double combination with AZT, efficacy was limited by the prompt appearance of resistance caused by one-step mutation at codon 106 or 181 of the RT gene. Only its use in triple combination (AZT+ddI+nevirapine) allowed this agent a place with drugs used for treatment of HIV infection. However it also showed limited efficacy.^{54,55}

The bioavailability of nevirapine is 90% and it is metabolized in liver. Its concentration in CSF is about 45% of the serum level.²⁶

Delavirdine:

It was the second NNRTI to be licensed by the FDA in 1997. Due to the pill burden and the required three times daily dosing, delavirdine is currently rarely prescribed. Delavirdine is not licensed in Europe where, in 1999, an application for licensure was rejected due to insufficient efficacy data. Nevertheless, delavirdine is likely to be as effective as the other NNRTIs.²⁶ In DLV 21, AZT+3TC+delavirdine was tested against AZT+3TC and AZT+delavirdine on 369 mostly treatment-naïve patients. After one year, 68% had a viral load below 50 copies/ml in the triple combination arm compared to less than 10% in the other two arms.⁵⁶

Its bioavailability is 60%-80% and it is metabolized in liver. The concentration in CSF is 0.5% of the serum concentration.²⁹

Efavirenz:

It was the third NNRTI to be approved, and the first for which it could be shown that NNRTIs were at least as effective and probably even better than PIs in untreated or only short term treatment-experienced patients. The 006 Study showed a superiority of efavirenz over indinavir.⁵⁷ Since then, efavirenz has been compared to other drugs in many studies and almost always did well. In ACTG 5095, efavirenz in combination with AZT+3TC was better than Abacavir.⁵⁸ In ACTG 384 it was better than nelfinavir.^{59,60}

❖ Protease inhibitors (PIs):

The HIV protease cuts the viral gag-pol polyprotein into its functional subunits. If the protease is inhibited and proteolytic splicing prevented, non-infectious virus particles will result. With the knowledge of the molecular structure of the viral protease, the first protease inhibitors (PIs) were designed in the early nineties. These substances were modified in such a way that they fit exactly into the active enzyme site of the HIV protease.

Saquinavir:

It was the first PI to be licensed for HIV therapy in 1995 and is still today one of the few substances whose efficacy has been proven based on clinical end points. Saquinavir is well tolerated. With no serious short-term problems, it is an attractive PI for patients who need a boosted PI-regimen. The earlier hard gel (Invirase™) and soft gel (Fortovase™)

capsules were replaced in 2005 by Invirase 500™ tablets, which significantly reduced the pill burden.²⁶

Ritonavir:

It was the first PI for which efficacy was proven on the basis of clinical endpoints.⁶¹ However, ritonavir is now obsolete as a single PI, since tolerability is too poor. Ritonavir is now only given to boost other PIs (positive use of drug-drug interaction). The “baby dose” used for this purpose (100 mg bid) is tolerated better.²⁶ Ritonavir used in low dose is a potent inhibitor of cytochrome 450 3A4 enzyme systems. When administered with other PIs, which are metabolized in this pathway, increased blood levels of the co-administered PIs are achieved. This helps to reduce the dose and modify schedules of the concomitant PI.²⁴

Indinavir:

It is one of the oldest PIs, which was initially very successful in large studies.^{62,63} Later, indinavir had mixed success, at least when unboosted: in the Atlantic Study, it was about as effective as nevirapine⁶⁴, but in the 006 Study it was clearly weaker than Efavirenz.⁵⁷ In the double blind, randomized CNAAB3005 Study, indinavir was more effective than abacavir, particularly in patients with high viral load at baseline.⁶⁵ Low protein binding (60%) seems to allow better CNS penetration than with other PIs. However indinavir now has only a minor role in current antiretroviral regimens.

Nelfinavir:

It was the fourth PI on the market in 1998 and was for a long time one of the most frequently used PIs. In the pivotal 511 Study, the use of nelfinavir in triple combination therapy was highly effective and well

tolerated. Sixty one percent of patients on nelfinavir (with AZT+3TC) had a viral load below 50 copies/ml at 48 weeks.⁶⁶ However, in patients with evidence of HIV protease inhibitor failure, the efficacy of nelfinavir appears to be limited.^{67,68}

The bioavailability of nelfinavir is 20%-80% and is higher when taken with food. It has plasma half-life of 5 hr, the longest half-life of all PIs.²⁹

Amprenavir:

It was the fifth PI to enter the European market, in 2000. Due to the further development of fosamprenavir, it was replaced in 2004. Only the suspension and the 50 mg tablet are available for children.²⁶

Atazanavir:

It was the first once-daily PI to be licensed in 2004. It is currently only licensed for treatment-experienced patients. In contrast to other PIs, atazanavir does not have a negative influence on lipid profiles,^{69,70} which is its main advantage besides the once-daily dosing. It has been shown that lipids improve when other PIs are replaced by atazanavir.⁷¹ One problem with atazanavir is that more than half the patients experience elevated bilirubin levels and some patients develop clinical jaundice.

Fosamprenavir:

It was licensed for treatment-naïve and -experienced patients in 2004. One advantage of the drug is that there are no restrictions with respect to food intake, and it can be taken on an empty stomach or with a meal.²⁶

Lopinavir/ritonavir (Lopinavir/r):

It is, since its licensing in April 2001, the first (and so far the only) PI with a fixed booster dose of ritonavir, which increases concentrations of lopinavir by more than 100 fold.²⁶ Large randomized studies are currently

underway to compare the soft capsules with the tablets. Lopinavir/r has a high genetic barrier to resistance, and it is likely that at least 6-8 cumulative PI resistance mutations are necessary for treatment failure.⁷² Lopinavir/r monotherapy is therefore currently being intensively investigated.

Tipranavir:

This first non-peptidic PI- was licensed in July 2005 for pre-treated patients.²⁶ It is recommended that it be taken with food. Tipranavir has good efficacy against PI-resistant viruses.⁷³ There is currently no license for use in therapy-naïve patients.

❖ Entry Inhibitors:

There are three crucial steps for entry of HIV into the CD4 cell:

1. Binding of HIV to the CD4 receptor,
2. Binding to co-receptors, and finally
3. Fusion of virus and cell.

Every step of HIV entry can theoretically be inhibited. All three drug classes, namely attachment inhibitors, co-receptor antagonists and fusion inhibitors (FIs) are currently summarized as entry inhibitors. In 2003, Fuzeon™- the first and so far only drug of this class- was licensed.

T-20 (Enfuvirtide, Fuzeon™):

Since May 2003, it has been licensed in Europe and the US for the treatment of HIV-1 infection in treatment-experienced adults and children over 6 years of age. It is preferable to treat patients within clinical studies, so that clinical experience with this new drug can be collected. As a relatively large peptide (36 amino acids) it has to be administered by

subcutaneous injection. T-20 binds to an intermediate structure of the HIV gp41-protein, which appears during fusion of HIV with the target cell. Unfortunately, this peptide fusion inhibitor lacks oral bioavailability, so that 2 injections per day are needed which cause important local side-effects. Moreover, the cost of synthesizing large peptides is very high, which excludes the use in developing countries such as India.²⁶

Maraviroc (Celsentri™, Selzentry™):

Maraviroc from Pfizer is currently the most promising CCR5 antagonist. In a double blind randomized study, in which 63 patients with R5-trope viruses received different doses of maraviroc, the median drop in virus load was 1.6 logs after 10-15 days on maraviroc 2 x 100 mg/day. In a double blind, randomized, Phase II pilot study on 113 pre-treated patients with X4-trope viruses, there was no visible significant difference in the reduction of viral load. Indeed, surprisingly, the CD4-cell count increased markedly. The license is expected this year.²⁶

HAART:

The terminology "Highly active antiretroviral therapy" (HAART) refers to use of combinations of 3 antiretroviral agents for treatment of HIV infection. To date, most clinical experience with use of HAART in treatment-naïve individuals has been based on 3 types of combination regimens: NNRTI-based (1 NNRTI+ 2 NRTI), PI-based (1-2 PI + 2 NRTI) and triple NRTI-based regimens. Most experience in India is with NNRTI based regimens.

Initial regimen for ART-naïve HIV-1 infected patients in India:

An NNRTI based regimen is recommended as first line therapy for most ART-naïve HIV infected patients. These regimens are potent and have shown comparable efficacy to unboosted PI based regimens and are superior to 3 NRTI based regimens. NNRTI-based regimens have the advantage of lower pill burden and are cheaper compared to most of the PI-based regimens. Use of NNRTI-based regimens as initial therapy can preserve the PIs for later use, reduce or delay patient exposure to some of the adverse events commonly associated with PIs. The major disadvantage is their low genetic barrier for development of resistance. These agents require only single mutation to confer resistance and cross resistance often develops across the entire class. As a result, patients who fail this initial regimen may lose the utility to other NNRTIs and/or may transmit NNRTI-resistant virus to others.²⁴

The choice of NRTI backbones:

The most important are TDF+3TC, TDF+FTC and ABC+3TC. These combinations have the advantage that they can be administered once daily: TDF+FTC and ABC+3TC can even be taken in a single tablet. Therefore, they have replaced the long-standing backbone of AZT+3TC.

Poor and non-recommended backbones:

Almost all guidelines now clearly recommend avoiding the previously popular d4T+ddI combination. Mitochondrial toxicity is too high, and it is inferior to AZT+3TC. AZT+ddI is contraindicated, as ddI has to be taken on an empty stomach (whilst AZT is tolerated better when taken with a meal), and also due to the greater risk of gastrointestinal side effects. TDF+ddI are relatively toxic and recently, many studies have shown lower

efficacy. TDF+ABC is likely to be problematic due to *rapid development of resistance*. AZT+d4T and FTC+3TC are antagonistic. 3-NRTI regimen of abacavir+ tenofovir+ lamivudine and didanosine+tenofovir+lamivudine should be avoided due to high rates of virological failure.²⁶

HIV and Pregnancy:²⁴

The risk of transmission of HIV from an infected mother is 14-32% if not breast-fed and is 25-48% if breast-fed. More than two-third of this transmission occurs during labor when baby is exposed to maternal genital secretions.

The goals of management of HIV in pregnancy are dual: managing the mother's HIV status and prevention of mother to child transmission (MTCT) of HIV. In selection of drug regimen the following points should be remembered:

1. Efavirenz should be avoided because of possible teratogenic effects in first trimester of pregnancy
2. Combination of d4T+ddI should be avoided because of risk of development of fatal lactic acidosis
3. Do not use nevirapine (NVP) as part of ART regimen if mother's CD4 count $>250/\text{mm}^3$ due to risk of fatal hepatotoxicity.

Hence, HIV infected pregnant women with CD4 counts $<250/\text{mm}^3$ should be offered NVP-based regimen which should be continued after delivery.

Interventions known to reduce risk of MTCT include: ART for mother and baby, elective Caesarian section (ECS) and avoidance of breast-feeding or exclusive breast-feeding.

A single dose of NVP at the onset of labor and within 72 hr of birth for the baby has been shown to reduce the risk of MTCT. However, there is evidence to suggest that resistance to single dose NVP is frequent⁷⁴ and this can compromise the mother's NVP-based regimen in the future.⁷⁵ Hence a combination of ZDV+3TC should be added to single dose NVP at the onset of labor and continued for 7 days after delivery to reduce risk of development of NVP resistance.

In mothers (with CD4 counts $>250/\text{mm}^3$) who can afford, a combination of standard 3 drug ART is recommended for reducing risk of MTCT. This option termed as START (Short-term antiretroviral therapy) intends to treat mothers with standard 3 drugs ART throughout the duration of pregnancy (except first trimester) and discontinuing shortly after delivery. Since NVP cannot be used in mothers with CD4 counts $>250/\text{mm}^3$ PI-based (Nelfinavir) regimen is recommended for START.

Post exposure prophylaxis (PEP):

Occupational transmission of HIV in health care settings has been documented. The transmission in healthcare settings can occur from HIV infected patients to other patients, from an infected patient to a healthcare worker (HCW) and from an infected HCW to a patient. Occupational exposures include percutaneous injury, for example, a needle-stick or cut with a sharp object (risk is approximately 0.3%), contact of mucous membrane or non-intact skin with blood, tissue or other body fluids that are potentially infectious (risk is 0.09%).

PEP should be initiated as quickly as possible, preferably within 1-2 hr post exposure and up to 36 hr. PEP should be discouraged more than 72

hr after exposure. It should be administered for 28 days. Two drugs PEP include 2 NRTIs combination therapy and 3 drugs PEP include 2 NRTIs + 1 boosted PI or EFV.²⁴

Limitations (Failure) of HAART:

The introduction of HAART has led to a significant reduction in AIDS-related morbidity and mortality. However, there are a number of problems associated with it, including, the difficulties of maintaining long-term adherence, drug-related toxicities and the development of drug resistance, all of which may lead to virological failure, which in turn leads to immunological failure and clinical progression.⁷⁶

1) Lack of adherence to HAART regimens:

Adherence is the ability to take prescribed drugs in the recommended dosages and schedules and following any special instructions, for example, empty stomach.

a) Complexity of the regimen:

One of the biggest challenges to successful adherence to HAART regimens is their complexity viz. high pill burden and frequent dosing.

Once daily and twice daily regimens are associated with significantly better compliance rates (73% and 70% respectively) than are 3-times-daily (52%) and 4-times-daily (42%). HAART regimens, in addition to requiring many daily doses with multiple pills, often impose very specific food requirements that must be observed in order to ensure adequate blood levels of the drug. Doses must be taken in precise relationship to meals and the amount of fluid intake are often quite exacting.

Although newer regimens have reduced the frequency of daily dosing, lowered the pill burden and minimized the special instructions regarding timing the dosing with meals, many PI-containing regimens still require patients to take more than 20 pills per day over multiple dosing time.

The presence of other conditions in addition to HIV infection, such as depression and alcohol abuse, can exacerbate the effects of regimen complexity on adherence. Similarly, patients with social problems, arising from an accumulation of stressful life events such as family separation and lack of a social support network, may have difficulty adhering to complex HAART regimens. Age, education and employment status have also been suggested to be influencing factors. Adherence can also be interrupted due to monetary issues as the treatment is expensive. Thus the incomplete and inadequate adherence of patients to HAART regimen permits the emergence of HIV variants that are resistant to current treatment.⁷⁷

b) Cost of the treatment:

Drug costs are noted as an important determinant of non-adherence. A recent study of patients visiting a non-government provider in south India, estimated the median annual cost of treatment of AIDS patients to be of the order of rupees 6000 over a reference period of 6 months, with the treatment of patients on antiretroviral drugs averaging to rupees 18,150 over the same period. Another study from Delhi, suggests similar estimates of about rupees 30000 for antiretroviral treatment over a period of 1 year and another rupees 10000 annually for the monitory tests. Thus a successful treatment of HIV still remains a challenge.⁷⁸

2) Adverse effects of ART:

Although effective drugs are available, several different side effects are associated with the use of anti-HIV drugs. Some of the most common types of side effects are described below:

a) Hepatotoxicity:

It is the general term for liver damage. There are several specific conditions that fall within the general category of hepatotoxicity. These include: hepatitis (inflammation of the liver), hepatic necrosis (death of liver cells) and hepatic steatosis (too much fat in liver), may be associated with a life-threatening condition called lactic acidosis. Symptoms of liver damage include nausea, vomiting, abdominal pain, loss of appetite, diarrhea and hepatomegaly (liver enlargement). All FDA-approved NRTIs, NNRTIs and PIs are associated with hepatotoxicity. NRTIs, especially stavudine, didanosine and zidovudine are associated with lactic acidosis and hepatic steatosis. NNRTIs, especially nevirapine, are associated with hepatitis and hepatic necrosis. PIs, especially ritonavir are associated with hepatotoxicity. Also, patients infected with both HIV and hepatitis C virus (HCV) may be at particular risk for developing hepatotoxicity while taking PIs.

b) Hyperglycemia:

It is an increase in the amount of glucose in the blood. Treatment with HIV PIs and infection with HCV increase the risk of hyperglycemia and diabetes in HIV patients.

c) Hyperlipidemia:

It is an increase in the amount of fat such as cholesterol and triglyceride in the blood. This increase can lead to heart disease and pancreatitis. PIs such as indinavir, nelfinavir, zidovudine are likely to cause hyperlipidemia.

d) Lactic Acidosis:

It is the life-threatening condition caused by high levels of lactate in blood (hyperlactatemia) and low blood pH. Low pH means that blood contains too much acid, which can be harmful to the cells of the body. NRTIs can cause hyperlactatemia by disrupting the function of mitochondria. This is known as mitochondrial toxicity. Severe lactatemia leads to lactic acidosis. Although all NRTIs are associated with hyperlactatemia and lactic acidosis, patients taking stavudine and didanosine seem to be at greater risk.

e) Lipodystrophy:

It is also called as fat redistribution. There are 2 different kinds of lipodystrophy: fat-wasting known as lipoatrophy. In this, fat is lost from particular areas of the body especially the arms, legs, face and buttock. Second type is fat accumulation, also known as hyperadiposity. In this, fat builds up in particular parts of the body especially the belly (also called "protease paunch" or "crixivan potbelly"), breasts, back of the neck and upper shoulders (often described as "buffalo hump"). Lipodystrophy may also lead to other metabolic disorders such as hyperlipidemia and hyperglycemia. Lipodystrophy in combination with hyperlipidemia and insulin resistance is called lipodystrophy syndrome. Lipodystrophy is linked to the concurrent use of NRTIs and PIs. Stavudine is associated with fat loss whereas PIs increase the risk of fat accumulation.

f) Osteonecrosis, Osteopenia and Osteoporosis:

Osteonecrosis means 'bone death'. It has been observed in the hip bone of some of the HIV patients. However, it is not clear if it occurs because of HIV infection itself or as side effect of the medications used for the treatment. Osteopenia is a condition in which the bones lose minerals such as calcium and phosphate and become less dense. This makes the bones weaker. When the bone loss becomes more severe the condition is referred to as osteoporosis. PIs are associated with increased risk of osteopenia and osteoporosis.

g) Skin rash:

Anti-HIV medications can cause mild skin rashes as well as serious even life-threatening rashes. The severe skin rashes include Stevens - Johnson syndrome (SJS) and Toxic Epidermal Necrolysis (TEN), which are the different forms of the same kind of skin rash. The symptoms of SJS and TEN include, flat or raised red spots on the skin that develop blisters in the center, blisters in the mouth, eyes, genitals or other moist areas of the body. NNRTIs cause the majority of skin rashes, with nevirapine causing the most severe rashes. NRTIs such as abacavir may cause a rash that is a symptom of a severe drug hypersensitivity (allergic) reaction. Where zalcitabine is the PI most likely to cause skin rash.^{79,80}

h) Neurological and Mental side effects:

Peripheral neuropathy is damage to the peripheral nerves, most often in the feet and hands. Such nerve damage may lead to tingling, burning sensations, pain, numbness or weakness. The condition is most often associated with the use of ddC, ddI and d4T, but may also be due to nerve damage caused by HIV disease itself. Other neurological side

effects include headache, dizziness and difficulties with speech and movement. Various anti-HIV drugs have been associated with mental side effects including confusion, anxiety, paranoia and depression.

i) Bone Marrow Suppression:

Some drugs including AZT can cause bone marrow toxicity. Damage to the bone marrow may lead to the loss of the ability to produce new blood cells. This can result in anemia, leukopenia, neutropenia and thrombocytopenia.

The occurrence of side effects plays a large role in adherence to drug regimens, which in turn can impact the development of drug resistance.

3) Drug-drug interactions:

Patients may receive other drugs for supportive care, treatment of opportunistic infections and immunomodulation. Hence, drug interactions are often unavoidable in HIV-infected patients because of the drug classes involved and the number of drugs prescribed. These drug-interactions alter the absorption, transport, distribution, metabolism or excretion of a drug that may lead to viral resistance or serious toxic effects resulting in treatment failure.⁸²

The NNRTIs and the PIs are metabolized by the cytochrome P450 3A4 group of enzymes in the liver and this enzyme is either induced or inhibited by other drugs. This can either decrease or increase the levels of NNRTIs or PIs. Drugs like rifampicin are potent inducers of the cytochrome enzyme systems. Concomitant administration of NNRTIs and PIs with rifampicin can lead to reduction of blood levels of the former and development of resistance and failure of ART.²⁴

4) HIV drug resistance:

Development of resistance to ARV drugs is common cause to ART failure. Resistance is the ability of the virus to replicate in the presence of ARV drugs. Inadequate adherence to the prescribed regimen, insufficient therapeutic blood levels due to poor absorption, drug-drug interactions, and sub-optimal dosing can lead to resistance and treatment failure.

Friedland and Williams⁸³ suggested that the relationship between adherence and the development of resistance to ARV drugs may be 'bimodal', in which both high and low levels of adherence pose a relative low risk of spawning drug resistance. Whereas the highest risk of virological failure and development of new drug resistance occurred in the 70-90% adherence range.⁸⁴

The genetic barrier for resistance varies from drug to drug. Drug-resistant mutants of HIV appear to result from a series of substitutions of key amino acids in the target enzyme. These mutations interfere with binding of the antiviral agent to the viral enzyme target site, thus rendering the drug inactive.

As in the late 1980s, monotherapy with the nucleoside analogue AZT was the only treatment available for HIV-infected persons, resistance was first observed among NRTIs. Each NRTI induces a set of genetic alterations generally in step-wise fashion, with primary mutations arising first and secondary mutations developing during continued therapy. The number of mutations required to induce clinical drug resistance also varies among agents.^{85,86,87} For example, multiple RT mutations are required for high level resistance to zidovudine, whereas Lamivudine resistance occurs with the presence of a single mutation at codon 184. Overlap and accumulation

of mutations are the reason for cross-resistance that can occur among different agents of this class. For instance, mutations conferring resistance to ddI have been identified at RT codons 65, 74, 184 and for ddC at 65, 69, 74, and 184. Thus resistance mutations for ddC are overlapping with those for ddI and consequently, cross-resistance is observed between both substances. Mutation at position 184 is also responsible for the resistance against 3TC. Abacavir requires accumulated mutations to evolve resistance. Multidrug resistant (MDR) strains have been isolated from patients on prolonged combination therapy with NRTIs.⁸⁸ The primary mutation conferring MDR is at position 151. This mutation is associated with loss of fitness of the corresponding virus variants, which is compensated by additional mutations at positions 62, 75, 77 and 116. These viruses are resistant to AZT, ddI, ddC, d4T and 3TC. In addition to substitution mutations, insert-deletion mutations have also been correlated with MDR. The 6-base pair (bp) insert at position 69 of the RT region is responsible for the development of MDR HIV-1 variants.^{89,90} In an Italian multicenter cohort, the prevalence of both Q151M and the T69S insert were evaluated in 177 patients who failed combination therapy.⁹¹ Resistance to NNRTIs can emerge after just a brief exposure (2-4 weeks) to the drug. NNRTIs bind to a hydrophobic cavity near the active site of RT and cause a conformational change to the enzyme. NNRTI-binding site are largely restricted to β sheets comprising codons 100 to 110 and 180 to 190. Major resistance mutations occur at positions 181 and 103. The Y181C mutation reduces NNRTI-binding affinity leading to drug resistance. The K103N mutation may act by preventing the formation of the hydrophobic binding pocket, thus reducing binding affinity indirectly. As

resistance generally is not specific for the particular NNRTI administered leads to cross-resistance to other compounds in this class.⁹²

HIV protease is responsible for cleavage of the gag and gag-pol polyprotein products, yielding the functional core proteins and essential enzymes required to produce mature HIV. Resistance associated with mutations in the protease gene may result from amino acid substitution at or near the active site, thus interfering with binding of the inhibitor because of conformational perturbations. Primary mutations are directly associated with resistance to particular PI and secondary mutation for another PI. The accumulation of secondary mutations is responsible for cross resistance among PIs. Single mutations confer only a modest reduction in drug-susceptibility (3-fold to 10-fold) and several mutations are required for development of high-level drug resistance. Accumulation of several mutations can yield decreases in susceptibility of up to 100 fold. Another mechanism affecting drug resistance involves mutations in the cleavage sites, lying outside the protease gene. Resistance-associated mutations in the protease gene may impair the efficiency of cleaving the polyprotein, and the cleavage site mutations compensate for this loss of activity. Hence cleavage site mutations are referred to as compensatory mutations. PIs are the only drug class for which resistance-associated amino acid substitutions external to the target protein have been identified.⁹³

In short, a single point mutation is sufficient to cause high-level NNRTI resistance. Conversely, resistance to PIs or to NRTIs (with exception of lamivudine) requires the selection of multiple resistance mutations and more time to evolve. Hence identification and development of

antiretroviral drug combinations that have a high pharmacokinetic and genetic barrier to resistance are important in the long-term management of HIV.

Managing ART failure:

When a patient fails a first-line regimen of 2 NRTIs+ 1 NNRTI, a PI-based regimen is recommended. PI-based regimen is recommended to be boosted with ritonavir (except nelfinavir). A potent boosted PI needs to be given with 2 NRTIs to make second-line regimen more robust. Three boosted PIs are currently available in India: lopinavir/ritonavir (LPV/r), saquinavir/ritonavir (SQV/r) and indinavir/ritonavir (IDV/r). LPV/r has better antiretroviral effects as compared to (SQV/r) and (IDV/r).²⁴

Other therapies:

To date, several immune-based approaches have also been investigated to complement antiretroviral therapy, to name a few: Cytokines such as Interleukin IL-2, IL-12, IL-7 and IL-15, Immunosuppressive drugs such as corticosteroids, hydroxyurea, cyclosporin A, thalidomide and related compounds that selectively target HIV-specific immunity such as tucarestat and murabutide. However, no clear evidence has yet emerged on the clinical impact of immunotherapy in management of HIV/AIDS.⁹⁴

Several approaches such as subunit vaccines, whole virus (killed) vaccines, attenuated virus vaccines and recombinant vaccines have been tried in the extensive effort to develop an effective AIDS vaccine. However, development has been hampered by extensive antigenic variation exhibited by HIV, the ability of HIV to exist as a provirus in host

cells, where it is inaccessible to the immune system and the lack of good animal model for AIDS.^{95,96}

In view of magnitude of AIDS pandemic, absence of protective vaccine, paucity of non-toxic therapy and resistance developed by the virus to different anti-HIV therapeutic drugs, there is urgent need for the development of new, specific and non-toxic drugs. To obtain lead molecules for this drug development, a number of plant products are being screened all over the world by scientists. Many compounds of plant origin such as alkaloids, carbohydrates, coumarins, flavonoids, tannins, lignins, lipids, proteins, terpenoids etc. have been identified that are reported to inhibit different stages in replicative cycle of HIV. Some of these plant-derived anti-HIV compounds like glycyrrhizin, papaverine, tricosanthin, castanospermin etc have been used in a limited number of patients suffering from AIDS.

Also, a pharmaceutical preparation containing extracts of *Terminalia arjuna*, *Phellodendron amurense*, *Terminalia chebula*, *Rhus javanica*, *Punica granatum*, *Geum japonicum*, *Syzygium aromaticum* and *Paeonia suffruticosa* or *Alpinia officinarum* for the treatment of AIDS has been patented.¹

Besides, promising activities have been shown by 3 natural product derived candidates in preclinical and early clinical trials. Sarawati MediChem Pharmaceuticals currently started phase II clinical trials of calanolide A for assessment of long-term anti-HIV activity of calanolide in combination with other anti-HIV agents. Another 2 lead molecules

which are licensed to Panacos Pharmaceuticals, 3-hydroxymethyl-4-methyl DCK (PA-334B) and DSB (PA-457), have also successfully completed preclinical studies. Recently Panacos has started phase I clinical studies of PA-457. These 3 clinical candidates have the potential to come up as drugs for treatment of HIV infection.⁹⁷

Some more plant products are in clinical trials and many more hope to follow this to give new drugs and lead molecules for drug-development for treatment of HIV infection.

For the present *in vitro* study, the plants were selected on citations in the literature on Indian medicinal plants and also those commonly used in folk medicine. Leaves powder of *Ocimum sanctum* (Tulas), Root powder of *Withania somnifera* (Ashwagandha), stem powder of *Tinospora cordifolia* (Gulvel) and the two mangrove species namely *Avicennia officinalis* and *Rhizophora mucronata* were used for the analysis. Shilajit, a herbomineral was also included for the *in vitro* study.

REVIEW
OF
LITERATURE

Many natural products are biologically active and have been used by man for thousands of years as traditional medicines. They have played a significant role as sources for lead compounds during the last century. Natural products based drugs derived from terrestrial origin are worth over \$ 25 billion annually. About 61% of 877 small molecules introduced worldwide during 1981-2002 can be traced to, or were inspired by natural products research, while approximately 30% of the best selling small molecules were either derived from or developed through a study of their resources. Presently, about 70% of antimicrobial and anticancer drugs approved for human use which originate from natural products alone or their analogues.⁹⁸

History:

The use of plants as medicine goes back to early man. The earliest known medical document is a 4000-year-old Sumerian clay tablet that records plant remedies for various illnesses. By the time of ancient Egyptian civilization, a great wealth of information already existed on medicinal plants. Among the many remedies prescribed were mandrake for pain relief and garlic for treatment of heart and circulatory disorders. This information, along with hundreds of other remedies, was preserved in the Ebers papyrus about 3500 years ago.

Ancient China is also a source of information about the early medicinal use of plants. The *Pun-tsao*, pharmacopoeia that was actually published around 1600, contained thousands of herbal cures that are attributed to the works of Shen-nung, China's legendary emperor who lived over 4000 years ago.

In India, herbal medicine dates back several thousand years to the Rig Veda, the collection of Hindu sacred verses. This has led to a system of health care known as Ayurvedic medicine. One useful plant from this body of knowledge is snakeroot, *Rauwolfia serpentina*, used for centuries for its sedative effects. Today the active components in snakeroot are widely used in Western medicine to treat high blood pressure.

Western medicine can be traced back to the Greek physician Hippocrates (460-377 BC), known as the Father of Medicine who used various herbal remedies in his treatments. Early Roman writing also influenced the development of Western medicine, especially the works of Dioscorides, a Roman military physician (1st century AD). He compiled information on over 600 plant species with medicinal value in *De Materia Medica*. It included descriptions and illustrations of the plants, along with directions on the preparation, uses and side effects of the drugs. After the arrival of Columbus, many New World plants became available to Europeans, and by the time of Henry VIII in England (1491-1547), an entire European or Western medical system that blended plant use and astrology had developed. For centuries, medicine in the West meant herbal medicine.

William Withering was the first in the medical field to scientifically investigate a folk remedy. His studies (1775-1785) of foxglove as a treatment for dropsy (congestive heart failure) set the standard for pharmaceutical chemistry.

In the nineteenth century, scientists began purifying the active extracts from medicinal plants. One breakthrough in pharmaceutical chemistry came when Friedrich Serturner isolated morphine from opium poppy (*Papaver somniferum*) in 1806. Continuing this progress, Justus von

Liebig, a German scientist became a leader in pioneering the field of pharmacology. With increased knowledge of active chemical ingredients, the first purely synthetic drugs based on natural products were formulated in the middle of the nineteenth century. In 1839, salicylic acid was identified as the active ingredient in a number of plants known for their pain-relieving qualities and was first synthesized in 1853. This led to the development of aspirin, which is the most widely used synthetic drug today.⁹⁹

Many such similar developments followed. Quinine from cinchona bark had its origin in the royal households of the South American Incas. In 1860, a German chemist Carl Koller isolated cocaine, the chemical responsible for the biological activity. As a local anaesthetic, it revolutionized several surgical and dental procedures. The jaborandi tree (*Pilocarpus jaborandi*) secretes alkaline-rich oil. Several substances are extracted from this aromatic oil, including the alkaloid pilocarpine, a weapon against the blinding disease, glaucoma. American Indians on the island of Guadeloupe used pineapple (*Ananas comosus*) poultices to reduce inflammation in wounds and other skin injuries, to aid digestion and to cure stomachache. In 1891, an enzyme that broke down proteins (bromelain) was isolated from fresh juice of pineapple and was found to break down blood clots. Other pharmaceuticals that have their origin in botanicals include atropine, hyoscine, digoxin, colchicine and emetine. Reserpine, an anti-hypertensive alkaloid (*Rauwolfia serpentina*) became available as a result of work carried out by Ciba-Geigy in India.¹⁰⁰

Why Herbal Medicine? :

Medicinal plants are important for pharmacological research and drug development not only as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. The process of synthetic drug discovery and development takes an average of 12 years, and any new drug requires the investment of an average of US\$ 230 million. It is seen that plant-based drugs take a comparatively much less time and expenses than synthetic drugs. Hence plant-based medicines would be cheaper. The worldwide market of herbal medicines is of the order of US\$ 60 billion to US\$ 80 billion and India is the second largest exporter of medicinal plants.¹⁰¹

Traditional medicine has served as a source of alternative medicine, new pharmaceuticals, and healthcare products. A number of complementary and alternative systems are popular in India, like Ayurveda, Homeopathy, Unani and Siddha, with Ayurveda being the most popular.¹⁰² Ayurveda is a traditional Indian medicinal system being practiced for decades. The ayurvedic knowledge database allows drug researchers to start from a well-tested and safe botanical material. Thus, the normal drug discovery course of 'laboratories to clinics' actually becomes from 'clinics to laboratories'- a true reverse pharmacology approach. Considerable research on pharmacognosy, phytochemistry, pharmacology and clinical therapeutics has been carried out on ayurvedic medicinal plants. Numerous molecules have come out of ayurvedic experimental base, examples include psoralens in vitiligo, holarrhena alkaloids in amoebiasis, guggulsterons as hypolipidemic agents, mucuna pruriens for Parkinson's disease, piperidines as bioavailability enhancers, baccosides in menta

retension, picrosides in hepatic protection, phyllanthins as antivirals, withanolides and many other steroidal lactones and glycosides as immunomodulators.³ It is documented that 80% of the world's population has faith in traditional medicine, particularly plant drugs for their primary healthcare because of better cultural acceptability, better compatibility with the human body and lesser side effects.¹⁰³

Herbal medicine scenario in India:

India is sitting on a gold mine of well-recorded and traditionally well-practiced knowledge of herbal medicine. This country is perhaps the largest producer of medicinal herbs and is rightly called botanical garden of the world.¹⁰⁴

India has progressive research institutes like Central Drug Research Institute (CDRI), Central Institute of Medicinal and Aromatic Plants and National Botanical Research Institute at Lucknow, Regional Research Laboratories (RRL) at Jammu, Bhubaneswar and Jorhat, National Chemical Laboratory (NCL) at Pune, which routinely undertake research on medicinal plants. Most of them are involved in standardizing the herbal medicines and isolating active compounds.

Reserpine (antihypertensive from *Rauwolfia*) is an extremely valuable contribution from Ayurvedic systems. Curcumin (anti-inflammatory from turmeric), Withaferin A (anti-inflammatory from ashwagandha), Kutkoside (hepatoprotective from kutki), Andrographolide (hepatoprotective from *andrographis*) and Vasicine (bronchodilator and expectorant from *vasaka*) are chemical entities with attractive scaffolds for drug discovery.

Ayurvedic preparations have been successfully evaluated for treatment of bronchial asthma, rheumatoid arthritis, and ischaemic heart disease. Piperine from pipali has come out as a bioenhancer in recent clinical evaluation. Botanicals like *Withania somnifera*, *Asparagus racemosus* have exhibited significant vaccine adjuvant activity in experimental systems, which have valuable applications in immunobiological industry. An IND application of Lupin Ltd. is in process and a US patent has been granted for development of herbal-based anti-psoriatic composition containing *Argemone mexicana*. Standardized fraction of guggulipid from *Commiphora wightii* developed by CDRI has been marketed (Guglip[®], Cipla Ltd.) for treating hyperlipidemia and atherosclerosis. RRL Jammu has commercialized *Boswellia serrata* gum resin as NSAID (non-Steroidal Anti-Inflammatory Drug) (Sallaki[®], Gufic). It is also hypolipidemic. A multicentric study by ICMR showed that a preparation from *Pterocarpus marsupium* was effective in reducing levels of blood glucose and glycosylated haemoglobin in patients with non-insulin-dependent diabetes mellitus. Analysis of most frequently used plant-based therapies in Ayurvedic system revealed that 43% of them have been tested on humans while 62% have been the subject of one or more animal studies. Among these drugs having sufficient clinical data are guggul, brahmi, ashwagandha, amlaki, guduchi, kutki, shatavari and shunthi.¹⁰⁵ Nicolas Piramal India Ltd. (NPIL) has build up a plant extract library having 6000 extracts prepared from around 2300 plant species collected from all over India. Such libraries could serve as a powerful tool and source of extracts to be screened for biological activities using high-throughput assays.¹⁰⁶ Some of the prominent commercial plant-derived medicinal compounds

include: colchicum, colchicine, betulinic acid, camptothecin, topotecan (Hycamtin®), CPT-11 (irinotecan, Camptosar®), 9-aminocamptothecin, delta-9-tetrahydrocannabinol (dronabinol, Marinol®), beta lapachone, lapachol, podophyllotoxin, etoposide, podophyllinic acid, vinblastine (Velban®), vincristine (leurocristine, Oncovin®), vindesine (Eldisine®), Fildesin®, vinorelbine (Navelbine®), docetaxel (Taxotere®), paclitaxel (Taxol®), tubocurarine, pilocarpine and scopolamine.¹⁰⁰

Antiviral activity of Herbal Medicine:

Many traditional medicinal plants have been reported to have strong antiviral activity and some of them have already been used to treat animals and people who suffer from viral infection. Research interests for antiviral agent development was started after the Second World War in Europe and in 1952 the Boots drug company at Nottingham, England examined the action of 288 plants against influenza A virus in embryonated eggs. They found that 12 of them suppressed virus amplification. During the last 25 years, there have been numerous broad-based screening programmes initiated in different parts of the globe to evaluate the antiviral activity of medicinal plants for *in vitro* and *in vivo* assays. Recently, considerable attention has been given to screening various species of medicinal plant extracts for possible anti-HIV activity.¹⁰¹

Traditional Medicine and HIV/AIDS:

HIV/AIDS has proved to be a devastating condition, as it affects the most productive segment of the population. This has promoted researchers to

share data on traditional medicine (plants) for AIDS management, as “Herbal Renaissance” is blooming across the world.

Au et al¹⁰⁸ screened the aqueous and methanol extracts of 20 herbs traditionally used in Chinese medicine for anti-HIV-1 integrase activity. It was found that most of the extracts showed strong inhibition of HIV-1 integrase. The inhibition was likely due to tannins and polyphenolics in the herbal extracts.

Similarly, extracts of 27 Chinese medicinal plants were tested for inhibitory activity against HIV in H9 cells. Eleven of the 27 extracts were found to be active.¹⁰⁹

In another study, Hussein et al¹¹⁰ screened 48 methanol and aqueous extracts from Sudanese medicinal plants for their inhibitory activity on viral replication. Nineteen extracts showed inhibitory effects on HIV-induced cytopathic effects (CPE) on MT-4 cells. The extracts were further screened against HIV-1 protease (PR) using an HPLC assay method. Of the tested extracts, the methanol extracts of *Acacia nilotica* (bark and pods), *Euphorbia granulata* (leaves), *Maytenus senegalensis* (stem-bark) and aqueous extracts of *A. nilotica* (pods) and *M. senegalensis* (stem-bark) showed considerable inhibitory effects against HIV-1 PR.

Bessong et al¹¹¹ evaluated 17 aqueous and methanol extracts from 9 South African medicinal plants for inhibitory properties against HIV-1 reverse transcriptase (RT). Isolated compounds were additionally evaluated on HIV-1 integrase (IN). The strongest inhibition against the RNA-dependent-DNA polymerase (RDDP) activity of RT was observed with the methanol extract of the stem-bark of *Peltophorum africanum* Sond., while the methanol extract of the roots of *Combretum molle* R.Br. ex G.

Don was the most inhibitory on the ribonuclease H (RNase H) activity. Gallotannin, bergenin and catechin were isolated from the methanol extract of the roots and stem-bark of *Peltophorum africanum*. The gallotannin inhibited the RDDP and RNase H functions of RT and also showed inhibitory activity against HIV-1 IN. Catechin showed no effect on RT but had a moderate activity on HIV-1 IN. Whereas bergenin was inactive on both enzymes.

Besides, Matsuse et al¹¹² tested Aqueous and methanolic extracts of 39 Panamanian medicinal plants for anti-HIV effects. The extracts were tested for the inhibition of HIV-induced cytopathic effects in cultured cells, HIV-reverse transcriptase (RT) and HIV-protease (PR) enzymes. The water extract of the branches of *Jatropha curcas* inhibited strongly the HIV-induced cytopathic effects with low cytotoxicity. On the other hand, the water extracts of the whole plant of *Chamaesyce hyssopifolia*, the leaves of *Cordia spinescens* and the aerial parts of *Hyptis lantanifolia*, and the methanol extract of the aerial parts of *Tetrapteris macrocarpa* were potent inhibitors of HIV-RT. Seven out of 39 plants were found to be moderate inhibitors of HIV-PR.

In another study, Min et al¹¹³ screened 49 methanol extracts of Korean Medicinal plants for their inhibitory activities against HIV-1 RT and HIV-protease. Regarding the HIV-1 reverse transcriptase, *Agrimonia pilosa* (whole plant), *Cornus kousa* (stem and leaf), *Limonium tetragonum* (root) and *Mallotus japonicus* (stem) showed significant inhibitory activity on RT. Whereas 4 plants, namely *Agrimonia pilosa* (whole plant), *Atractylodes japonica* (root), *Clematis heracleifolia* (whole plant) and *Syneilesis*

palmata (whole plant), were appreciably active against recombinant HIV-1 protease.

India's Opportunity:

There is a need to unleash a total war on AIDS and a wonderful weapon for it in India would be traditional medicine in combination with modern medicine and molecular biology. Ayurveda remains one of the most ancient medical systems widely used in India. It aims at holistic management of health and disease and has a sound philosophical and experimental basis.¹¹⁴

Plants form a dominant part of Ayurvedic pharmacopoeia. There are 2 ways in which AIDS can be managed by plants aid. The first one is direct HIV virus in-activators such as *Punica granatum* (Dalimba), *Glycyrrhiza glabra* (Yashtimadhu), *Azadirchta indica* (neem) and *Grifula frondosa*.¹¹⁵ The other approach is immunity promoters such as tubers of *Asparagus racemosus*, roots of *Withania somnifera*, stems of *Tinospora cordifolia* and tubers of *Ipomoea mauritiana*.¹¹⁶

Apart from antiviral effect, traditional medicine is also used to treat opportunistic infections, neoplasm etc. and to obtain relief from the side-effects of conventional retroviral drugs.

It remains the main healing system for millions of people in India, where Ayurvedic practioners are treating more and more HIV-positive patients. The Government of Gujarat has established 2 centers for the treatment of AIDS using Ayurvedic principles. One centre is in Surat and the other in Jamnagar. In both these places practioners of modern medicine observe and clinically examine the patients. However, the treatment is given

according to Ayurvedic principles and the experience so far has been very encouraging. Also, a clinical study entitled "Role of Ayurvedic drugs in HIV treatment: A preliminary study on HIV-positive patients" has been persuaded at Pune. The study has revealed statistically significant weight gain in the treated group as compared to the control group.¹¹⁵ Thus opportunities for developing modern phytomedicines based on leads from Ayurveda are indeed vast.

Interpretation of HIV/AIDS as Ojakshay in Ayurveda:

In an ancient Ayurvedic text, Madhana Nidan, written around 700 A.D., the author, Madhavakara foretells a disease that will come to India. From its description, the disease appears to be HIV/AIDS. Its cure was said to be Shilajit.¹¹⁷

Causes:

The main cause is deficient life-sap (ojas), which causes an extremely weakened immune system. Ojas is the final vital component from the diet. There are eight drops of this ojas that help the heart in its regular functioning and vitality. One ounce of ojas goes to the brain and regularizes the brain activity and the remaining goes to the rest of the body. In this way, all the activities can be performed properly and gracefully.¹¹⁵ It is this sap, that covers and protects the immune system from harm. When some foreign body attacks the immune system, the ojas or life-sap covers, protects and eventually repairs the damage. Ojas is lost or diminished by excess sex, improper diet, junk food, drugs, excess worry, thinking and insomnia as they can dry or burn up ojas.¹¹⁷

Symptoms:

Vayu and Pitta are primary factors, but Kapha may represent symptoms of congestion and lung disorders.

Therapies:

1. The main herb suggested in Madhan Nidan is Shilajit (tridoshic-for all doshas). It boosts the immune system (ojas) and is antiviral.
2. Vayu / Immune boosting herbs: Shatavari, Ashwagandha, Kapikachhu and Chyavan prash build the ojas and balance Vayu.
3. Pitta reducing herbs:
 - a) Blood purifiers: Gotu kola, Manjishtha, Turmeric
 - b) Liver cleansers: Gotu Kola, Bhringaraj, Musta and Sandalwood (Antibacterial/ antiseptic)
 - c) Gokshura may be used to treat burning or infections of the urinary tract. It is also good for seminal debility and nerve disorders.
 - d) Herbal antibiotics like Guduchi and Turmeric can also be used to treat infections.

4. Kapha reducing herbs: hot spices are used to treat congestion.

Ayurveda also mentions importance of diet. A Vayu and Pitta-reducing diet and lifestyle are required; spicy, sour, bitter and astringent tastes are not recommended. Foods that are wholesome, pure (Sattwic) and ojas building are suggested. For example, boiled milk, yogurt, ghee, whole cane sugar, maple syrup and raw honey, almonds and sesame seeds.¹¹⁷ Charaka gives a very elaborate description of ghee. According to him, when a drug has proved ineffective in certain conditions, it will prove efficacious if prepared with ghee.¹¹⁵

Besides, the gold ash formula, Suvarna Vasant Malti is said to be excellent for all immune disorders. For AIDS, 2 pills, one in the morning with half tsp. honey and one in the evening is recommended.¹¹⁷ As gold is not only a medicine in itself but also makes other medicines doubly effective if prescribed in combination.¹¹⁵ Thus Ayurveda can play a significant role in HIV therapy.

In general, Ayurveda advocates the Rasayana group of drugs for staying healthy and disease free. Rasayanas are the preparations made either from a single plant or a combination of several plants are well known for their immunomodulatory properties.

Effect of Immunomodulatory agents:

Immunomodulatory agents can enhance or inhibit the immunological responsiveness of an organism by interfering with its regulatory mechanisms. These may be antigen independent and may directly induce production of mediators and effector molecules by the immunocompetent cells. The primary target of immunomodulatory compounds is believed to be the macrophages, which play a key role in the generation of an immune response. The activated macrophages display not only increased phagocytosis and intra-cellular killing of pathogens by producing effector molecules like free radicals and nitric oxide, but also produce cytokines like tumor necrosis factor (TNF)- α and interleukin (IL)-1, IL-6, IL-12 etc. these cytokines may in turn activate T cells or NK cells.

The immunomodulatory agents may selectively activate either cell mediated or humoral immunity by stimulating either Th1 or Th2 type of

cell response, respectively. It is now realized that enhancement of Th1 type of T-cell response may be of therapeutic significance for a variety of intracellular pathogens, like mycobacteria, protozoan parasites and viruses (HIV); whereas Th2 type of response may be beneficial against extracellular pathogens. Ayurvedic rasayana drugs may play major role in this regard. Rasayana therapy is a multidimensional curative treatment which at molecular level, acts as anti-inflammatory, oxidative immunomodulatory agent and as an anti-glycosylation end-product.¹¹⁸

Rasayana Therapy:

An entire section of Materia Medica of Ayurveda termed 'Rasayana' is devoted to enhancement of body's resistance. In practice, this is achieved by using extracts of various plant materials called rasayanas. In terms of Charaka, by using rasayana therapy, one attains youth, gets sharp memory and intellect, freedom from diseases, lustrous complexion and the 'strength of horse'.

At the root of Ayurveda is the connection between macrocosm and the microcosm. The universe is made up of five elements: earth, air, fire, water and space (or ether). Human beings are composed of those same elements. These 5 elements interact and in humans, they occur as the three doshas (Vata, Pitta and Kapha), each with specific characteristics and functions. According to Ayurvedic theory, a harmonious balance between these 3 humors of the body is needed for positive health; imbalance of these may cause disease(s).

Vata means 'wind' and this dosha is composed of the elements air and space. Disruption in Vata occurs as gas leading to pain. Vata is located in

the brain, heart, ears, bones, lungs, thighs and colon. Pitta means 'bile' and this dosha is composed of the elements fire and water. Disruption in Pitta occurs as acid and bile, leading to inflammation. Pitta is located in liver, spleen, sweat glands, small intestine, blood, lymph system and eyes. Kapha means 'phlegm' and this dosha is composed of the elements earth and water. Disruption of Kapha occurs as liquid and mucus, which lead to swelling and sometimes discharge. Kapha is located in the head, nose, mouth, joints, tongue, throat, lymph system and stomach.

There are 7 dhatus or tissues namely, rasa (plasma and lymph), rakta (blood), mamsa (muscle), meda (fat), asthi (bone), majja (bone marrow) and shukra (semen and sexual fluids). Formed by assimilation of dietary items, the tissues influence the characteristics and behavior of the doshas. The dhatus are arranged in hierarchical fashion- rasadhatu being the primordial tissue. The rasadhatu has been likened to the plasma. The tissues receive nutrients from the rasadhatu, picking up the components they need and influence the working of subsequent tissues. Drugs that improve the quality of this rasadhatu and thereby of the entire body are Rasayanas.

A list of 34 plants has been identified as Rasayanas in the Ayurvedic system of medicine. Dr. R. N. Chopra has mentioned other 68 plants with similar properties. To name a few: *Allium sativum*, *Aloe vera*, *Asparagus racemosus*, *Azadirachta indica*, *Curcuma longa*, *Tinospora cordifolia*, *Withania somnifera*, *Ocimum sanctum*, *Panax ginseng*, *Picrorhiza kurroa* etc. A herbomineral Shilajit is also considered as Rasayana drug.^{118,119,120} Of these plants, *O. sanctum*, *W. somnifera*, *T. cordifolia* and the herbomineral Shilajit were selected for the present *in vitro* study.

These botanicals have array of compounds such as isoflavonoids, indole, phytosterols, polysaccharides, sesquiterpenes, alkaloids, glucans and tannins with antimicrobial and immunomodulating potential. For example, crude extract of *Tinospora cordifolia* (TC) has immunostimulant, anticancer and cytoprotective activities, where cytoprotective activities are attributed to polysaccharides and immunostimulant activity is attributed to diterpenoids. The possible mechanism of immunomodulatory activity was elucidated as activation of macrophages, leading to increase granulocyte-macrophage colony-stimulating factor (GM-CSF), leading to leukocytosis and improved neutrophil function. Whereas, polysaccharide derived from TC resulted in activation of NK-cells, complement system, Th1-pathway cytokines, coupled with low nitric oxide synthesis.¹²¹

In short, botanical immunodrugs could provide a unique opportunity to bioprospect diverse and synergistic chemical moieties, which combination might act on multiple targets and improve the therapeutic spectrum.

Mangrove Plants:

Besides medicinal plants, Mangrove plants are also used in folkloric medicine for treatment of several diseases.¹²² "Mangrove" is a combination of the Portuguese word "*Mangue*" and English word "grove". Mangroves were first mentioned in 325 BC, by the Greek explorer Nearchus.¹ Mangroves occur in 121 countries covering 15 million ha worldwide. As of 2005, India harbors the largest mangroves in the world and India alone contributes for 3% of the global mangrove habitat.¹²⁴ Sundarbans is the largest mangrove forest in the world. It is shared by both India and Bangladesh.

The Indian part of the Sundarbans is located in the western part of the Ganges-Brahmaputra Delta. India has about 4,482 square km under mangroves. About 59% of this is found along the east coast (Bay of Bengal), 23% occurs on the west coast (Arabian Sea) and 18% is found on the Andaman and Nicobar Islands.¹²³ The Pichavaram mangrove forest near Chidambaram, South India is the second largest mangrove forest in the world.¹²⁵

Mangrove forests include taxonomically diverse plants exhibiting common adaptive features. This diverse group has been categorized as true mangroves and mangrove associates. Mangrove associated fauna include invertebrates (belonging to Porifera, Bryozoa, Mollusca, Arthropoda etc.) marine fish, amphibians (frog such as *Rana tigrina*, *R. cyanophlyctis* etc) reptiles (salt-water crocodile, snakes, lizards, turtles and terrapins), birds and mammals (monkeys, flying foxes, fishing cats, Royal Bengal tiger etc.).¹²³

The following listing gives the number of species of mangroves in each listed plant genus and family.

Major components:¹²⁵

Family	Genus, number of species	Common name
Avicenniaceae or Verbanaceae	<i>Avicennia</i> 9	Black mangrove
Rhizophoraceae	<i>Bruguiera</i> 6; <i>Ceriops</i> 2; <i>Kandelia</i> 1; <i>Rhizophora</i> 8	Red mangrove
Combretaceae	<i>Conocarpus</i> 1; <i>Laguncularia</i> 11; <i>Lumnitzera</i> 2; <i>Conocarpus</i> 1; <i>Laguncularia</i> 11; <i>Lumnitzera</i> 2	White and Buttonwood mangrove
Arecaceae	<i>Nypa</i> 1	Mangrove palm
Lythraceae	<i>Sonneratia</i> 5	Mangrove apple

Black mangroves are moderately salt-tolerant. The wood of these trees is dark-brown to nearly black. Red mangroves are highly salt-tolerant plant groups living very close to seawater. White mangroves get their name from their smooth white bark and buttonwood mangroves are shrubby, low branching and multi-trunked. The name is derived from its red-brown cone-like fruits.¹²³

Minor components:¹²⁵

Family	Genus, number of species
Acanthaceae	<i>Acanthus</i> , 1; <i>Bravaisia</i> , 2
Bombacaceae	<i>Camptostemon</i> , 2
Cyperaceae	<i>Fimbristylis</i> , 1
Euphorbiaceae	<i>Excoecaria</i> , 2
Lecythidaceae	<i>Barringtonia</i> , 6
Lythraceae	<i>Pemphis</i> , 1
Meliaceae	<i>Xylocarpus</i> , 2
Myrsinaceae	<i>Aegiceras</i> , 2
Myrtaceae	<i>Osbornia</i> , 1
Pellicieraceae	<i>Pelliciera</i> , 1
Plumbaginaceae	<i>Aegialitis</i> , 2
Pteridaceae	<i>Acrostichum</i> , 3
Rubiaceae	<i>Scyphiphora</i> , 1
Sterculiaceae	<i>Heritiera</i> , 3

Thus the term mangrove is used to designate halophytic (salt loving) and salt-resistant marine tidal forests comprising of trees, shrubs, palms and ground ferns. Mangroves are highly productive ecosystem with various important economic and environmental functions. The uses of mangrove fall in two major categories: Firstly, the indirect use of mangrove ecosystem in the form of vital ecological functions such as control coastal erosion and protection of coastal land, stabilization of sediment and natural purification of coastal water from pollution. Secondly, the

economic benefits which are many and varied. Apart from prawn fisheries many other species of economic importance are associated with mangroves. These include crabs, shrimp, oysters, lobsters and fish. Traditionally, mangroves have been exploited for firewood and charcoal and their uses include construction of dwellings, furniture, boats and fishing gear around the Indian Ocean and everywhere in the Far East. They are the important source of tannins for dyeing, leather production and oil drilling.

Two basic factors justify the study of chemical constituents of mangrove plants. Firstly, mangroves are one of the easiest tropical forest types to grow. They have the ability to grow where no other vascular plant can. They exist under stressful conditions such as saline environment, high concentration of moisture, high and low tides of water and abundant living microorganisms and insects. These have imposed several modifications in these plants to establish water and salt economy. There are modifications in other physiological processes such as carbohydrate metabolism or polyphenol synthesis and due to these reasons, they may have chemical compounds, which protect them from these destructive elements. The second reason is that recently extracts from mangroves have proven activity against human, animal and plant pathogens.¹²⁶

Apart from this, numerous bioactive compounds are derived from mangroves. Skin disorders and sores, including Leprosy, may be treated with ashes and bark infusions of certain species. Headaches, rheumatism, snakebites, boils, ulcers, diarrhea, hemorrhages and many medical conditions are traditionally treated with mangrove plants.¹²⁷ Also extracts from mangrove plants such as *Aegiceras corniculatum*, *Ceriops decandra*

Excoecaria gallocha, *Rhizophora apiculata* and *Rhizophora lamarckii* have been tested for *in vitro* anti-HIV activity.¹²⁸ Besides, Premnathan et al¹²⁹ tested extracts of seaweeds, seagrasses and mangroves from the southeast coast of India for *in vitro* antiviral activity against Newcastle disease, vaccinia, Semliki Forest, encephalomyocarditis and hepatitis viruses. Among them 2 mangrove plant extracts, leaf of *Bruguiera cylindrica* and bark of *Rhizophora mucronata* showed activity against 2 of the viruses tested.

Drugs of Herbomineral origin:

Even though, majority of natural drugs are derived from plant and animal origins, a few of them, obtained from mineral sources, are of paramount significance as pharmaceutical aids. The drugs of herbo-mineral origin like Shilajit are very effective tonics and stimulants.¹³⁰ It has been mentioned in *Sushrut Samhita* that in the month of May-June the sap or latex juice of the plant emerges as a gummy exudation from the rocks of mountains due to the strong heat of the sun. The Sanskrit meaning of Shilajit is 'Conqueror of mountain and destroyer of weakness'. There are number of hypotheses about the origin of Shilajit. One such research claims that the bryophytes present in the vicinity of Shilajit-exuding rocks are responsible for the formation of Shilajit. The bryophytes reveal the occurrence of minerals and metals in their tissues such as copper, silver, zinc, iron, lead etc. which are similar to the elements present in Shilajit. Shilajit has been in use as a folk medicine over 3000 years as a rejuvenator and adaptogen. It has been said that there is hardly any curable disease that can not be controlled or cured with the aid of shilajit. It is perhaps the best rasayan

Ayurveda has prescribed. There are 4 different varieties of shilajit which have been described in *Charaka Samhita*, namely savarna, rajat, tamra and lauha shilajit. Savarna is a gold shilajit and is red in color. Tamra is copper shilajit and is blue in color. Rajat is a silver shilajit and is white in color while the lauha shilajit is an iron-containing shilajit and is brownish black in color. Tamra and savarna shilajit are not found commonly but the last variety, i.e. lauha shilajit is commonly found in Himalayan ranges and is supposed to be the most effective according to the therapeutic point of view. In traditional medicine, it is prescribed to treat genitourinary disorder, jaundice, gallstone, kidney stones, digestive disorders, enlarged spleen, epilepsy, nervous disorder, chronic bronchitis and anemia. Modern scientific research has also systematically validated a number of properties of shilajit and has proven that shilajit is truly a panacea of oriental medicine.^{131,132}

Molecular targets of Antiretroviral Therapy (ART):

The replication cycle of HIV comprises a number of steps that have been considered adequate targets for chemotherapeutic interventions. Some of the prominent targets are,

❖ Entry Inhibitors:

HIV is an enveloped virus and its envelope protein complex (Env) controls the key process of viral entry. Env is a complex composed of transmembrane gp41 subunit and a non-covalently associated surface gp120 subunit. Infection is initiated by the binding of the virion gp120 Envelope protein to CD4 molecule present on some T-cells, macrophages and microglial cells. This interaction induces a conformational change that

promotes secondary gp120 binding to the co-receptor CCR5 or CXCR4. CCR5 is the co-receptor for HIV-1 strains that infect macrophages (Mac-tropic or R5 strains), while CXCR4 is the co-receptor for HIV-1 strains that infect T-cells (T-tropic or X4 strains). Subsequently, gp41 undergoes conformational changes that mediate fusion of the viral membrane with the target cell membrane. HIV entry is therefore a complex process involving multiple protein interactions, each of which can become potential target for the development of new HIV entry blockers.

Sulfated polysaccharides are potent inhibitors of HIV-1 and HIV-2 replication *in vitro*. They exert their anti-HIV activity by shielding off the positively charged amino acids in the V3 loop of the viral envelope glycoprotein gp120. The V3 loop is necessary for virus attachment to the cell surface heparan sulphate, a primary binding site, before more specific binding occurs to the CD4 receptor of CD4⁺ cells.¹³³

The non-plant origin inhibitors include heparin, dextran, dextrin sulfate, pentosan polysulfate and mannan sulfate. They inhibit virus adsorption to the cells and thus inhibit syncytium (giant cell) formation between HIV-1 infected and normal CD4⁺ T-cells.

The mannose-specific plant lectins from *Cymbidium* hybrid, *Epipactis helleborine*, *Hippeastrum* hybrid and *Listeria ovata* and the N-acetylglucosamine-specific plant lectins from *Urtica dioica* likely interfere with the virus-cell fusion process and it is assumed that they interact with specific glycosylation sites within the viral glycoproteins gp120 and/or gp41, particularly those sites that are rich in mannose or N-acetylglucose.^{134,135}

❖ **Reverse Transcriptase Inhibitors:**

Once HIV enters the cell, the enzymes within the nucleoprotein complex are activated and begin the viral reproductive cycle. The HIV-reverse transcriptase (RT) controls 3 consecutive functions, i.e., RNA transcriptase to DNA, degradation of RNA template by RNase H and duplication of the remaining DNA strand. Hence RT activity is considered as one of the most important targets.

Hydroxymaprounic acid isolated from *Maprounea Africana*¹³⁶, Caffeic acid isolated from *Hyssop officinalis*¹³⁷ and Swertifrancheside isolated from *Swertia franchetiana* have exhibited HIV-RT inhibitory activity.¹³⁸

❖ **Integrase Inhibitors:**

The enzyme integrase is considered as an attractive target as it catalyzes the integration of viral DNA into host DNA.

Potential integrase inhibitors may include Curcumin isolated from *Curcuma longa*¹³⁹, Quercetin isolated from *Quercus rubra*¹⁴⁰ and a protein GAP-31 isolated from *Gelonium multiflorum*.¹⁴¹

❖ **Protease inhibitors:**

HIV protease is responsible for cleavage of the gag and gag-pol polyprotein products, yielding the functional core proteins and essential enzymes (reverse transcriptase, integrase, protease) required to produce mature HIV. Therefore this enzyme became a promising target for ART.

Extracts of Indonesian and Egyptian folk medicines viz. *Rumex cyperius* (fruits), *Boswellia carterii* (seeds), *Centaurea scoparius* (roots), *Datura stramonium* (seeds), *Nigella sativa* (seeds) and *Terminalia horrida* (resin) are reported to exhibit HIV-PR inhibiting activity.¹⁴²

❖ **Budding (Assembly/Release) Inhibitors:**

Hypericin and pseudohypericin isolated from the genus *Hypericum* are reported to have the capacity to block viral assembly or release or directly inactivate the assembled virions.¹⁴³

Major groups of Anti-HIV compounds from plants:

Plants have an almost limitless ability to synthesize aromatic substances (Phytochemicals) as secondary metabolites that serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. These antimicrobial Phytochemicals, many of which serve as anti-HIV compounds can be divided into following categories,

1. Phenolics and Polyphenols:¹⁴⁴

a) Simple Phenols and Phenolic acids:

Some of the simplest bioactive Phytochemicals consist of a single substituted phenolic ring. The site(s) and number of hydroxyl groups on the phenol group are thought to be related to their relative toxicity to microorganisms. The mechanisms thought to be responsible include, enzyme inhibition by the oxidized compounds possibly through reaction with Sulfhydryl groups or through more nonspecific interactions with proteins.

Gallic acid and Galloyl glucoses isolated from *Terminalia chebula* exhibited HIV integrase inhibitory activity.⁹⁷

b) Quinones:

Quinones are aromatic rings with 2 ketone substitutions. They are known to complex irreversibly with nucleophilic amino acids in proteins, often leading to inactivation of protein and loss of function. Probable targets in

the microbial cell are surface-exposed adhesins, cell wall polypeptides and membrane bound enzymes. Quinines may also render substrates unavailable to the microorganisms. Thus, the potential range of quinone antimicrobial effects is great.

A trimeric naphthoquinone, conocurvone isolated from *Canospermum incurvum* showed potent anti-HIV activity. Conocurvone inhibited cytopathic effects and replication of HIV in T-cells.¹⁴⁵

c) Flavones, Flavonoids and Flavonols:

Flavones are the phenolic structures containing one carbonyl group. The addition of 3-hydroxyl group yields a flavonol, where as Flavonoids occur as a C₆-C₃ unit linked to an aromatic ring. Their activity is probably due to their ability to complex with extracellular and soluble proteins and to complex with bacterial cell walls.

Isolicoflavonol, glycyrrhisflavone and licochalcone isolated from *Glycyrrhiza glabra* have been reported to inhibit HIV cytopathicity and giant cell formation.¹⁴⁶

d) Tannins:

“Tannin” is a general descriptive name for a group of polymeric phenolic substances capable of tanning leather or precipitating gelatin from solution (Astringency). They are divided into 2 groups, hydrolysable and condensed tannins. Hydrolysable tannins are based on gallic acid, while condensed tannins are derived from flavonoid monomers. Many human physiological activities such as stimulation of phagocytic cells, host-mediated tumor activity and a wide range of anti-infective actions have been assigned to tannins. Their mode of antimicrobial action may be

related to their ability to inactivate microbial adhesins, enzymes, cell envelope transport proteins etc.

Methanolic extract obtained from the fruit of *Phyllanthus emblica* has shown inhibition of HIV Reverse Transcriptase (RT) and putranjivain A. Ellagitannin has been isolated as active principle responsible for this activity.¹⁴⁷

e) Coumarins:

Coumarins are phenolic substances made of fused benzene and α -pyrone rings. Their fame has come mainly from their antithrombotic, anti-inflammatory and vasodilatory activities.

Calanolide A, Calanolide B and its dihydroderivative isolated from different species of the genus *Callophyllum* have shown inhibition of HIV replication. All 3 Calanolides inhibited the laboratory adapted HIV-1 variants, the clinical viral isolates inclusive of the diverse clades (A-F syncytium-inducing and non-syncytium inducing isolates and T-tropic and M-tropic isolates. Calanolide A is currently in phase II clinical trial as Nucleoside Reverse Transcriptase Inhibitor (NNRTI).¹⁴⁸

2. Terpenoids and Essential oils:¹⁴⁴

Phenolic compounds possessing a C_3 side chain at lower level of oxidation and containing no oxygen are classified as essential oils. The fragrance of a plant is carried in the essential oil fraction. They are called terpenes, the general chemical structure is $C_{10}H_{16}$ and they occur as diterpene triterpenes, and tetraterpens (C_{20} , C_{30} , C_{40}) as well as hemiterpenes (C_5) and sesquiterpenes (C_{15}). Terpenes or terpenoids are active against

bacteria, fungi, viruses and protozoa. The mechanism of action speculated to involve membrane disruption by the lipophilic compounds. Diterpene lactone, andrographolide isolated from *Andrographis panicula* inhibited cell to cell transmission, viral replication and syncytia formation in HIV infected cells.¹⁴⁹

3. Alkaloids:¹⁴⁴

Heterocyclic nitrogen compounds are called alkaloids. The mechanism of action is attributed to their ability to intercalate with DNA.

Michellamine B isolated from leaves of *Ancistrocladus karupensis* acts both at an early stage of the HIV life cycle by inhibiting Reverse Transcriptase as well as at later stages by inhibiting cellular fusion and syncytium formation.¹⁵⁰

4. Lectins and Polypeptides:¹⁴⁴

They are often positively-charged and contain disulfide bonds. The mechanism of action may be the formation of ion channels in the microbial membrane or competitive inhibition of adhesion of microbial proteins to host polysaccharide receptors.

Seed protein MAP-30 isolated from *Momordica charantia* and protein TA 29 isolated from roots of *Trichosanthes kirilowii* have shown dose dependent inhibition of cell free HIV infection and replication with cytotoxic effects.^{151,152,153}

Figure-1(a): POTENTIAL PLANT-DERIVED ANTI-HIV LEADS^{154,155}

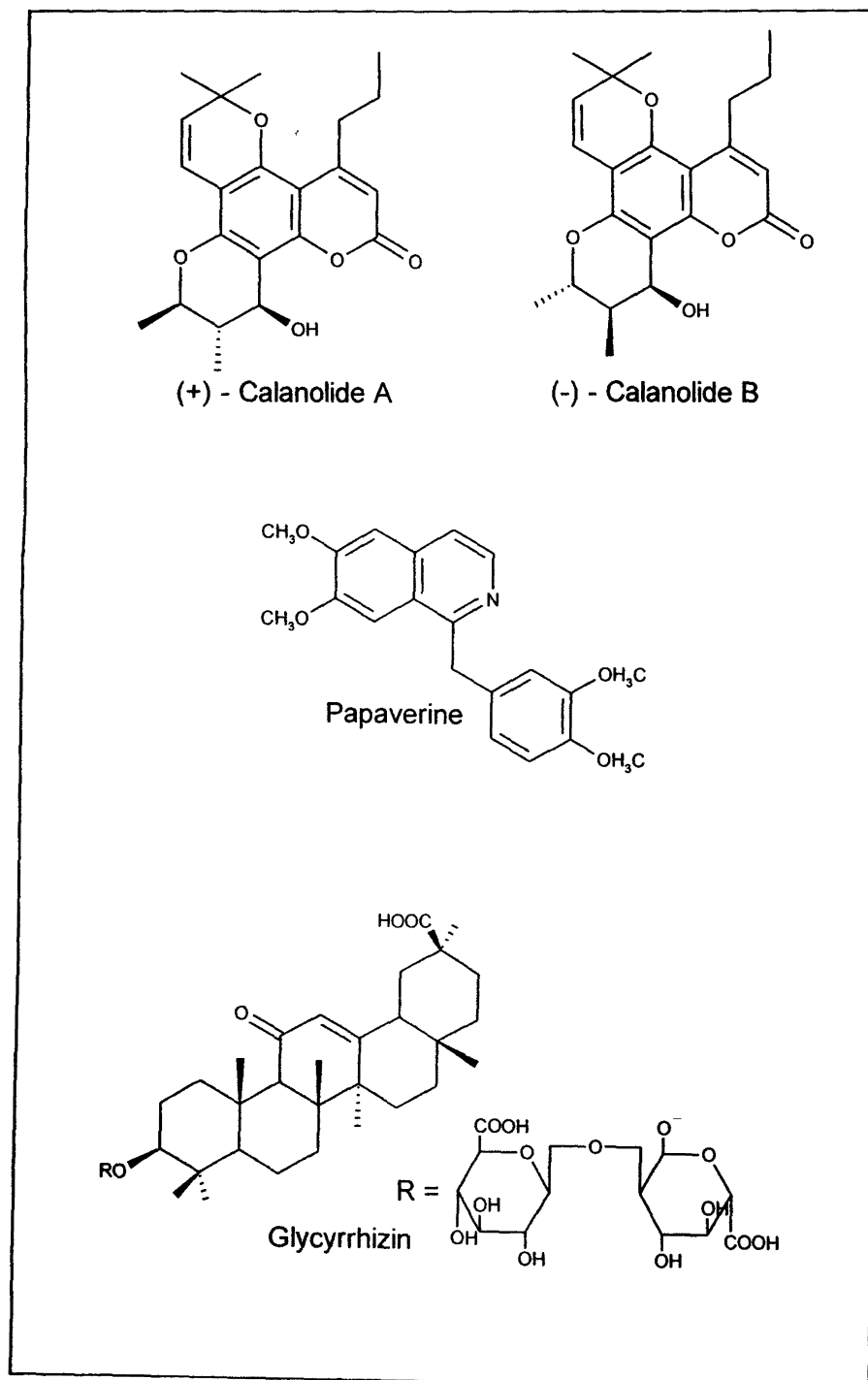
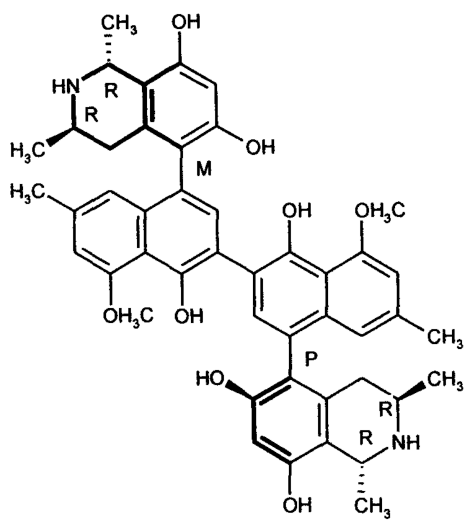
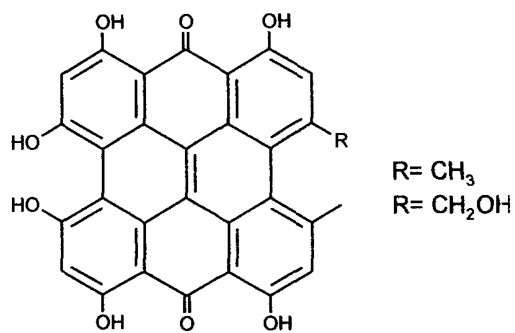


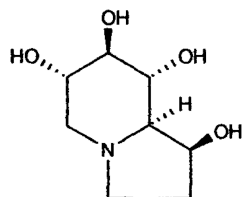
Figure-1(b): POTENTIAL PLANT-DERIVED ANTI-HIV LEADS ^{154,155}



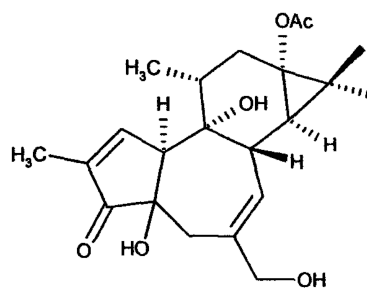
Michellamine B



Hypericin and Pseudohypericin



Castanospermine



Prostratin

There are a number of comprehensive reviews of the more promising anti-HIV compounds discovered in plants.

In one such review, Cos P. et al¹⁵⁶ has categorized anti-HIV phytochemicals or plant extracts according to their putative mechanism of action. According to the review, plant substances can possibly influence the progression of HIV/AIDS in a number of ways, including inhibition of virus entry into the target cell, inhibition of viral enzymes such as Reverse Transcriptase (RT), Integrase and Protease or inhibition of cellular factors such as NF-Kappa B and TNF- α that affect viral replication.

Bharate S.¹⁵⁷ has listed 52 medicinal plants and their products with anti-HIV activity. To name a few: *Andrographis paniculata*, *Annona glabra*, *Croton tiglium*, *Curcuma longa*, *Terminalia bellerica*, *Terminalia chebula* etc.

According to De Clercq¹⁵⁸ the replicative cycle of HIV comprised of ten steps that may be adequate targets for chemotherapeutical intervention. Most of the substances that have been identified as anti-HIV agents can be assigned to one of these 10 classes of HIV inhibitors based on the stage at which they interfere with the HIV replicative cycle.

Another review listed plants with possible anti-HIV activity, their active principles or extracts, the assay used and the mechanism of action. It includes *Areca catechu* (Betel nut) Linn., *Camellia sinensis* Linn., *Papaver somniferum* Linn., *Phyllanthus emblica* Linn. and *Terminalia arjuna*.¹⁵⁹

Singh et al.⁹⁷ has discussed anti-HIV natural products from plants, marine organisms and from microbial origin. The review also included miscellaneous anti-HIV agents such as Bovine milk proteins, Leguminous anti-fungal proteins and Lysozyme. Briefly, bovine milk contains a number

of proteins such as lactoferrin, lactoperoxidase, glycolactin, lactalbumin, lactoglobulin and casein. These proteins inhibited HIV-1 RT, Protease and Integrase to different extents. Lactoferrin has been reported to have inhibitory activity against HIV-1 RT whereas α -lactalbumin, β -lactoglobulin and casein inhibited HIV-1 protease and integrase to an appreciable extent.¹⁶⁰

Anti-fungal proteins are defense molecules produced by diverse organisms including seeds of leguminous plants. Examples include Cowpea β -antifungal protein was found to have high potency in inhibiting HIV-protease and integrase while Cowpea α -antifungal protein was potent in inhibiting HIV-1 RT and integrase.¹⁶¹

Lysozyme is a potent AIDS-fighting protein naturally found in tears, saliva and urine of pregnant women and could help in more effective treatments for AIDS. It has been suspected that lysozyme works with another anti-AIDS protein found in urine called ribonuclease to break down the genetic material of the HIV and prevent the virus from replicating. Lysozyme found in mother's milk, white blood cells and chicken egg white also showed powerful anti-AIDS activity; whereas ribonuclease from pancreas of cows also possesses anti-HIV properties.¹⁶²

Besides, Kusumoto et al.¹⁶³ screened 39 crude drugs used in Indian traditional medicine (Ayurveda) for their inhibitory effects on HIV-protease (PR). The most potent inhibition was shown by *A. catechu* extract, from which some procyanidins were isolated. One of them, Arecatannin B1 showed significant HIV-1 PR inhibitory activity.

In general, as stated by Berghe et al¹⁶⁴, following stages are involved in the development of antiviral agents from plants,

1. Selection, initial collection and identification of plants.
2. Preparation of crude extracts for antiviral screening.
3. Screening of plant extracts for *in vitro* antiviral activity.
4. Bio-assay guided fractionation leading to the isolation of pure active compounds.
5. Determination of the *in vitro* antiviral activity and toxicological evaluation of the pure active compounds.
6. Structure elucidation of active products.
7. Confirmation of antiviral activity by *in vivo* assays.
8. Large-scale re-isolation and/or synthesis. Structure-activity relationship studies and determination of the mode of action of the active product on the biosynthesis of the virus.
9. Classical pharmacological evaluation, formulation and toxicological studies.
10. Clinical trials.

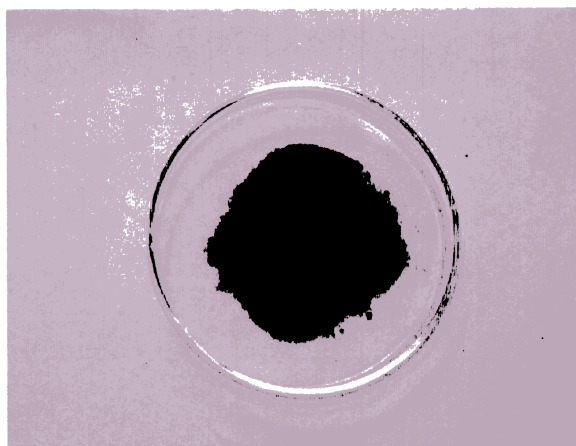
In the present study, we analyzed twenty three extracts prepared from five plants namely *Ocimum sanctum*, *Withania somnifera*, *Tinospora cordifolia*, *Avicennia officinalis*, *Rhizophora mucronata* and Shilajit a herbomineral for their specific inhibitory activity against HIV *in vitro*.

***Ocimum sanctum* Linn.**



Leaves of *O. sanctum*

[Cited: www.dkimages.com]



Leaves powder of *O. sanctum*

Family	Labiatae
Vernacular Name	Tulas, Tulsi
Habitat ¹⁶⁵	This small shrub is found throughout India
Chemical constituents ¹⁶⁶	Volatile and non-volatile compounds. The volatiles are mainly composed of Eugenol, methyl eugenol, carophyllene, pinene, camphene etc. and non-volatiles active are mainly ursolic acid, apigenin and luteolin

Biological activities:**Antimicrobial effects:**

Essential oil of Tulsi has antibacterial, antifungal and antiviral properties. It inhibits the growth of *E. coli*, *B. anthracis*, *M. tuberculosis* etc. Its antitubercular activity is one-tenth the potency of streptomycin and one-fourth that of isoniazid.¹⁶⁷ The efficacy of its crude extract was compared with steroids (dexamethasone) in the treatment with acute viral encephalitis. The survival in the *O. sanctum* treated group was significantly higher ($p < 0.05$).¹⁶⁸

Antimalarial effect:

Essential oil of *O. sanctum* has been reported to possess 100% larvicidal activity against the *Culex* mosquitoes.¹⁶⁷

Antiallergic and Immunomodulatory activity:

Essential oil of *O. sanctum* was found to have anti-allergic properties. When administered to laboratory animals, the compound was found to inhibit mast cell degranulation and histamine release in the presence of allergen. These studies reveal the potential role of *O. sanctum* extracts in the management of immunological disorders including allergies and asthma.¹⁶⁷

Anti-diabetic effect:

A randomized, placebo-controlled cross-over single blind trial on 40 human volunteers suffering from Type II diabetes was performed. During which the 4 week trial, subjects alternatively received a daily dose of 2.5 g of Tulsi leaves powder or a placebo for 2 week periods. The result

showed 17.6% reduction in fasting blood glucose and 7.3% decline in postprandial blood glucose on treatment with Tulsi as compared to the blood glucose levels during treatment with placebo.¹⁶⁷

Anti-stress or Adaptogenic effect:

Adaptogen is a term used to describe agents that increase the non-specific resistance of organisms against a variety of biological, physical and chemical stressors. Extracts from the plant have been found to reduce stress i.e. noise pollution, in rats. The ethanolic extract of *O. sanctum* reversed the changes in plasma levels of corticosterone induced by exposure to both acute and chronic noise-stress, indicating the anti-stress property of the plant against noise.^{167,168}

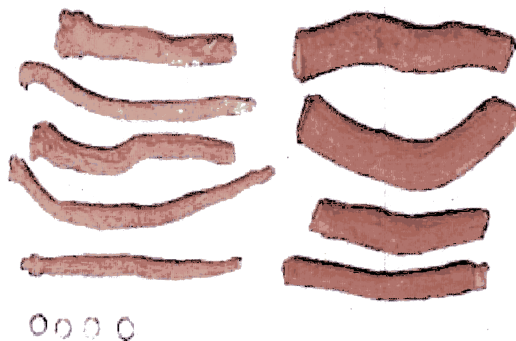
Anti-Cancer activity:

Extract of *O. sanctum* was tested for anti-cancer activity. Though this plant extract did not completely inhibit the development of solid tumor, it induced significant lag in tumor development.¹²⁰

Radio-protective effect:

The radio-protective effect of leaf extract of *O. sanctum* in combination with WR-2721 has been investigated. The mice were injected intraperitoneally with *O. sanctum* and then the whole body was exposed to gamma radiation. The protective effect of water extract of *O. sanctum* was greater than the aqueous ethanol extract in protecting mice against 11 Gy of Co-60 gamma irradiation.¹²⁰

***Withania somnifera* Dunal**



Roots of *W. somnifera*

[Cited: www.herbalnet.org]



Root powder of *W. somnifera*

Family	Solanaceae
Vernacular Name	Ashwagandha
Habitat ¹⁶⁹	This shrub is in Himalayas; 6000 feet, common in Bombay, western India, sometimes in Bengal
Chemical constituents ¹⁷⁰	Alkaloids (isopelletierine, anaferine), steroidal lactones (withanolides, withaferins), saponins containing an additional acyl group (sitoindoside VII & VIII), and withanolides with a glucose at carbon 27 (sitoindoside IX & X). it is rich in iron.

Biological activities:

Anti-tumor property:

In one study *W. somnifera* (WS) was evaluated for its anti-tumor effect in urethane-induced lung adenomas in adult male albino mice. Simultaneous administration of ethanol extract of *W. somnifera* and urethane reduced tumor incidence significantly. The histological appearance of the lungs of animals protected by *W. somnifera* was similar to those observed in the lungs of control animals. In addition to protection from carcinogenic effects, WS treatment also reversed the adverse effects of urethane on total leukocyte count, lymphocyte count, body weight and mortality.¹⁷⁰

Anti-stress effect:

The alcohol extract of WS reduced stress-induced increases in blood urea nitrogen levels, blood lactic acid and adrenal hypertrophy in rats.¹⁷⁰

Antioxidant effect:

The active principles of WS, sitoindosides VII-X and withaferin A (Glycowithnolides), have been tested for antioxidant activity using the major free-radical scavenging enzymes, superoxide dismutase (SOD), catalase (CAT) and glutathione peroxidase (GPX) levels in the rat brain. Dose-related increases in all enzymes were observed and the increases were comparable to those seen with deprenyl (a known antioxidant) administration.¹⁷⁰

Anti-inflammatory effect:

The effect of root powder of WS was evaluated on paw swelling and bony degenerative changes in Freund's adjuvant-induced arthritis in rats. WS

caused significant reduction in both paw swelling and degenerative changes as observed by radiological examination. The reductions were better than those produced by the reference drug, hydrocortisone.¹⁷⁰

Immunomodulatory property:

Glycowithnolides and a mixture of sitoindosides IX-X isolated from WS were evaluated for their immunomodulatory and central nervous system effects (anti-stress, memory and learning) in Swiss mice and Wistar strain albino rats. Both materials produced statistically significant mobilization and activation of peritoneal macrophages, phagocytosis and increased activity of the lysosomal enzymes. Both compounds also produced significant anti-stress activity in albino mice and rats and augmented learning acquisition and memory retention in both young and old rats.¹⁷¹

Hematopoietic effect:

Administration of WS extract was found to significantly reduce leukopenia induced by cyclophosphamide (CTX) treatment in Swiss albino mice. It was observed that, in the CTX-plus-WS mice, the cellularity (13.1×10^6 cells/femur) of bone marrow was significantly ($P < 0.001$) increased compared to the CTX-alone treated group (8×10^6 cells/femur).¹⁷²

Effect on the endocrine system:

The studies were conducted to evaluate the efficacy of root extract of WS in regulating thyroid function in mice. The treatment significantly increased the serum levels of 3,3',5-triiodothyronine (T3) and tetraiodothyronine (T4). Thus the results suggested that, it stimulates thyroidal activity.¹⁷⁰

Rejuvenating effect:

In a clinical trial, WS purified powder was given 3 g/day for one year to 101 normal healthy male volunteers, age 50-59 years. All subjects showed significantly increased hemoglobin and RBC count, and improvement in hair melanin and seated stature. They also showed decreased erythrocyte sedimentation (SED) rate. Thus the study indicated that WS may prove useful as a general health tonic.¹⁷⁰

***Tinospora cordifolia* Willd.**



Stem of *T. cordifolia*

[Cited: www.sirisimpex.com]



Stem powder of *T. cordifolia*

Family	Menispermaceae
Vernacular Name	Gulvel, Guduchi
Habitat ¹⁶⁹	Himalayas, throughout Madras presidency districts
Chemical constituents ¹⁷³	Alkaloids, diterpenoid lactones, glycosides, steroids, sesquiterpenoid, phenolics, aliphatic compounds and polysaccharides

Biological activities:

Anti-Diabetic activity:

It is reported that the daily administration of either alcoholic or aqueous extract of *T. cordifolia* [TC] decreases the blood glucose level and increases glucose tolerance in rodents. Aqueous extract also caused a reduction in blood sugar in alloxan-induced hyperglycemia in rats and rabbits.¹⁷³

Anti-stress activity:

The anti-stress and tonic property of the plant was clinically tested and it was found that it brought about good response in children with moderate degree of behavior disorders and mental deficit. It has also significantly improved the I.Q. levels.¹⁷³

Hepatoprotective activity:

The hepatoprotective action of *T. cordifolia* was reported in one of the experiment in which goats treated with *T. cordifolia* have shown significant clinical and hemato-biochemical improvement in CCl₄-induced hepatopathy.¹⁷³

Anti-viral activity:

Extract of *T. cordifolia* has also exhibited *in vitro* inactivating property against Hepatitis B and E surface antigen in 48-72 hr.¹⁷³

Anti-inflammatory effect:

The dried stem of *T. cordifolia* produced significant anti-inflammatory effect in both acute and sub-acute models of inflammation and TC was

found to be more effective than acetylsalicylic acid in acute inflammation.¹⁷⁴

Antioxidant activity:

The aqueous extract of roots of *T. cordifolia* has shown the antioxidant action in alloxan diabetes rats. The administration of the extract for 6 weeks resulted in a significant reduction of serum and tissue cholesterol, phospholipids and free fatty acids in alloxan diabetic rats.¹⁷³

Anti-neoplastic agent:

It has been found that *T. cordifolia* killed the HeLa cells very effectively *in vitro*. In this study, exposure of HeLa cells to *T. cordifolia* extract (methanol, aqueous and methylene chloride) resulted in a dose dependent but significant increase in cell killing when compared to non-drug treated controls.¹⁷⁵

Anti-tubercular activity:

Ether extract of stem of *T. cordifolia* has inhibited the *in vitro* growth of *Mycobacterium tuberculosis* at 1:50,000 dilution.¹⁷³

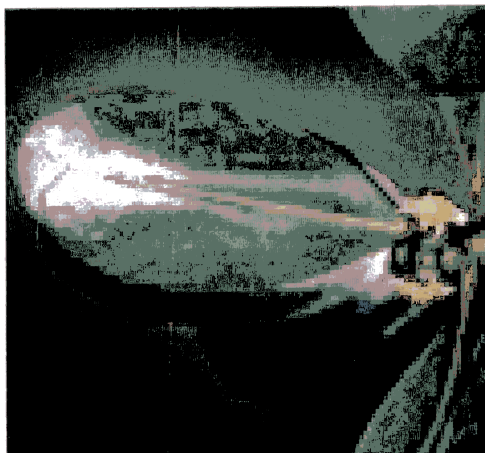
Immunomodulatory activity:

It has been reported that pretreatment with *T. cordifolia* reduced mortality in mice injected with *E. coli* intra-peritoneally, abdominal sepsis and Candida infection. It has been observed that it stimulates macrophages and enhanced their phagocytic activity and intracellular killing activity.¹¹⁸

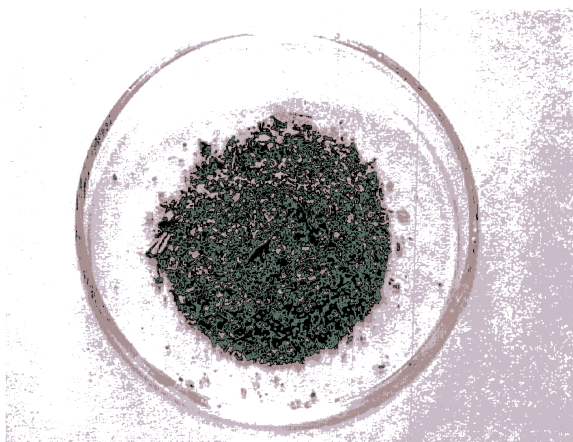
Other activities:

Its ethanolic extract has exhibited significant anti-pyretic activity in experimental rats.¹⁷³

***Avicennia officinalis* Linn.**



Leaves of *A. officinalis*
[Cited: www.naturia.per.sg]



Leaves powder of *A. officinalis*

Family	Verbanaceae
Vernacular Name	Tivar
Habitat ¹⁷⁶	Salt marshes & tidal forests of India
Chemical constituents ^{176,177,178}	Mainly Tannin, lapachol, betulin, betulinic acid, lupeol, lupenone, ursolic acid, and β -sitosterol

Uses / Biological activities:

Bark- astringent¹⁷⁶

Root- aphrodisiac¹⁷⁶

Resin from bark- contraceptive, antidote for snake-bite¹⁷⁷

Pulp of unripe fruit- in healing skin lesions of small pox¹⁷⁷

Fruit and immature seed as cicatrizing of ulcers and abscesses¹⁷⁷

Fifty percent ethanol extract of aerial parts showed anti-protozoal activity.¹⁷⁹

Kernels contain a naphthaquinone derivative, lapachol which possess anti-tumor property.^{177,178}

A. officinalis is also effective for joint pain and bronchial asthma.¹²⁴

Decoction of *A. officinalis* leaves is used to treat stomach and urinary disorders.¹²³

***Rhizophora mucronata* Lam.**



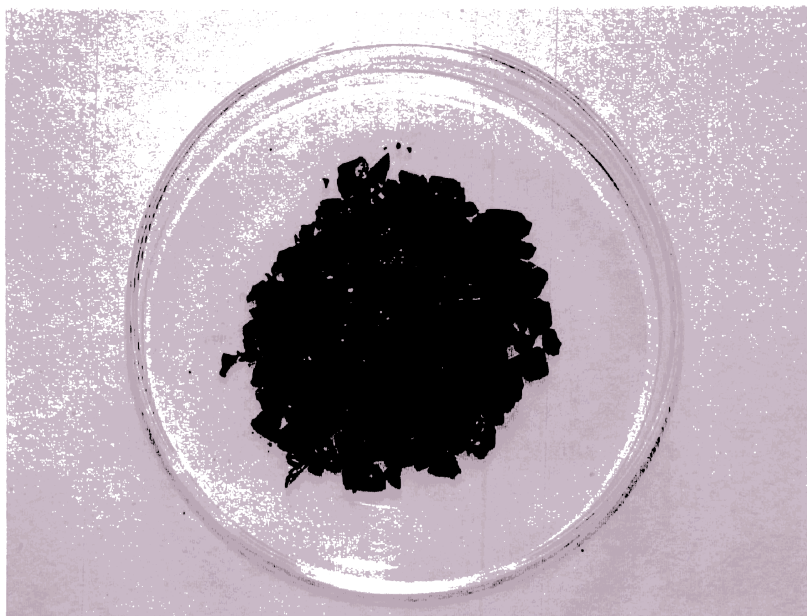
Leaves of *R. mucronata*



Leaves powder of *R. mucronata*

Family	Rhizophoraceae
Vernacular Name	Kandel
Habitat ¹⁷⁶	Along muddy shores and tidal creeks of India
Chemical constituents ¹⁷⁶	Tannin
Uses/Biological activities ^{176,177,124}	Bark- astringent, anti-diabetic, hemorrhage and angina

Shilajit



Granules of pure Shilajit

Latin Name	Asphaltum
Habitat ¹³¹	Shilajit is a pale-brown to blackish-brown exudation, of variable consistency, from layers of rocks in Himayalan ranges of the Indian subcontinent. It is also found in Russia, Tibet, Norway and other countries, where it is collected in small quantities from steep rock faces at altitudes between 100 and 5000 m.
Chemical constituents ¹³¹	Major organic mass of shilajit comprised humus (60-80%) along with other components such as benzoic acid, hippuric acid, fatty acid, ichthyol, ellagic acid, resin, triterpenes, sterol, aromatic carboxylic acid, 3,4-benzocoumarins, amino acids, and phenolic lipids. The major physiological action of shilajit was found to be due to the presence of bioactive dibenzo-alpha-pyrones along with humic and fulvic acids.

Biological activities:

Anti-ulcerogenic and anti-inflammatory activity:

It was found that Shilajit increased the carbohydrate/protein ratio and decreased the gastric ulcer index, indicating an increased mucus barrier. Shilajit was also found to have potent anti-inflammatory activity in all three models of acute, sub-acute and chronic inflammation. Shilajit is perhaps the first agent to possess both anti-ulcerogenic and anti-inflammatory activities.¹³¹

Antioxidant activity:

The effect of shilajit on lipid peroxidation and glutathione content in rat liver homogenates was also investigated. It was found that it inhibited lipid peroxidation induced by cumene hydroperoxide and ADP/Fe⁺⁺ complex in a dose dependent manner. Shilajit also decreased the rate of oxidation of reduced glutathione content and inhibited the ongoing lipid peroxidation which was induced by these agents immediately after its addition to the incubation system.¹³¹

Anti-diabetic activity:

Diabetes mellitus was produced in male albino rats by the administration of streptozotocin (STZ). STZ-induced hyperglycemia may be due to decrease in pancreatic islet superoxide dismutase activity, led to an accumulation of free radicals and damage of beta cells. It was found that Shilajit prevented both these effects of STZ possibly by its action as a free radical scavenger.¹³¹

Memory enhancement and anxiolytic activity:

The effect of Shilajit was also investigated for anxiolytic activity in Charles Foster strain albino rats. The observed neurochemical studies on shilajit indicated a decrease in rat brain 5-hydroxytryptamine turnover, associated with an increase in dopaminergic activity leading to an increase in memory and anxiolytic activity in albino rats.¹⁸⁰

Anti-stress activity:

Shilajit collected from India, Nepal, Pakistan and Russia and organic constituents isolated from them were studied for their anti-stress effect in albino mice. It was found that Shilajit from India, Nepal and a combination of total ethyl acetate extract and fulvic acids extracted from Indian Shilajit produced a statistically significant improvement in forced swimming induced immobility in albino mice.¹⁸¹

Anti-allergic activity:

The effect of shilajit and its main active constituents fulvic acids, 4'-methoxy-6-carbomethoxybiphenyl and 3,8-dihydroxy-dibenzo-alpha-pyrone were studied in relation to the degranulation and disruption of mast cell against noxious stimuli. Shilajit and its active constituents provided significant protection to antigen-induced degranulation or sensitized mast cells, markedly inhibited the antigen induced spasm of sensitized guinea-pig ileum and prevented mast cell disruption.¹³¹

Immunomodulatory activity:

Shilajit as an immunomodulatory agent was studied in mice that were given either Shilajit extract or a placebo. The white blood cell activity was

studied and monitored prior to and at intervals after receiving the shilajit extract or a placebo. It was found that the white cell activity increased as the dose of Shilajit extract and time of exposure was increased. Shilajit and its combined constituents elicited and activated murine peritoneal macrophages and activated splenocytes of tumor-bearing animals at early and later stages of tumor growth.¹³¹

Rationale:

Human immunodeficiency virus (HIV), the causative agent of acquired immunodeficiency syndrome (AIDS), is currently the most significant infectious pathogen with devastating consequences. HIV produces gradual effects on the body's defense mechanism thereby leading to cancers and opportunistic infections involving multiple systems of the body such as immune, gastrointestinal, genitourinary, endocrine, dermatologic and nervous system.

Although the development of potent antiviral drugs has allowed HIV-infected people to live longer, it is clear that these drugs do not cure the infection. Moreover, there prolonged use often engenders significant toxicity and results in the emergence of drug-resistant viruses. Due to there high cost and the current lack of adequate infrastructure for delivery, these drugs are not widely available in the developing world, where HIV is spreading the fastest. In the ongoing efforts to develop a vaccine, certain approaches are showing early promise in monkeys. However, the persistence of HIV infection coupled with its high rate of spontaneous mutation raises concerns about eventual viral immune escape. Most importantly, the ability of HIV to establish latent reservoirs early in the course of infection ensures the persistence of this pathogen even in the face of intensive drug therapy and vigorous antiviral immune responses.

Besides, HIV/AIDS affects individuals in prime-working age group which has a large negative effect on growth in national income. It also affects savings of government as owing to increased spending on treatment and prevention related to HIV/AIDS reduces budgets for road construction and

maintenance and other infrastructure services. Moreover, Mother to child transmission is resulting in increasing number of orphans and children living with HIV that makes HIV/AIDS more of social, emotional problem than mere economical burden. India has the world's second largest burden of HIV infected persons. It is clear by now that the effects of HIV/AIDS are not limited to the realm of medicine.

Consequently there is a need to develop safe, effective and economical alternative to drugs presently approved for systematic treatment of AIDS. Traditional knowledge will serve as a powerful search engine and most importantly, will greatly facilitate intentional, focused and safe natural products research to rediscover the drug discovery process. Almost, 70% modern medicines are derived from natural products. Ayurveda remains one of the most ancient and yet living traditions practiced widely in India and has a sound philosophical, experiential and experimental basis. Ayurvedic medicines use largely herbal and herbomineral preparations to treat a variety of chronic disease conditions. Besides, mangrove plants are also used in folklore medicine for treatment of numerous ailments. The beneficial effect of these to HIV/AIDS patients could be due to one or combination of several factors: direct inhibition of HIV replication, boosting of immune system or having inhibitory properties against one or several opportunistic infections.

There is therefore good reason for *in vitro* testing of selected plant material and the herbomineral for anti-HIV activity.

AIMS
AND
OBJECTIVES

The aims and objectives of the present *in vitro* study were as follows:

1. To collect the raw material of the five selected medicinal plants namely, root powder of *Withania somnifera* Dunal, Stem powder of *Tinospora cordifolia* Willd., leaves powder of *Ocimum sanctum* Linn. Also, leaves of two mangrove plants namely, *Avicennia officinalis* Linn. and *Rhizophora mucronata* Lam. and a herbomineral Shilajit.
2. To carry out preliminary phytochemical screening of the raw material of the selected plants and the herbomineral.
3. To prepare different extracts with the help of following methods and to calculate their percentage yields.
 - ❖ By standard Soxhlet extraction method using non-polar and polar solvents.
 - ❖ Aqueous extraction by Decoction method.
4. To prepare cells and virus stock for the *in vitro* study.
5. To carry out preliminary screening of plant extracts using,
 - ❖ XTT Formazan Assay
 - ❖ p24 Inhibition Assay
6. To evaluate specific inhibition of viral target molecule in the presence or absence of plant extracts by performing,
 - ❖ Reverse Transcriptase (RT) Inhibition Assay
 - ❖ gp120 Binding Inhibition Assay

7. To evaluate the antiviral effect of extracts by using following parameters,
 - ❖ Dose-response study
 - ❖ Effect on clinical isolates
 - ❖ Time-course study
 - ❖ Combinatorial effect
8. To carry out preliminary TLC study of active extracts.

MATERIALS

AND

METHODS

1. Collection of the material:

a) Procurement of Raw Plant Material:

Root powder of *Withania somnifera* and stem powder of *Tinospora cordifolia* were procured from Zandu Pharmaceuticals, Mumbai. Leaves powder of *Ocimum sanctum* was purchased from Atul Medical Stores, Vile Parle (E), Mumbai.

b) Collection of Mangrove plants:

Avicennia officinalis was collected from Mahim Creek, Mumbai whereas *Rhizophora mucronata* was collected from Vikroli highway, Mumbai. The leaves were washed, sun-dried and powdered in a grinder mixer.

c) Shilajit was purchased from Vaidya (Dr.) Rajeev K. Kanitkar, Vile Parle (E), Mumbai.

The plant material and the herbomineral were identified and authenticated by Dr. J. M. Pathak, Zandu Pharmaceutical, Mumbai.

2. Phytochemical Screening: ¹⁸²

Materials:

Ethanol (Sterling Chemicals and Alcohols PVT Ltd.)

Hydrochloric acid (HCl) (Qualigens)

Mercuric Chloride (Sarabhai M. Chemicals)

Potassium Iodide (Qualigens)

Ferric Chloride (LOBA CHEMIE)

NaOH (Sarabhai M. Chemicals)

Conc. Sulphuric acid (Qualigens)

Chloroform (SRL)

Ammonia Solution (100%) (Qualigens)

Acetic acid Glacial (SRL)

Acetic anhydride (Sarabhai M. Chemicals)

Method:

Qualitative phytochemical analysis of the crude powder of 4 plants and a herbomineral was determined as follows:

1) Test for resins:

To 0.5 g of material 5 ml of boiling Ethanol was added. This was filtered through Whatman No.1 filter paper and the filtrate diluted with 4 ml of 1% aqueous HCl. The formation of resinous precipitate indicated the presence of resins.

2) Test for Alkaloids:

0.5 g material was added to 1% aqueous HCl on a steam bath. This was filtered and 1 ml of the filtrate treated with a few drops of Mayer's

reagent.¹⁸³ Formation of creamish precipitate was indication of presence of alkaloids.

3) Test for Tannin:

0.5 g of material was stirred with 10 ml of distilled water. This was filtered and a few ml of 5% ferric chloride was added to the filtrate. A deep green coloration showed the presence of tannin.

4) Test for Saponin:

0.5 g of material was shaken with water in a test tube. Frothing which persist on warming was taken as evidence for presence of saponin.

5) Test for Flavonoids:

0.5 g of material was dissolved in 2 ml dilute NaOH solution and filtered. A few drops of conc. sulfuric acid were then added to the filtrate. The presence of flavonoid was indicated when the solution became colorless.

6) Borntrager's test for Anthraquinone:

0.5 g of material was taken into a dry test tube and 5 ml of chloroform was added and shaken for 5 min. This was filtered and the filtrate shaken with an equal volume of 100% ammonia solution. A pink, violet or red color in ammoniacal layer (lower layer) indicated presence of free anthraquinones.

7) Keller Killiani test for cardiac Glycoside:

0.5 g of material was dissolved in 2 ml of glacial acetic acid containing one drop of ferric chloride solution and filtered. The filtrate was then underlayered with 1 ml of conc. sulfuric acid. A brown ring obtained at the interphase indicated the presence of deoxy sugar typical of cardenolides.

8) Salkowski test for Steroidal ring:

0.5 g of material was dissolved in 2 ml of chloroform and filtered. Sulphuric acid was carefully added to form lower layer. A reddish brown color at the interface indicated the presence of steroidal ring.

9) Liebermann-Burchard test for steroidal Terpenes:

0.5 g of material was dissolved in 2 ml of chloroform and filtered. To the filtrate 1 ml of acetic anhydride and 2 drops of conc. sulfuric acid were added. A pink color which changes to bluish green on standing indicated the presence of steroidal terpenes.

10) Test for Carbohydrates:

0.5 g of material was heated with 1 ml of conc. sulfuric acid. Blackening and effervescence occurred indicating the presence of carbohydrate.

3. Preparation of extracts:

Materials:

Soxhlet apparatus and RB flasks (Reviera)

Petroleum ether (60-80⁰C) (SRL)

Ethanol (Sterling Chemicals and Alcohols PVT Ltd.)

DMSO and RPMI 1640 (Himedia)

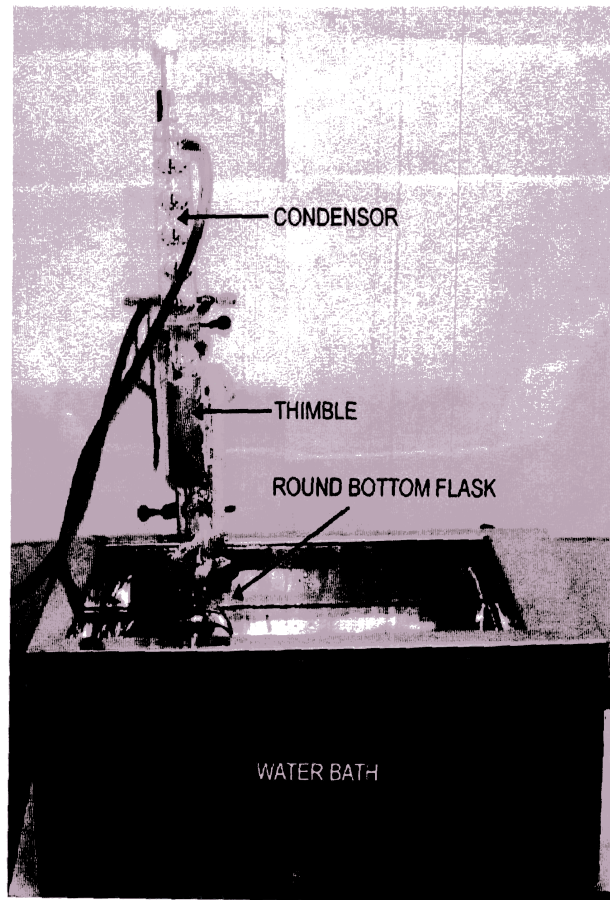
Method:

a) Soxhlet extraction using AR organic solvents:

A weighed quantity of the material was introduced in a thimble made of filter paper into wide central tube of the extractor. Organic solvent was poured into the central portion, from where it percolated into the flask. The extractor along with the flask was fitted to the condenser. The water bath with constant boiling water was used.

The material was first extracted in Soxhlet apparatus with Petroleum Ether (60-80⁰C) and then with Ethanol in succession. A separate extraction of plant material was carried out with Ethanol alone. The extraction was carried out at boiling point of petroleum ether and ethanol. The completion of extraction was indicated by colorless liquid in siphon tube. The excess of solvent in the flask was collected and the solvent from the extracts was removed using water bath. The percentage yield of individual extractives was then calculated.

Figure-1: Soxhlet Extraction Apparatus



b) Aqueous extraction: ¹⁸⁴

Aqueous extracts were prepared by plain decoction method. To 5 g of powder 50 ml of distilled water was added in 80°C in stoppered flask. It was shaken well and allowed to stand for 10 min. after cooling 2 g of Kieselglehr was added and filtered. The filtrate was transferred to a trade-evaporating dish and water was evaporated on a boiling water bath, dried for 30 min and residue was weighed. The percent of water-soluble extractives with respect to the air-dried drug was then calculated.

Thus there were four extracts of each plant namely, Ethanol (Direct), Petroleum ether, Ethanol (Successive) and Aqueous.

c) Reconstitution of Extracts:

Petroleum ether and Ethanol extracts were dissolved in DMSO (not more than 1%) and then diluted with RPMI 1640 medium, whereas aqueous extracts were reconstituted in RPMI 1640 medium alone.

Each extract was reconstituted to prepare stock of 25 mg/ml. The stock solutions were then filtered through 0.22 µm membrane filter and stored at 4°C until further use.

4. Preparation of Peripheral Blood Mononuclear Cells (PBMCs):

Materials:

Sterile Centrifuge tubes (15ml) (Laxbro)

EDTA vacutainer (BD Biosciences)

Histopaque-1077 (Sigma-Aldrich)

Sterile RPMI 1640 (HIMEDIA)

Fetal Calf Serum (FCS) (HIMEDIA)

PHA (Sigma)

Gentamycine (Nicolas)

Interleukine-2 (IL-2) (Sigma)

Tissue culture flasks (25 cm²) (Tarsons)

Method:

a) Collection of blood samples:

Ten ml of blood samples were collected in EDTA vacutainer from the known HIV seropositive drug-naïve (Non-ART) and drug-treated (ART) individuals. Written consent was obtained before blood collection.

Similarly, ten ml of blood sample was collected in EDTA vacutainer from the healthy individual.

b) Confirmation of sero-status:

The rapid HIV card test (PMC medical Pvt. Ltd.) was performed in order to confirm the sero-status of the 2 patients. For this, small amount of blood sample was put on the card and allowed to absorb. The developer was then added. The reaction was then incubated for five minutes and

observed for the development of pink band. The band developed was compared with the control provided in the kit.

c) Separation of peripheral blood mononuclear cells (PBMCs):

Separation of peripheral blood mononuclear cells was carried out using density gradient centrifugation as per the protocol mentioned.¹⁸⁵ The procedure was carried out as follows:

Three ml of Ficoll-hypaque (Density 1.077 g/ml) was taken in sterile centrifuge tubes. Four ml of the anticoagulated blood sample collected was then slowly overlaid on to density gradient in each tube. The tubes were then centrifuged at 1500 rpm for 20 min. at room temperature. The buff color band of separated PBMCs, obtained on top of Ficoll layer from each tube was then collected in separate tubes. The harvested cells were then washed with sterile RPMI 1640 twice.

The same procedure was followed for separating PBMCs from HIV positive Blood samples.

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5. Preparation of Virus stock:

For the preparation of HIV stocks, the PBMCs of the HIV-infected patient were co-cultivated with that of the PBMCs isolated from healthy individual.¹⁸⁵

a) Preparation of PHA-blasts:

The cell viability of the harvested, washed healthy donor's cells was determined using 0.4% trypan blue stain. For this 0.1 ml cell suspension was mixed with 0.2 ml of trypan blue, and kept for one minute. The cell viability was determined using Haemocytometer.

The cells then inoculated in 25 cm² tissue culture flasks with sterile RPMI 1640 medium containing 10% Fetal Calf Serum (FCS), 1µg/ml PHA mitogen and 50 µg/ml gentamycine at a cell concentration of 3-5 X 10⁶ cells/ml. the cells were cultured in upright position for 3 days at 37⁰ C in humidified 5% CO₂ atmosphere (Forma Scientific water jacketed incubator). After the incubation, the cells were centrifuged at 1200 rpm for 10 min and the pellet was then resuspended in sterile RPMI 1640 medium containing 10% Fetal Calf Serum (FCS), 10 U/ml Interleukine-2 (IL-2) and 50 µg/ml gentamycine at a cell concentration of 2 X 10⁶ cells/ml.

b) Actual co-cultivation:

Five ml of the PHA-stimulated donor PBMCs were mixed with 3 ml of the patient's PBMCs in IL-2 medium at concentration of 2 X 10⁶ cells/ml in 25 cm² tissue culture flasks. The culture bottles were incubated overnight at 37⁰ C in the incubator standing under 45⁰ angle, to allow the cells to settle

in the corner of the flaks. Further incubation was carried out in upright position.

c) Collection of virus stock:

On day 4 of co-cultivation, the cells were centrifuged at 1200 rpm for 15 min. The culture supernatant was collected and stored at -70° C. A small quantity was tested for the presence of virus by reverse transcriptase assay. The cell pellet was resuspended in fresh IL-2 medium and incubated at 37° C in humidified 5% CO₂ atmosphere.

On day 7 of co-cultivation, the culture supernatant was collected as described earlier. The half cell pellet was then mixed with the 3-day old PHA-stimulated donor PBMCs (5 X 10⁶ cells/ml) and suspended in fresh IL-2 medium. The cultures were maintained for 4 weeks for Non-ART isolate (virus isolated from drug-naïve patient) and 2 weeks for ART isolate (virus isolated from drug-treated patient) to prepare sufficient stock. The same protocol was followed through out for collection of culture supernatants on 4th and 7th day of each week.

d) Determination of virus titre:

The culture supernatants collected at regular time intervals on 4th day and 7th day each week of culturing, were tested for the presence of virus particle by performing, reverse transcriptase assay.

Reverse transcriptase assay: ¹³⁶

Materials:

Protease, Nuclease free Water (Bangalore Genei)

Tris-HCl (SRL)

KCl (HIMEDIA)

MgCl₂ (HIMEDIA)

NP-40 (USB)

EGTA (HIMEDIA)

DTT (USB)

dTTP (Bangalore Genei)

Glutathione (HIMEDIA)

BSA (HIMEDIA)

POLY(rA).p(dT)₁₂₋₁₈ (Amersham Biosciences)

DE-81 Whatman filter Discs (B Patel and Company)

Na₂HPO₄.7H₂O (HIMEDIA)

Scintillation Vials (Tarsons)

Scintillation Cocktail (SRL)

Scintillation counter (Packard)

Sterile eppendorf tubes (Tarsons)

Method:

For this assay, all the reagents were prepared in DNase-RNase-free water and filter sterilized through bacteria proof filter.

The reaction mixture (Final volume: 100μl) contained the following: 50 mM Tris (pH 7.8), 150 mM KCl, 5 mM MgCl₂, 0.05% NP-40, 0.5 mM EGTA, 5 mM DTT, 20 μM dTTP, 0.3 M Glutathione, 2.5 μg/ml BSA, 0.5 μCi of ³H-TTP, 2.5 μg/ml POLY(rA).p(dT)₁₂₋₁₈.

The 100 μ l of the reaction mixture taken in sterile eppendorf tube was inoculated with 10 μ l of culture supernatant. The mixture was then incubated for 3 hrs at 37°C. The reaction was terminated by addition of 25 μ l of 0.1 M EGTA followed by chilling the mixture on ice. 100 μ l of each reaction mixture was then spotted uniformly onto DE-81 Whatman filter and kept at ambient temperature for 15 min. The dried filters were washed four times with 5% aqueous $\text{Na}_2\text{HPO}_4 \cdot 7\text{H}_2\text{O}$ followed by two more washings with doubly distilled water. Finally, the filters were thoroughly dried and subjected to scintillation counting.

Non-ART isolate (virus isolated from the drug-naïve patient) was used for preliminary assays such as XTT formazan and p24 inhibition assay, also to evaluate effect of extracts on RT of Non-ART isolate and to check the combinatorial effect of extracts. While, ART isolate (virus isolated from drug-treated patient) was used only to evaluate effect of extracts on RT of ART isolate.

6. Preliminary Screening by:

a) XTT Formazan Assay: ¹⁸⁶

Materials:

Sterile 96 well plates with lid (Laxbro)

XTT sodium salt (Sigma)

PMS (SRL)

ELISA Reader (Labsystems Multiskan Ascent)

Tissue culture medium and supplements as stated earlier

Method:

Antiviral and cytotoxic effects of the extracts were evaluated simultaneously with the XTT Formazan assay.

Principle:

The compound, sodium 3-[1-(phenylamino)-carbonyl]-3,4-tetrazolium-bis(4-methoxy-6-nitro)benzene-sulfonic acid hydrate (XTT), a light yellowish tetrazolium reagent, can be metabolically reduced by mitochondrial dehydrogenase in viable cells to a water-soluble, brown-colored formazan product. Phenazine methosulfate (PMS), an electron-coupling agent, can significantly enhance the production of XTT Formazan. The amount of XTT Formazan produced is correlated with the number of viable cells. Several T-cell lines are sensitive to the lysis caused by HIV replication. Anti-HIV agents protect these cells from lysis. Therefore, XTT plus PMS has been widely used for screening anti-HIV agents.

Procedure:

Six five-fold dilutions of each of the plant extracts were made in sterile RPMI 1640 without phenol red (10mg/ml, 2mg/ml, 0.4mg/ml, 0.08mg/ml,

0.016mg/ml and 0.0032mg/ml). 50 µl of each of the dilutions were added in two different sets, to the flat bottom 96 well plate.

The assay was accomplished with 3-day old phytohemagglutinin (PHA) stimulated PBMCs. The cells were cultured in RPMI 1640 without phenol red containing 2 mM L-glutamine, supplemented with 10% Fetal Bovine Serum, 10 U/ml Interleukine-2 and 50 µg/ml gentamycine. The cells were centrifuged and resuspended in fresh medium at a concentration of 2×10^6 cells/ml. Fifty microlitre of the resuspended cells were added to the wells of both the sets. To the first set, 25 µl of the virus supernatant was added to determine the antiviral effect of the extracts. To the second set, 25 µl of the sterile RPMI 1640 without phenol red was added and this set was considered for cytotoxicity study. The third set was kept as extract control comprising of each of the dilutions and the growth medium only. Proper cell controls were kept for the infected and uninfected cells. AZT was included as standard reference compound. The plate was then incubated at 37°C in an atmosphere of 5% CO₂ for 5 days. At the end of incubation period, the mixture of XTT (1mg/ml) and 0.02mM of N-methyl phenazonium methosulfate (25 µl) was added to all the wells. The plates were further incubated for 4 hr and then agitated for 1 hr using plate shaker. The plates were then read at 450 nm to check the cell viability spectrophotometrically with the help of Labsystem Multiscan Ascent ELISA reader.

The percentage inhibition was calculated as, $[(A_{tv} - A_{cv}) / (A_{cd} - A_{cv})] \times 100$, where A_{tv} is the Absorbance of test compounds with virus infected cells, A_{cv} is the Absorbance of virus control (untreated infected cells) and A_{cd} is the Absorbance of cell control (untreated uninfected cells), respectively.¹⁸⁷

The percentage cytotoxicity was calculated as, $[(A-B)/A] \times 100$ where, A and B are the Optical Density (OD) of untreated and of treated cells, respectively.¹⁸⁸

Form above calculation, the result was expressed as 50% Cytotoxic Concentration (CC_{50}) and 50% Effective Concentration (EC_{50}). The selectivity index¹⁸⁷ was also calculated as ratio of CC_{50}/EC_{50} .

b) HIV p24 Inhibition Assay:¹⁸⁹

Materials:

24 well plates (Corning Incorporated Costar)

p24 ELISA kit (ZeptoMetrix Corporation)

ELISA Reader (Labsystems Multiskan Ascent)

Method:

The effect of extracts on HIV replication was determined by viral core protein p24 expression using commercially available ELISA kit.

As viral productions peaks at day 4, p24 expression was assayed in cell-free supernatants harvested at day 4.

Principle:

The culture medium was incubated in microtiter wells precoated with anti-p24 antibody and assayed for p24 antigen by biotin-labeled anti-p24 antibody followed by incubation with streptavidine-peroxidase conjugate. The amount of captured p24 was determined by measuring the absorbance at 450 nm of tetramethylbenzidine (TMB) substrate.

Procedure:

The assay was accomplished with 3-day old phytohemagglutinin (PHA)-stimulated PBMCs. The cells were cultured in RPMI 1640 containing 2 mM L-glutamine, supplemented with 10% Fetal Bovine Serum, 10 U/ml Interleukine-2 and 50 µg/ml gentamycine. The cells were centrifuged and resuspended in fresh medium at a concentration of 2×10^6 cells/ml. Six five-fold dilutions of each of the plant extracts were made in sterile RPMI 1640 (10mg/ml, 2mg/ml, 0.4mg/ml, 0.08mg/ml, 0.016mg/ml and 0.0032mg/ml).

Two hundred microliter of PBMCs were seeded in 24-well plates with or without various dilutions of each of the extract. The cells were then inoculated with 100µl of virus and incubated at 37°C in an atmosphere of 5% CO₂ for 4 days. The cells were harvested on day 4 post-infection and cell free supernatants were collected for the determination of p24 production by commercial ELISA kit according to manufacturer's instructions. AZT was included as standard reference compound. The percentage inhibition was calculated as¹⁹⁰,

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{Sample}}) / A_{\text{control}}] \times 100.$$

Where, A_{control} is Optical Density (OD) of control (without extract) and

A_{Sample} is Optical Density of Sample. (Extract and/or AZT)

7. Anti-HIV Assays:

a) Reverse Transcriptase (RT) Inhibition Assay:¹³⁶

Materials:

HIV Reverse Transcriptase enzyme (Ambion)

AZT (Sigma)

Other reagents as stated earlier

Method:

In this assay, the effect of extracts was investigated for their ability to inhibit RNA-dependent-DNA-polymerase (RDDP) activity of HIV RT.

Principle:

The inhibition of RDDP activity was measured by evaluating the incorporation of methyl-3H thymidine triphosphate (Methyl [3H]TTP) by RT using polyadenylic acid-oligodeoxythymidilic acid [poly(rA).p(dT)₁₂₋₁₈] as a template-primer in the presence and absence of extracts.

Procedure:

The HIV reverse transcriptase (RT) enzyme inhibition due to each of the extract was determined by adding 25 µl of each extract (Final concentration 5 mg/ml) to the reaction mixture.

The reaction mixture (Final volume: 100µl) contained the following: 50 mM Tris (pH 7.8), 150 mM KCl, 5 mM MgCl₂, 0.05% NP-40, 0.5 mM EGTA, 5 mM DTT, 20 µM dTTP, 0.3 M Glutathione, 2.5 µg/ml BSA, 0.5 µCi of ³H TTP, 2.5 µg/ml POLY(rA).p(dT)₁₂₋₁₈.

The reaction was started by addition of 0.5 Units of recombinant reverse transcriptase enzyme (Ambion). The mixture was then incubated for 3 hr

at 37°C. The reaction was terminated by addition of 25 µl of 0.1 M EGTA followed by chilling the mixture on ice. 100 µl of each reaction mixture was then spotted uniformly onto DE-81 Whatman filter and kept at ambient temperature for 15 min. The dried filters were washed four times with 5% aqueous Na₂HPO₄.7H₂O followed by two more washings with doubly distilled water. Finally, the filters were thoroughly dried and subjected to scintillation counting. Negative control, RPMI 1640 medium without extracts, was set up in parallel. AZT was used as positive control. The percentage inhibition was calculated as¹⁴⁷,
$$\text{Inhibition (\%)} = [(\text{CPM of Negative control} - \text{CPM of Test}) / \text{CPM of Negative control}] \times 100.$$

b) gp120 Binding Inhibition Assay:¹⁹¹

Materials:

gp120 Capture ELISA kit (ImmunoDiagnostics, Inc.)

Heparin sodium salt (SRL)

ELISA reader (Labsystems Multiskan Ascent)

Method:

Binding of gp120 to CD4 was analyzed using a commercially available gp120 Capture ELISA kit.

Principle:

In this assay, CD4 molecules have been immobilized on a 96-well microtiter plate. The coated plate is then used to capture free HIV-1 gp120 supplied in the kit. Quantitation of captured gp120 is done using detector reagent provided in the kit.

Procedure:

To determine whether extracts could interfere with the binding of CD4 to gp120 by interaction with soluble gp120, each extract (Final concentration 5 mg/ml) was mixed with 25 ng of purified gp120 in a total volume of 100 μ l and incubated at room temperature for 1 hr. This mixture was then added to microtiter plate wells coated with CD4 ligand and incubated at room temperature for 1 hr. The solutions were aspirated and washed 3 times with washing buffer. The extent of gp120 binding was assessed by using detector reagent provided in the kit according to the manufacturer's instructions. Negative control without extracts, was set up in parallel. Heparin was included as positive control.

The percentage inhibition was calculated as,¹⁹⁰

$$\text{Inhibition (\%)} = [(A_{\text{control}} - A_{\text{Sample}}) / A_{\text{control}}] \times 100.$$

Where, A_{control} is Optical Density (OD) of Negative control and

A_{Sample} is Optical Density of Sample (extract and/or heparin)

8. Assessment of Antiviral activity by:

a) Dose-response study:

Six five-fold dilutions of each extract were prepared as described earlier. The HIV reverse transcriptase enzyme inhibition due to each of the concentration of extracts was determined using HIV RT inhibition assay as described previously.¹³⁶ Briefly, 25 µl of each of the concentration was added to the reaction mixture containing 0.5 Units of recombinant reverse transcriptase enzyme (Ambion) and incubated the mixture for 3 hrs. Further assay procedure comprising spotting of the samples on anionic filter membranes, after terminating the reactions and washing of filter membranes was the same as described previously. Extent of HIV RT inhibition was evaluated by scintillation counting. AZT was included as positive control in the study.

b) Effect on clinical isolates:

The analysis was carried out to determine the activity of each extract against two different clinical samples. The reverse transcriptase of *in vitro* cultivated virus stock from blood samples of HIV seropositive drug-naïve (Non-ART) and drug-treated (ART) individual was considered for this study. The reverse transcriptase inhibition assay was carried out as described earlier.¹³⁶

c) Time-course study:

In order to investigate the mechanism of inhibition of gp120/CD4 binding, a study was conducted to evaluate the time-course effect by adding each extract before binding and after binding of gp120 to CD4. The assay was

performed using commercial gp120 Capture ELISA kit (ImmunoDiagnostics, Inc.).

To determine whether the extracts could bind to the immobilized CD4 and thus interfere with the interaction with gp120, CD4-coated wells were washed 3 times with washing buffer and incubated with 100µl of each of the extract (Final concentration 5 mg/ml) for 1 hr at room temperature. The extracts-treated wells were then washed 3 times and incubated with 25 ng of gp120 for 1 hr at room temperature. The degree of bound gp120 was measured using detector reagent provided in the kit.¹⁹¹

In separate experiment, the extracts were added after 1 hr pre-incubation of gp120 (25 ng) in CD4-coated plates. After gp120-preadsorbed plates were washed 3 times, each extract (Final concentration 5 mg/ml) was incubated in the plates for another 1 hr at room temperature, followed by detection of bound gp120 by detector reagent supplied in the kit according to the manufacturer's instructions.¹⁹²

d) Combinatorial effect:

For this study, reverse transcriptase inhibition assay was performed as described earlier¹³⁶ and Non-ART isolate was used as virus source.

To evaluate the combinatorial effect, different combinations of aqueous extracts of 5 plants and the herbomineral were prepared by mixing them in equal proportion (12.5 µl) in separate eppendrof tubes and a mixture of all the 6 extracts (4.2 µl each) together was also prepared to study their effect on HIV-RT. The effect of combinations was determined by comparing the CPM values obtained after scintillation analysis with that of Negative control. AZT was included as positive control.

9. Preliminary Thin Layer Chromatography (TLC) Study:

Materials:

Petroleum ether (60-80⁰C) (SRL)

Ethanol (Sterling Chemicals and Alcohols PVT Ltd.)

Benzene (SRL)

Chloroform (SRL)

Ethyl acetate (SRL)

Iodine resublimed (CDH)

Method:

In order to estimate number of components that can possibly be obtained from each crude extract, the extracts were first analyzed on Thin Layer Chromatography. The TLC was run using different permutations and combinations of solvents. This was essential to achieve best resolution of extracts on chromatogram. TLC was run by standard method as described below,

Only extracts of *A. officinalis*, *R. mucronata* and Shilajit were subjected to TLC study, as sufficient study of TLC profiles has been carried out earlier on other plants.

Alumina sheet coated with silica gel was cut into pieces (plates) of size (7.5 cm x 2.5 cm) and used for spotting the extracts.

The solvents and the combinations used for the separation are as follows,

Single solvents used:

Petroleum ether, Ethanol, Benzene, Chloroform and Ethyl acetate.

Combinations used:

- Petroleum ether and Ethanol in the ratio (80:20), (60:40), (40:60), (20:80)
- Benzene and Chloroform in the ratio (80:20), (60:40), (40:60), (20:80)

The solvent was first introduced in a small chamber i.e. staining jar with a lid. The lid was then closed in order to ensure complete saturation of atmosphere in jar with solvent vapours. This helped in faster separation and formation of straight solvent front. The spotted plates before placing in chamber were dried at room temperature in order to evaporate solvent. Separation was carried out by ascending TLC in which sample spot was above the solvent level.

Once the solvent reached to the required level, the plates were removed from the jar and dried at room temperature in order to remove the solvent of chromatogram. The dried chromatogram was then visualized after exposing it to iodine vapours.

R_f values were calculated as follows,¹⁹³

$$R_f = \frac{\text{Distance traveled by the spot}}{\text{Distance traveled by the solvent front}}$$

RESULTS

1. Selected Plants:

Indigenous plants and a herbomineral selected in the present study are listed in the following table.

Table-1 : Indigenous Plants and Herbomineral under study

Plant / Herbomineral	Family	Vernacular Name	Part Used
<i>Ocimum sanctum</i> Linn.	Labiatae	Tulas	Leaves
<i>Withania somnifera</i> Dunal	Solanaceae	Ashwagandha	Root
<i>Tinospora cordifolia</i> Willd.	Menispermaceae	Gulvel	Stem
<i>Avicennia officinalis</i> Linn.	Verbenaceae	Tivar	Leaves
<i>Rhizophora mucronata</i> Lam.	Rhizophoraceae	Kandel	Leaves
Shilajit	-	Shilajit	Granules

2. Preliminary Phytochemical Screening:

The phytochemical analysis of *O. sanctum* showed presence of resins, alkaloids, tannin and steroidal terpenes. *W. somnifera* and *T. cordifolia* revealed presence of alkaloids, saponin and flavonoids. *T. cordifolia* also showed presence of resins. *A. officinalis* showed presence of resins and tannin. The phytochemical analysis of Shilajit revealed presence of tannin and saponin. In general, presence of carbohydrates and cardiac glycosides was noted in all the plants and the herbomineral. However, anthraquinone was not detected in any of the test material.

**Table-2: Phytochemical analysis of selected plants and
Herbomineral**

	Os	Ws	Tc	Ao	shilajit
Resins	+	-	+	+	-
Alkaloids	+	+	+	-	-
Tannin	+	-	-	+	+
Saponin	-	+	+	-	+
Flavonoids	-	+	-	-	-
Anthraquinone	-	-	-	-	-
Cardiac Glycosides	+	+	+	+	+
Steroidal Ring	+	+	+	+	-
Steroidal Terpenes	+	-	-	-	-
Carbohydrates	+	+	+	+	+

Note: + = Present

- = Absent

Os = *O. sanctum*

Ws = *W. somnifera*

Tc = *T. cordifolia*

Ao = *A. officinalis*

3. Yield of crude extracts:

The selected plant parts were extracted with Distilled Water, Non-polar and Polar solvents. The percent yield of individual extractives was then calculated. (Table-3)

Table-3: Percentage yield of the extractives

Plant / Herbomineral	Yield (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
<i>O. sanctum</i>	8.13	1.68	6.88	4.4
<i>W. somnifera</i>	9.44	0.47	7.08	7.0
<i>T. cordifolia</i>	5.55	1.15	4.63	5.8
<i>A. officinalis</i>	18.56	1.47	14.73	16.0
<i>R. mucronata</i>	22.40	5.98	19.60	24.77
Shilajit	20.86	0.07	17.44	66.0

As shown in Table-3, percent yield of Petroleum Ether extract of Shilajit was very low to pursue further work. Thus, total 23 extracts were subjected to *in vitro* anti-HIV activity.

4. Virus Cultivation:

a) Confirmation of sero-status of HIV patient:

The card test performed to confirm, the sero-status of the HIV positive individuals, revealed development of a single pink band corresponding to the HIV-1.

This indicated that the infection was due to HIV-1 only.

b) Collection of virus stocks:

The virus stocks collected in the form of culture supernatant every fourth and seventh day of each week during co-cultivation were tested for the presence of viral reverse transcriptase by performing reverse transcriptase assay with the help of liquid scintillation counter.

Table-4: Determination of virus titre by Reverse Transcriptase assay

Culture supernatant		CPM	
Week	Day	Non-ART Clinical Isolate	ART Clinical Isolate
First	4 th day	1585	586
	7 th day	1171	393
Second	4 th day	1875	849
	7 th day	1399	468
Third	4 th day	1409	-
	7 th day	1149	-
Fourth	4 th day	1319	-
	7 th day	1082	-

Note: Non-ART: Virus isolated from drug-naïve patient

ART: Virus isolated from drug-treated patient (IDV+d4T+NVP)

In general, the maximum reverse transcriptase activity was observed on fourth day, especially in second week of co-cultivation for both the clinical isolates.

5. Preliminary Screening Assays:

a) XTT Formazan Assay:

For this assay, the result is expressed as 50% Cytotoxic Concentration (CC_{50}) and 50% Effective Concentration (EC_{50}). The selectivity index was also calculated as ratio of CC_{50}/EC_{50} . As per the table-5, CC_{50} value of the extracts ranged from 0.4 mg/ml to 0.0032mg/ml. While, EC_{50} value ranged from 0.08 mg/ml to 0.0032 mg/ml. Aqueous extract of *O. sanctum* showed the highest Selectivity Index (SI) of 125. Other three extracts of *O. sanctum* and aqueous extract of *T. cordifolia* showed SI of 25. Petroleum ether extract of *T. cordifolia* showed SI of 5 while, Ethanol (Direct) and Ethanol (Successive) extracts showed SI as 1. In case of *W. somnifera*, Ethanol (Direct), Ethanol (Successive) and Aqueous extract showed SI of 1, whereas Petroleum ether extract showed SI as 5. Extracts of mangrove plants namely, *A. officinalis* and *R. mucronata* showed SI of 1 and 5 respectively. The herbomineral Shilajit also showed SI of 5. AZT was included as positive control which showed SI as 25.

Table-5 : Comparative study of Cytotoxicity and Antiviral activity

Plant / Herbomineral	Extracts	CC₅₀ (mg/ml)	EC₅₀ (mg/ml)	Selectivity Index (SI)
<i>O. Sanctum</i>	Ethanol (Direct)	>0.08	<0.0032	25
	Petroleum Ether	>0.08	<0.0032	25
	Ethanol (Successive)	>0.08	<0.0032	25
	Aqueous	>0.4	<0.0032	125
<i>W. somnifera</i>	Ethanol (Direct)	>0.016	<0.016	1
	Petroleum Ether	>0.08	<0.016	5
	Ethanol (Successive)	>0.016	<0.016	1
	Aqueous	>0.08	<0.08	1
<i>T. cordifolia</i>	Ethanol (Direct)	>0.016	<0.016	1
	Petroleum Ether	>0.016	<0.0032	5
	Ethanol (Successive)	>0.016	<0.016	1
	Aqueous	>0.08	<0.0032	25
<i>A. officinalis</i>	Ethanol (Direct)	>0.0032	<0.0032	1
	Petroleum Ether	>0.0032	<0.0032	1
	Ethanol (Successive)	>0.0032	<0.0032	1
	Aqueous	>0.016	<0.016	1
<i>R. mucronata</i>	Ethanol (Direct)	>0.016	<0.0032	5
	Petroleum Ether	>0.016	<0.0032	5
	Ethanol (Successive)	>0.016	<0.0032	5
	Aqueous	>0.08	<0.016	5
Shilajit	Ethanol (Direct)	>0.016	<0.0032	5
	Petroleum Ether	NA	NA	NA
	Ethanol (Successive)	>0.016	<0.0032	5
	Aqueous	>0.016	<0.0032	5
Control	AZT	>0.008	<0.00032	25

Note: CC₅₀ : 50% Cytotoxic ConcentrationEC₅₀ : 50% Effective ConcentrationSelectivity Index (SI) : CC₅₀ / EC₅₀

NA : Not Applicable

b) HIV p24 Inhibition Assay:

i) HIV p24 Inhibition by Extracts of *O. sanctum*

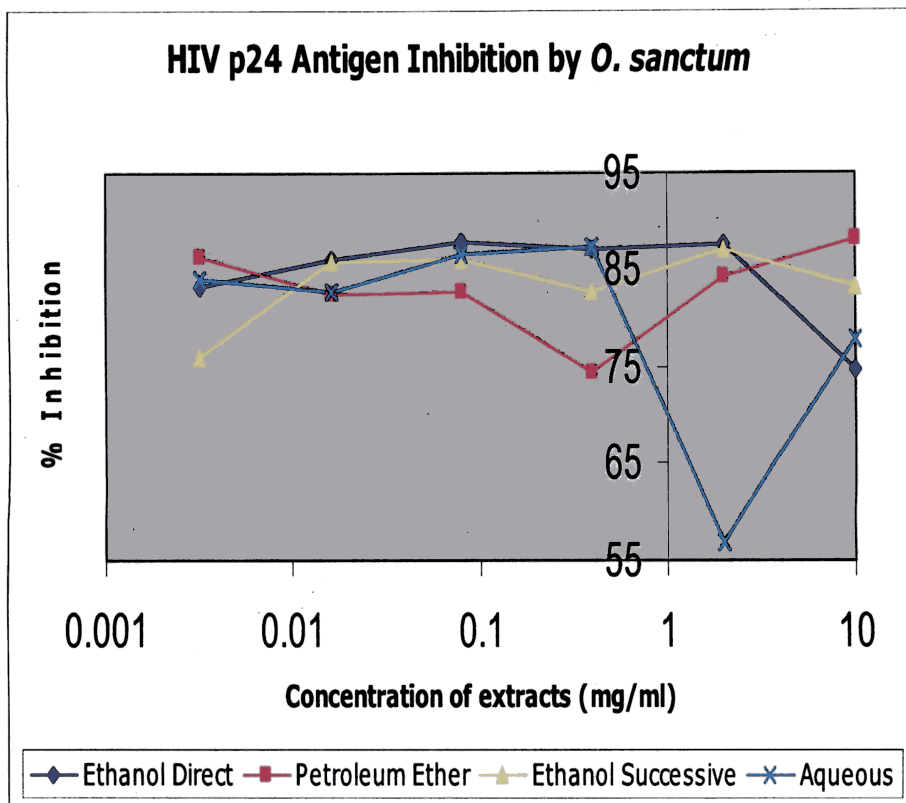
In general, all the extracts showed significant ($\geq 50\%$ Inhibition) inhibition of HIV p24 antigen. Petroleum ether extract of *O. sanctum* showed the highest inhibition of 88.24% at a concentration of 10 mg/ml. Whereas the lowest inhibition of 56.70% was noted with Aqueous extract at a concentration of 2 mg/ml. Among other extracts of *O. sanctum*, Ethanol (Direct) extract showed the highest inhibition (88.07%) at 0.08 mg/ml, Ethanol (Successive) extract at 2 mg/ml (87.25%) and Aqueous extract at 0.4 mg/ml (87.42%).

Table-6: Effect of Extracts of *O. sanctum* on HIV p24 Antigen

Conc. (mg/ml)	HIV p24 Inhibition (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
10	74.51 $\pm$ 1.80	88.24 $\pm$ 0.71	83.33 $\pm$ 1.05	77.78 $\pm$ 1.34
2	87.58 $\pm$ 0.65	84.31 $\pm$ 0.99	87.25 $\pm$ 0.91	56.70 $\pm$ 2.94
0.4	87.09 $\pm$ 0.69	74.35 $\pm$ 1.35	82.84 $\pm$ 1.09	87.42 $\pm$ 0.77
0.08	88.07 $\pm$ 0.72	82.84 $\pm$ 0.98	86.11 $\pm$ 1.22	86.76 $\pm$ 0.81
0.016	86.11 $\pm$ 1.10	82.35 $\pm$ 1.13	85.78 $\pm$ 0.77	82.68 $\pm$ 0.99
0.0032	83.17 $\pm$ 1.07	86.44 $\pm$ 0.83	75.82 $\pm$ 1.48	83.99 $\pm$ 1.24
Control AZT (0.0032)	76.31 $\pm$ 3.54			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

Graph 5 (b)-i: HIV p24 Inhibition by Extracts of *O. Sanctum*



ii) HIV p24 Inhibition by Extracts of *W. somnifera*

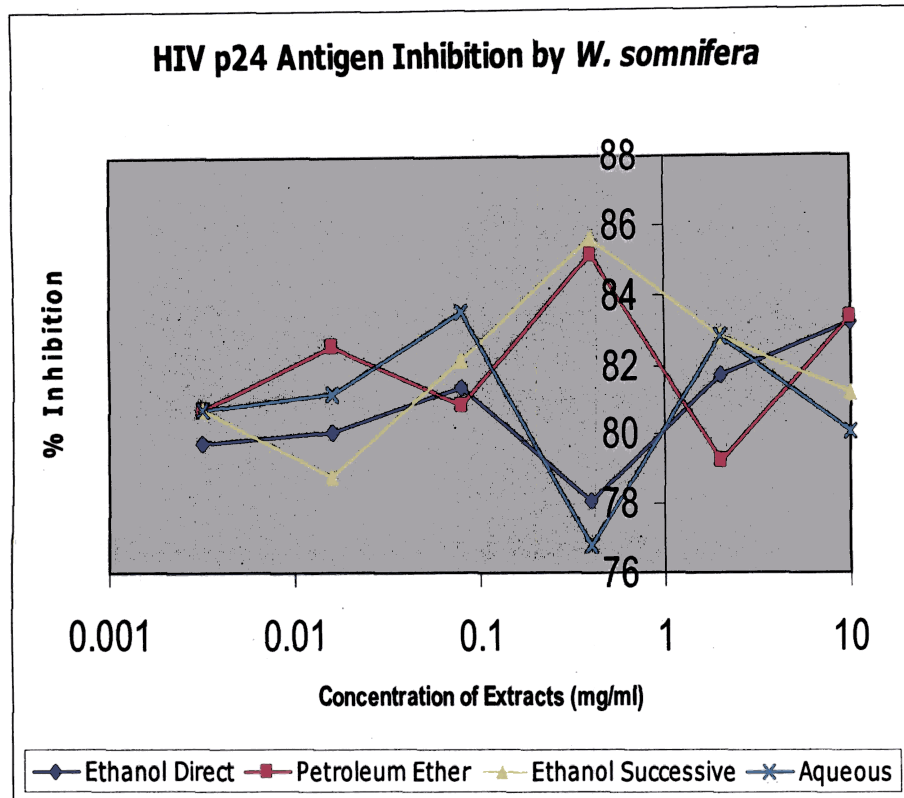
The highest HIV p24 antigen inhibition of 85.62% was seen with Ethanol (successive) extract of *W. somnifera* at a concentration of 0.4 mg/ml. However, Aqueous extract of *W. Somnifera* showed the lowest inhibition of 76.80% at same concentration. Among other extracts of *W. somnifera*; Ethanol (Direct) extract showed the highest inhibition (83.17%) at 1 mg/ml, Petroleum ether extract at 0.4 mg/ml (85.13%) and Aqueous extract at 0.08 mg/ml (83.50%). In general, all the extracts significantly ($\geq 50\%$ Inhibition) inhibited HIV p24 antigen.

Table-7: Effect of Extracts of *W. somnifera* on HIV p24 Antigen

Conc. (mg/ml)	HIV p24 Inhibition (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
10	83.17 $\pm$ 1.20	83.33 $\pm$ 1.05	81.21 $\pm$ 1.21	80.07 $\pm$ 1.41
2	81.70 $\pm$ 1.41	79.25 $\pm$ 1.47	82.84 $\pm$ 1.32	82.84 $\pm$ 1.21
0.4	78.10 $\pm$ 1.55	85.13 $\pm$ 1.16	85.62 $\pm$ 1.02	76.80 $\pm$ 1.87
0.08	81.37 $\pm$ 1.43	80.88 $\pm$ 1.24	82.19 $\pm$ 1.26	83.50 $\pm$ 1.67
0.016	80.07 $\pm$ 1.41	82.52 $\pm$ 1.24	78.76 $\pm$ 1.62	81.21 $\pm$ 1.44
0.0032	79.74 $\pm$ 1.54	80.72 $\pm$ 1.36	80.72 $\pm$ 1.46	80.72 $\pm$ 1.48
Control AZT (0.0032)	76.31 $\pm$ 3.54			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

5 (b)-ii: HIV p24 Inhibition by Extracts of *W. somnifera*



iii) HIV p24 Inhibition by Extracts of *T. cordifolia*

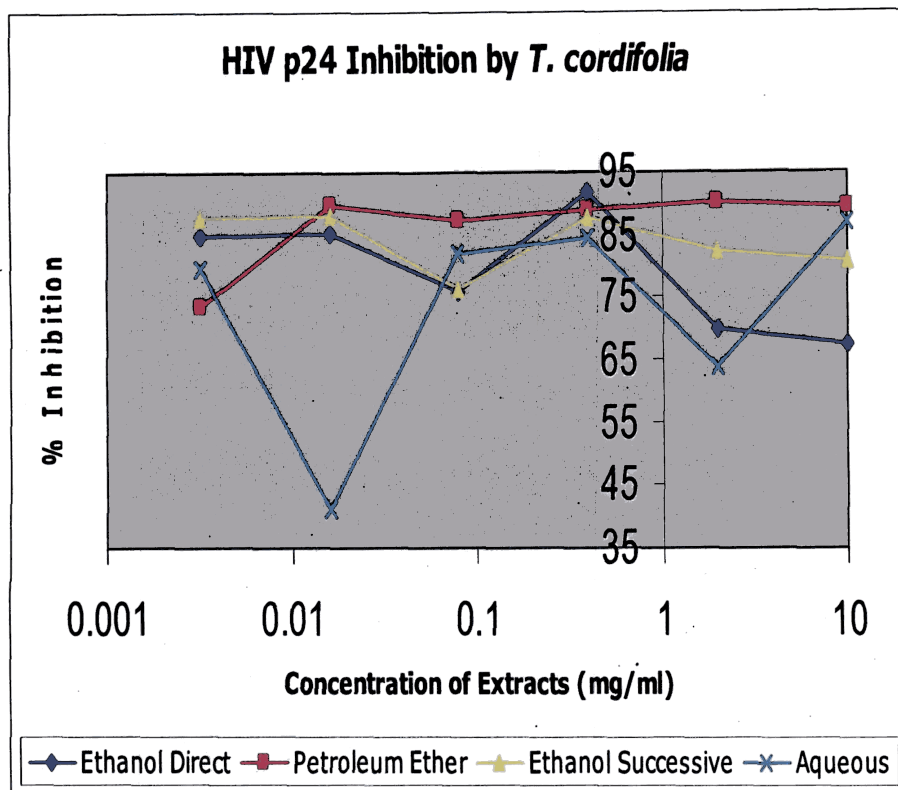
Ethanol (Direct) extract of *T. cordifolia* showed the highest inhibition of HIV p24 antigen with 91.67% at a concentration of 0.4 mg/ml. The lowest inhibition of 41.18% was noted with Aqueous extract at a concentration of 0.016 mg/ml. Among other extracts of *T. cordifolia*, Petroleum ether extract showed the highest inhibition (90.20%) at 2 mg/ml, Ethanol (Successive) extract at 0.016 mg/ml (88.24%) and Aqueous extract at 0.4 mg/ml (84.48%).

Table-8: Effect of Extracts of *T. cordifolia* on HIV p24 Antigen

Conc. (mg/ml)	HIV p24 Inhibition (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
10	67.48 ± 3.92	89.38 ± 2.50	80.88 ± 2.94	86.76 ± 0.35
2	69.93 ± 2.36	90.20 ± 2.09	82.35 ± 1.43	63.73 ± 0.57
0.4	91.67 ± 0.12	88.89 ± 2.81	87.75 ± 2.50	84.48 ± 2.38
0.08	75.82 ± 0.61	87.42 ± 0.04	76.47 ± 2.28	81.86 ± 5.20
0.016	85.46 ± 1.87	89.54 ± 0.04	88.24 ± 2.18	41.18 ± 0.13
0.0032	84.97 ± 1.72	73.53 ± 2.88	87.75 ± 1.57	79.74 ± 3.20
Control AZT (0.0032)	76.31 ± 3.54			

Note: Percent inhibition ≥50% is considered as significant.

5 (b)-iii: HIV p24 Inhibition by Extracts of *T. cordifolia*



iv) HIV p24 Inhibition by Extracts of *A. officinalis*

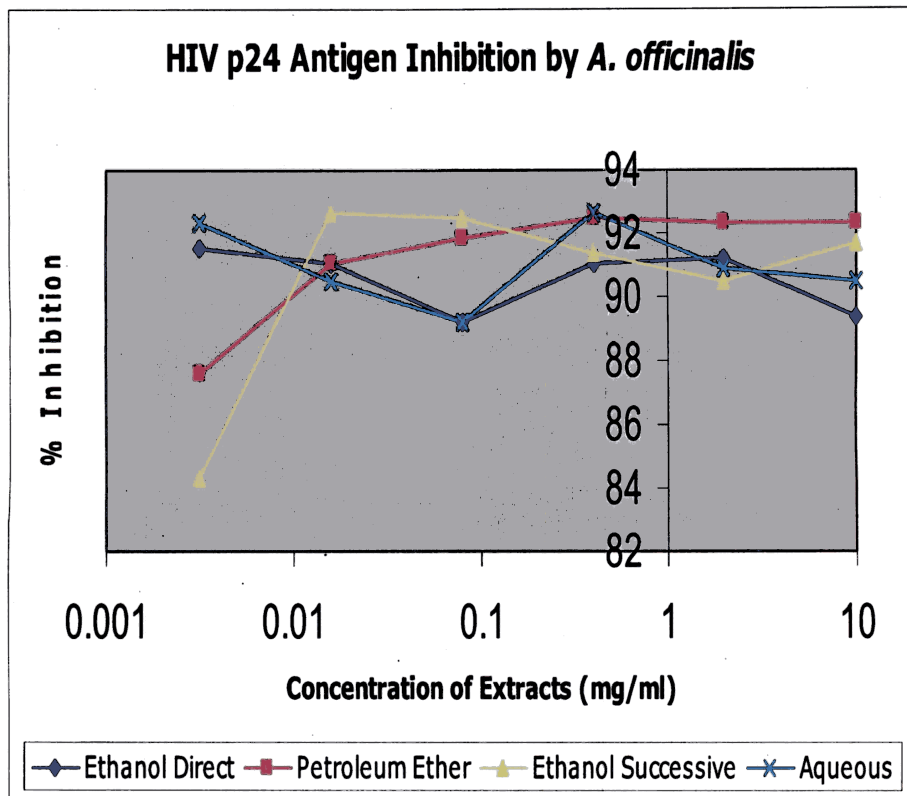
The highest HIV p24 antigen inhibition was seen with Ethanol (Successive) extract of *A. officinalis* at a concentration of 0.016 mg/ml with 92.65%. Aqueous extract of *A. officinalis* also showed similar activity (92.65%) at a concentration of 0.4 mg/ml. Among other two extracts of *A. officinalis*, Ethanol (Direct) extract showed the highest inhibition (91.50%) at 0.0032mg/ml and Petroleum ether extract at 0.4 mg/ml (92.48%). In general, all the extracts of *A. officinalis* significantly ($\geq 50\%$ Inhibition) inhibited HIV p24 antigen at all given concentrations that ranged from 84% to 92%.

Table-9: Effect of Extracts of *A. officinalis* on HIV p24 Antigen

Conc. (mg/ml)	HIV p24 Inhibition (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
10	89.38 $\pm$ 0.28	92.32 $\pm$ 0.31	91.67 $\pm$ 0.23	90.52 $\pm$ 0.55
2	91.18 $\pm$ 0.39	92.32 $\pm$ 0.31	90.52 $\pm$ 0.32	90.85 $\pm$ 0.42
0.4	91.01 $\pm$ 0.40	92.48 $\pm$ 0.41	91.34 $\pm$ 0.26	92.65 $\pm$ 0.28
0.08	89.22 $\pm$ 0.41	91.83 $\pm$ 0.35	92.48 $\pm$ 0.30	89.22 $\pm$ 0.41
0.016	91.01 $\pm$ 0.17	91.01 $\pm$ 0.51	92.65 $\pm$ 0.40	90.52 $\pm$ 0.32
0.0032	91.50 $\pm$ 0.37	87.58 $\pm$ 0.42	84.31 $\pm$ 0.76	92.32 $\pm$ 0.31
Control AZT (0.0032)	76.31 $\pm$ 3.54			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

5 (b)-iv: HIV p24 Inhibition by Extracts of *A. officinalis*



v) HIV p24 Inhibition by Extracts of *R. mucronata*

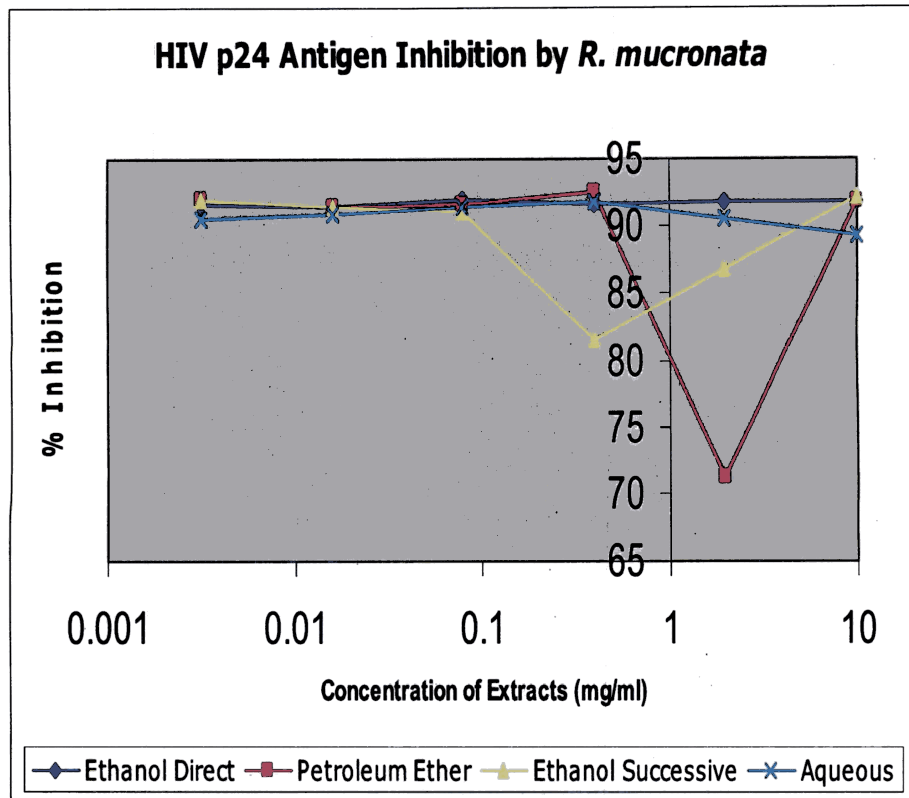
Petroleum ether extract of *R. mucronata* showed the highest inhibition of HIV p24 antigen with 92.48% at a concentration of 0.4 mg/ml. However, the same extract showed the lowest inhibition of 71.24% at a concentration of 2 mg/ml. Among other extracts of *R. mucronata*, Ethanol (Successive) extract showed the highest inhibition (92.16%) at 10 mg/ml and Aqueous extract at 0.4 mg/ml (91.83%). HIV p24 inhibition by Ethanol (Direct) extract was noted in the range of 91.50% to 91.99%.

Table-10: Effect of Extracts of *R. mucronata* on HIV p24 Antigen Inhibition

Conc. (mg/ml)	HIV p24 Inhibition (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
10	91.83 ± 0.35	91.83 ± 0.23	92.16 ± 0.21	89.38 ± 0.1
2	91.83 ± 0.45	71.24 ± 1.68	86.76 ± 0.47	90.52 ± 0.1
0.4	91.67 ± 0.23	92.48 ± 0.41	81.54 ± 0.61	91.83 ± 0.1
0.08	91.99 ± 0.33	91.67 ± 0.36	91.18 ± 0.39	91.50 ± 0.1
0.016	91.50 ± 0.37	91.50 ± 0.48	91.50 ± 0.25	90.85 ± 0.1
0.0032	91.67 ± 0.23	91.99 ± 0.33	91.99 ± 0.21	90.52 ± 0.1
Control AZT (0.0032)	76.31 ± 3.54			

Note: Percent inhibition ≥50% is considered as significant.

5 (b)-v: HIV p24 Inhibition by Extracts of *R. mucronata*



vi) HIV p24 Inhibition by Extracts of Shilajit

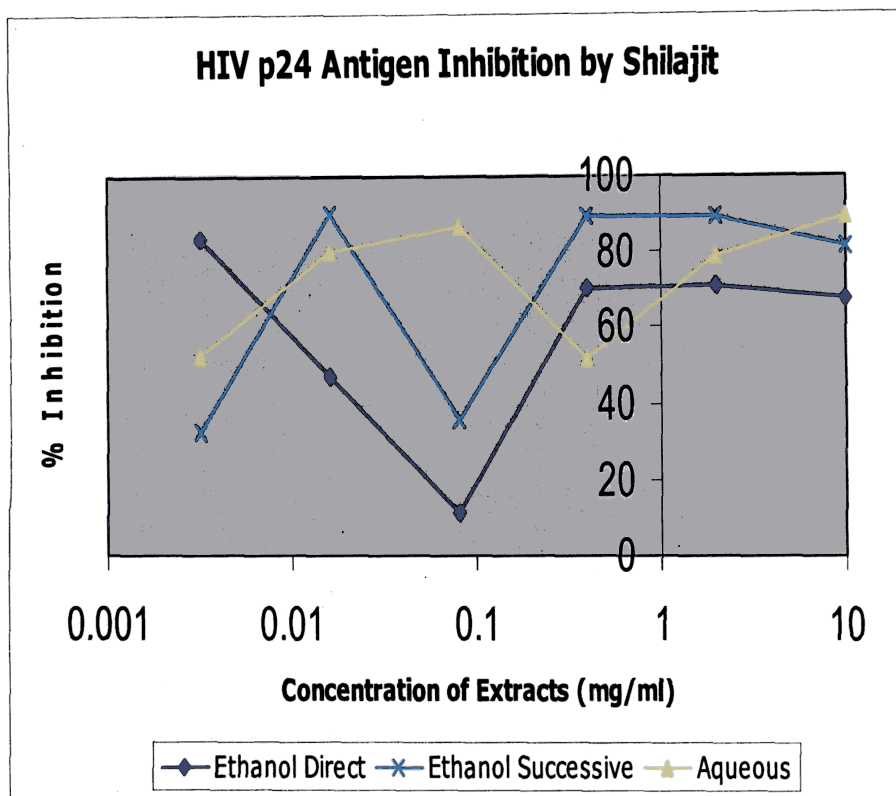
Ethanol (Successive) extract of Shilajit showed the highest inhibition of HIV p24 antigen with 90.20% at a concentration of 0.016 mg/ml, followed by Aqueous extract that showed 89.71% inhibition at a concentration of 10 mg/ml. While, the lowest inhibition of 11.44% was noted with Ethanol (Direct) extract at a concentration of 0.08 mg/ml. However it showed the highest inhibition of 83.50% at a concentration of 0.0032 mg/ml.

Table-11: Effect of Extracts of Shilajit on HIV p24 Antigen

Conc. (mg/ml)	HIV p24 Inhibition (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
10	67.48 ± 1.29	NA	81.21 ± 1.57	89.71 ± 0.15
2	70.75 ± 0.79	NA	89.71 ± 1.25	78.59 ± 0.59
0.4	70.10 ± 1.07	NA	89.71 ± 1.94	51.47 ± 2.16
0.08	11.44 ± 3.59	NA	35.95 ± 2.79	86.60 ± 0.25
0.016	46.73 ± 0.29	NA	90.20 ± 0.10	80.23 ± 0.59
0.0032	83.50 ± 2.20	NA	32.68 ± 3.49	52.12 ± 2.34
Control AZT (0.0032)	76.31 ± 3.54			

Note: Percent inhibition ≥50% is considered as significant.

5 (b)-vi: HIV p24 Inhibition by Extracts of Shilajit



6. Anti-HIV Assays:

a) Reverse Transcriptase (RT) Inhibition Assay:

As per Table-12, nineteen of twenty three extracts showed significant inhibition ($\geq 50\%$ Inhibition) of recombinant HIV-RT. Among Ethanol (Direct), Ethanol (Successive) and Aqueous extracts, Shilajit showed highest inhibition of RNA Dependent DNA Polymerase (RDDP) function of recombinant HIV-RT. Whereas among Petroleum ether extracts *R. mucronata* showed highest inhibition followed by *O. sanctum*. AZT was included as positive control that showed 71.04% inhibition.

Table-12: Inhibition of HIV-RT by Extracts

Plant / Herbo- mineral	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
Os	82.96 $\pm$ 1.20	79.92 $\pm$ 1.22	76.22 $\pm$ 0.96	85.04 $\pm$ 0.04
Ws	18.77 $\pm$ 1.84	68.71 $\pm$ 1.23	46.19 $\pm$ 1.77	32.30 $\pm$ 5.50
Tc	57.27 $\pm$ 1.57	64.48 $\pm$ 0.50	54.29 $\pm$ 1.24	66.03 $\pm$ 0.88
Ao	43.92 $\pm$ 4.06	74.79 $\pm$ 3.47	82.00 $\pm$ 0.26	82.18 $\pm$ 1.72
Rm	81.17 $\pm$ 0.02	87.07 $\pm$ 1.85	84.74 $\pm$ 0.05	82.48 $\pm$ 1.93
Shilajit	83.55 $\pm$ 1.04	NA	89.45 $\pm$ 1.08	86.35 $\pm$ 1.22
AZT [control] (0.0016 mg/ml)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

Os = *O. sanctum*

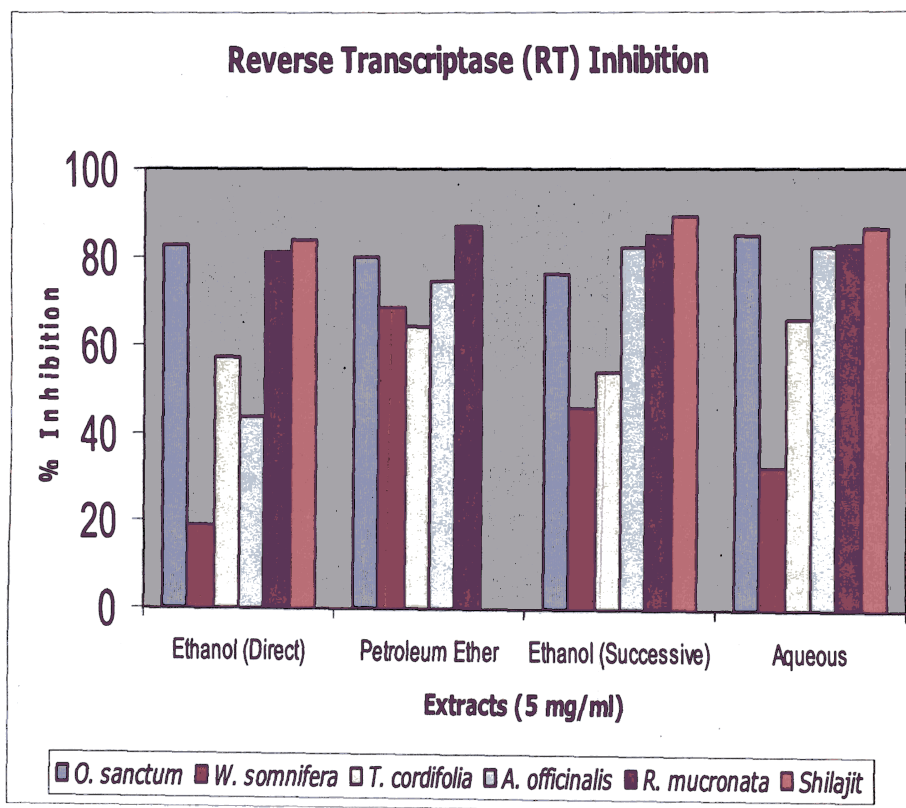
Ws = *W. somnifera*

Tc = *T. cordifolia*

Ao = *A. officinalis*

Rm = *R. mucronata*

Graph 6 (a): Inhibition of HIV-RT by Extracts



b) gp120 Binding Inhibition Assay:

As illustrated in Table-13, among Ethanol (Direct), Petroleum ether and Aqueous extracts, *A. officinalis* showed the highest inhibition where as among Ethanol (successive) extracts, *R. mucronata* showed highest inhibition followed by *T. cordifolia*. In general, only nine of twenty three extracts significantly ($\geq 50\%$ Inhibition) inhibited gp120 binding. Ethanol (Direct) and Petroleum ether extracts of *O. sanctum* and Ethanol (successive) and Aqueous extracts of *W. somnifera* showed no inhibition. Heparin a known gp120/CD4 binding inhibitor was included as positive control that showed 75.96% inhibition.

Table-13: gp120 Binding Inhibition by Extracts

Plant / Herbo- mineral	gp120 Binding Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
Os	0	0	36.85 $\pm$ 0.29	21.24 $\pm$ 0.88
Ws	23.15 $\pm$ 0.71	1.12 $\pm$ 0.63	0	0
Tc	39.44 $\pm$ 0.67	78.54 $\pm$ 0.16	80.22 $\pm$ 0.38	41.91 $\pm$ 0.57
Ao	85.17 $\pm$ 0.19	81.01 $\pm$ 0.30	78.65 $\pm$ 0.23	69.33 $\pm$ 0.37
Rm	49.10 $\pm$ 0.52	69.33 $\pm$ 0.22	81.69 $\pm$ 0.13	60.11 $\pm$ 0.69
Shilajit	35.62 $\pm$ 0.62	NA	28.88 $\pm$ 0.75	26.40 $\pm$ 0.92
Heparin [control] (12.5 U)	75.96 $\pm$ 0.25			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

Os = *O. sanctum*

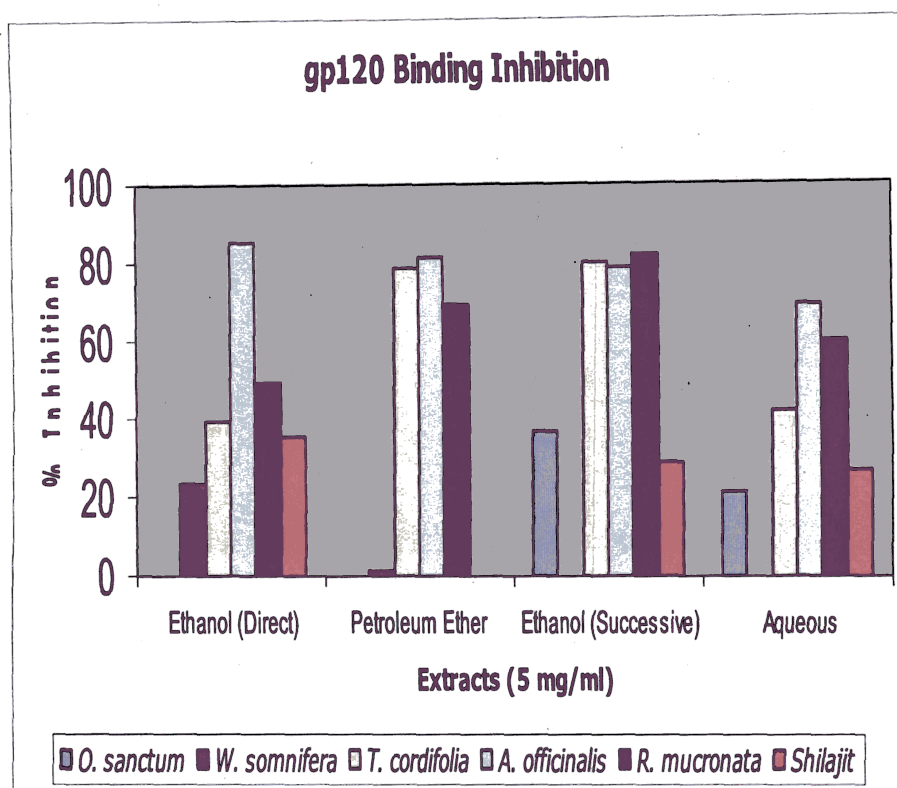
Ws = *W. Somnifera*

Tc = *T. cordifolia*

Ao = *A. officinalis*

Rm = *R. mucronata*

Graph 6 (b): gp120 Binding Inhibition by Extracts



7. a) Dose-Response Study:

i) Reverse transcriptase Inhibition by extracts of *O. sanctum*:

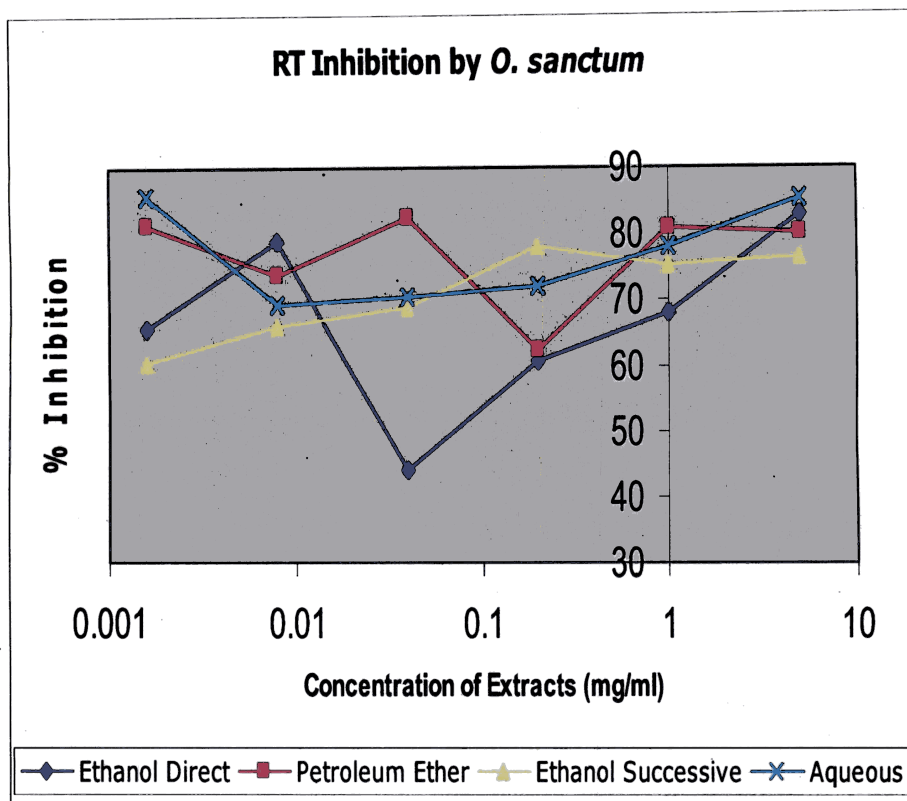
In general, all the extracts showed significant ($\geq 50\%$ inhibition) inhibition of RDDP function of recombinant HIV reverse transcriptase, except Ethanol (Direct) extract that showed 44.04% inhibition at a concentration of 0.04 mg/ml. Aqueous extract of *O. sanctum* showed the highest inhibition of 85.76% at a concentration of 0.0016 mg/ml. Among other extracts, Ethanol (Direct) extract showed the highest inhibition of 82.96% at a concentration of 5 mg/ml, Petroleum ether at 0.04 mg/ml (82.42%) and Ethanol (Successive) extract at 0.2 mg/ml (78.07%).

Table-14: Effect of extracts of *O. sanctum* on HIV-RT

Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
5	82.96 $\pm$ 1.20	79.92 $\pm$ 1.22	76.22 $\pm$ 0.96	85.04 $\pm$ 0.04
1	68.06 $\pm$ 0.66	80.81 $\pm$ 0.62	75.09 $\pm$ 1.48	77.89 $\pm$ 0.67
0.2	60.73 $\pm$ 3.19	62.40 $\pm$ 1.90	78.07 $\pm$ 1.26	72.17 $\pm$ 1.28
0.04	44.04 $\pm$ 6.92	82.42 $\pm$ 0.18	68.95 $\pm$ 0.34	70.50 $\pm$ 0.03
0.008	78.90 $\pm$ 1.45	73.42 $\pm$ 0.17	65.91 $\pm$ 1.66	69.19 $\pm$ 1.34
0.0016	65.61 $\pm$ 1.46	81.35 $\pm$ 0.31	60.55 $\pm$ 1.25	85.76 $\pm$ 0.86
AZT [control] (0.0016)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

**Graph 7 (a)-i: Reverse Transcriptase Inhibition by
*O. sanctum***



ii) Reverse transcriptase Inhibition by extracts of *W. somnifera*:

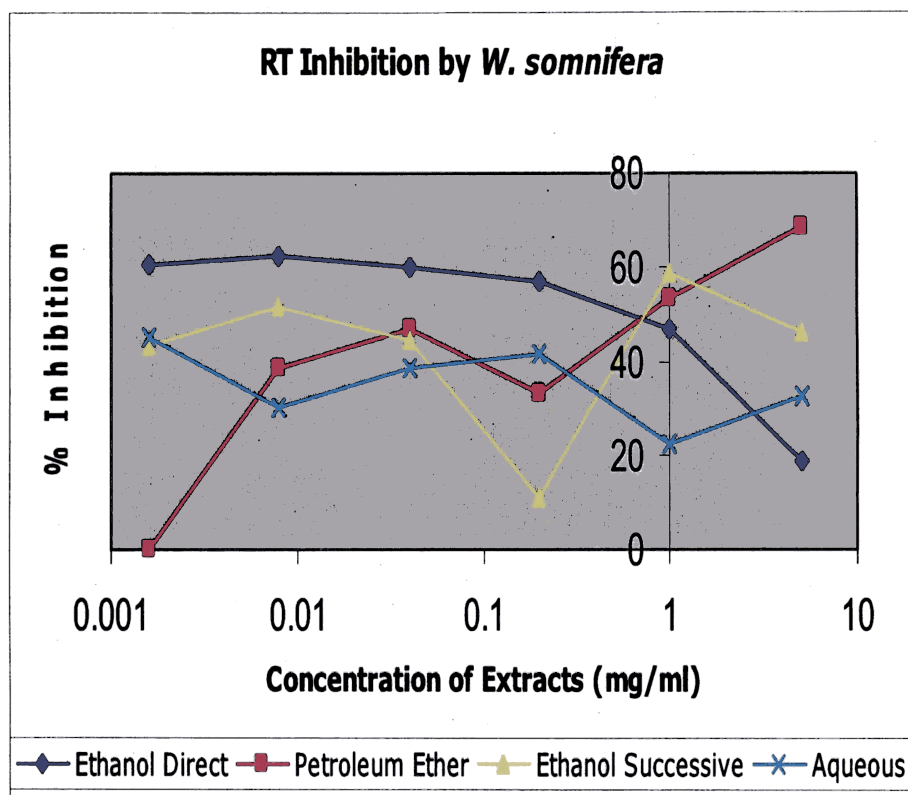
Petroleum ether extract of *W. somnifera* showed the highest inhibition of 68.71% at a concentration of 5 mg/ml. But, it showed no inhibition at a concentration of 0.0016 mg/ml. In general, only four concentrations [0.2 mg/ml to 0.0016 mg/ml] of Ethanol (Direct), two concentrations each of Petroleum Ether [5 mg/ml and 1 mg/ml] and Ethanol (Successive) [1 mg/ml and 0.008 mg/ml] extracts significantly inhibited ($\geq 50\%$ Inhibition) recombinant HIV-RT. Aqueous extract showed no significant inhibition at any concentration tested.

Table-15: Effect of extracts of *W. somnifera* on HIV-RT

Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
5	18.77 $\pm$ 1.84	68.71 $\pm$ 1.23	46.19 $\pm$ 1.77	32.30 $\pm$ 5.50
1	46.78 $\pm$ 2.15	53.58 $\pm$ 1.46	59.00 $\pm$ 1.29	22.05 $\pm$ 3.16
0.2	57.27 $\pm$ 1.23	33.37 $\pm$ 3.08	11.08 $\pm$ 3.88	41.24 $\pm$ 3.14
0.04	60.43 $\pm$ 2.09	46.72 $\pm$ 0.42	44.40 $\pm$ 3.16	38.74 $\pm$ 2.09
0.008	62.46 $\pm$ 1.10	38.68 $\pm$ 1.37	51.61 $\pm$ 0.33	30.15 $\pm$ 4.74
0.0016	60.67 $\pm$ 1.36	0	43.21 $\pm$ 1.41	45.17 $\pm$ 2.85
AZT [control] (0.0016)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

Graph 7 (a)-ii: Reverse Transcriptase Inhibition by *W. somnifera*



iii) Reverse transcriptase Inhibition by extracts of *T. cordifolia*:

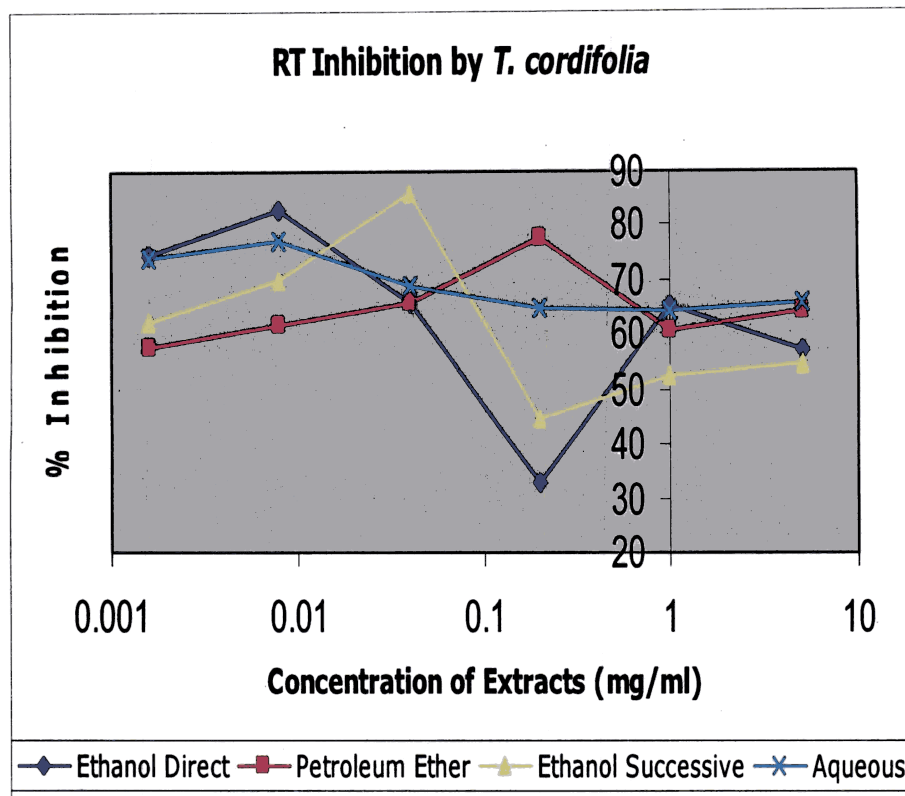
Ethanol (successive) extract of *T. cordifolia* showed the highest inhibition (85.88%) of the viral enzyme at a concentration of 0.04 mg/ml. Ethanol (Direct) and Aqueous extracts showed the highest inhibition at 0.008 mg/ml with 82.54% and 77% respectively. While Petroleum ether extract showed the highest inhibition of 77.53% at a concentration of 0.2 mg/ml. In general, all the extracts showed significant inhibition ($\geq 50\%$ Inhibition) of the RDDP function of HIV-RT, except Ethanol (Direct) and Ethanol (Successive) extracts at concentration of 0.2 mg/ml.

Table-16: Effect of extracts of *T. cordifolia* on HIV-RT

Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
5	57.27 $\pm$ 1.57	64.48 $\pm$ 0.50	54.29 $\pm$ 1.24	66.03 $\pm$ 0.88
1	65.49 $\pm$ 1.04	60.79 $\pm$ 1.00	52.32 $\pm$ 2.72	64.30 $\pm$ 2.28
0.2	32.66 $\pm$ 2.45	77.53 $\pm$ 2.31	44.64 $\pm$ 0.50	64.90 $\pm$ 0.58
0.04	65.91 $\pm$ 0.27	65.97 $\pm$ 1.53	85.88 $\pm$ 0.47	68.71 $\pm$ 2.78
0.008	82.54 $\pm$ 0.97	61.50 $\pm$ 0.45	69.73 $\pm$ 0.45	77.00 $\pm$ 1.83
0.0016	74.67 $\pm$ 0.99	57.75 $\pm$ 2.48	62.28 $\pm$ 1.01	74.08 $\pm$ 0.70
AZT [control] (0.0016)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

**Graph 7 (a)-iii: Reverse Transcriptase Inhibition by
*T. cordifolia***



iv) Reverse transcriptase Inhibition by extracts of *A. officinalis*:

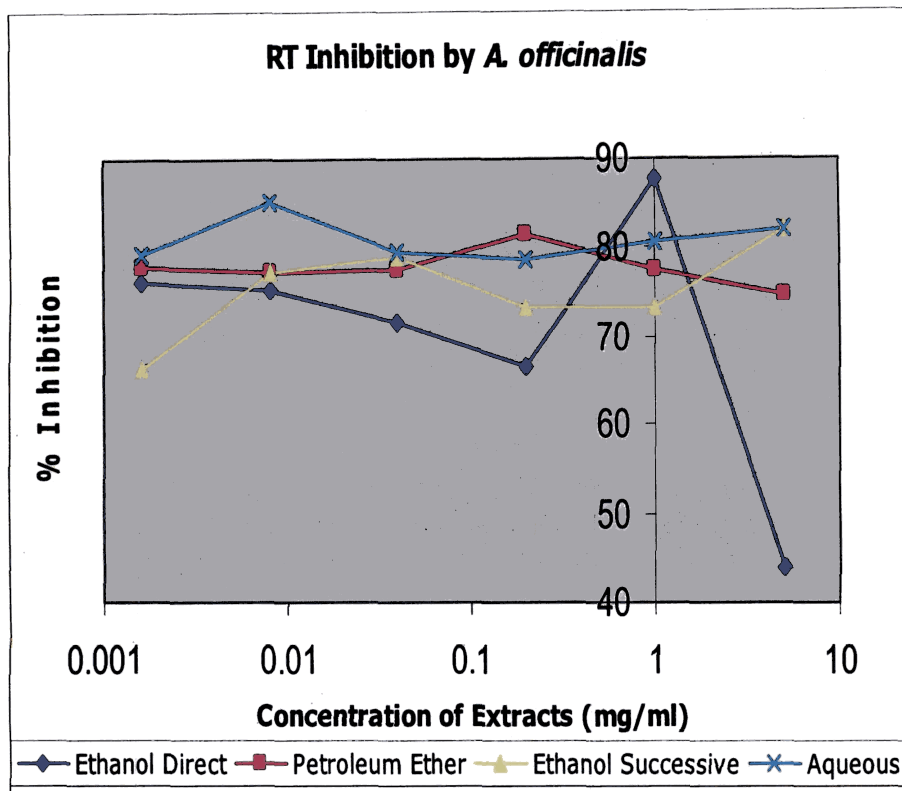
As per the table-17, the highest RT inhibition was seen by Ethanol (Direct) extract of *A. officinalis* with 87.84% at a concentration of 1 mg/ml. Petroleum ether extract showed the highest inhibition of 81.59% at a concentration of 0.2 mg/ml. Ethanol (Successive) extract showed the highest inhibition at a concentration of 5 mg/ml with 82.00%. Whereas, Aqueous extract showed the highest inhibition of 85.10% at a concentration of 0.008 mg/ml. In general, all the extracts showed significant inhibition ($\geq 50\%$ Inhibition) of the RDDP function of HIV-RT, except Ethanol (Direct) extract at a concentration of 5 mg/ml.

Table-17: Effect of extracts of *A. officinalis* on HIV-RT

Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
5	43.92 $\pm$ 4.06	74.79 $\pm$ 3.47	82.00 $\pm$ 0.26	82.18 $\pm$ 1.73
1	87.84 $\pm$ 0.57	77.65 $\pm$ 0.08	73.36 $\pm$ 0.17	80.69 $\pm$ 1.55
0.2	66.51 $\pm$ 2.23	81.59 $\pm$ 1.10	73.30 $\pm$ 2.06	78.84 $\pm$ 0.19
0.04	71.69 $\pm$ 0.35	77.71 $\pm$ 0.46	78.90 $\pm$ 2.60	79.62 $\pm$ 0.68
0.008	75.45 $\pm$ 0.59	77.41 $\pm$ 0.56	77.23 $\pm$ 0.81	85.10 $\pm$ 0.48
0.0016	76.10 $\pm$ 1.68	77.83 $\pm$ 0.54	66.39 $\pm$ 1.77	79.32 $\pm$ 1.08
AZT [control] (0.0016)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

**Graph 7(a)-iv: Reverse Transcriptase Inhibition by
*A. officinalis***



v) Reverse transcriptase Inhibition by extracts of *R. mucronata*:

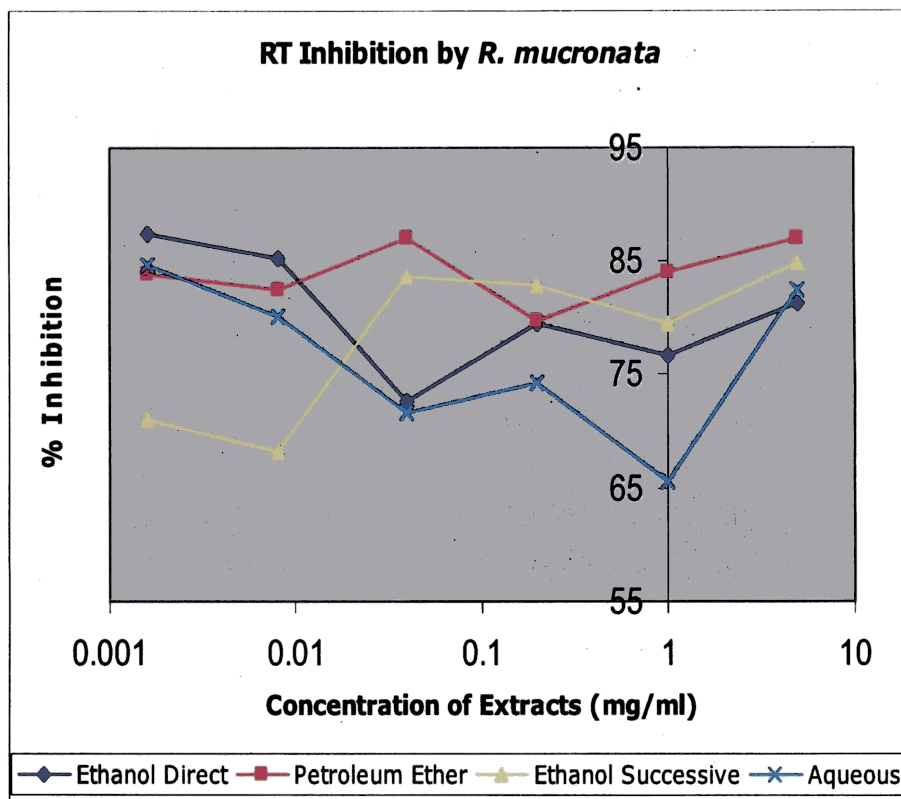
All the extracts of *R. mucronata* significantly ($\geq 50\%$ Inhibition) inhibited RDDP function of HIV-RT at all given concentrations. Ethanol (Direct) and Aqueous extracts showed the highest inhibition of 87.31% and 84.51% respectively at a concentration of 0.0016 mg/ml. Whereas, Petroleum ether and Ethanol (Successive) extracts showed the highest inhibition at a concentration of 5 mg/ml with 87.07% and 84.74% respectively.

Table-18: Effect of extracts of *R. mucronata* on HIV-RT

Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
5	81.17 $\pm$ 0.02	87.07 $\pm$ 1.85	84.74 $\pm$ 0.05	82.48 $\pm$ 1.93
1	76.58 $\pm$ 0.61	83.97 $\pm$ 0.62	79.32 $\pm$ 1.48	65.67 $\pm$ 2.38
0.2	79.50 $\pm$ 1.57	79.68 $\pm$ 0.37	82.72 $\pm$ 0.52	74.14 $\pm$ 0.66
0.04	72.59 $\pm$ 1.28	87.01 $\pm$ 1.09	83.67 $\pm$ 1.15	71.51 $\pm$ 0.72
0.008	85.16 $\pm$ 1.21	82.36 $\pm$ 0.84	68.18 $\pm$ 1.95	80.10 $\pm$ 0.68
0.0016	87.31 $\pm$ 1.34	83.73 $\pm$ 0.28	70.92 $\pm$ 0.16	84.51 $\pm$ 1.98
AZT [control] (0.0016)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

**Graph 7 (a)-v: Reverse Transcriptase Inhibition by
*R. mucronata***



vi) Reverse transcriptase Inhibition by extracts of Shilajit:

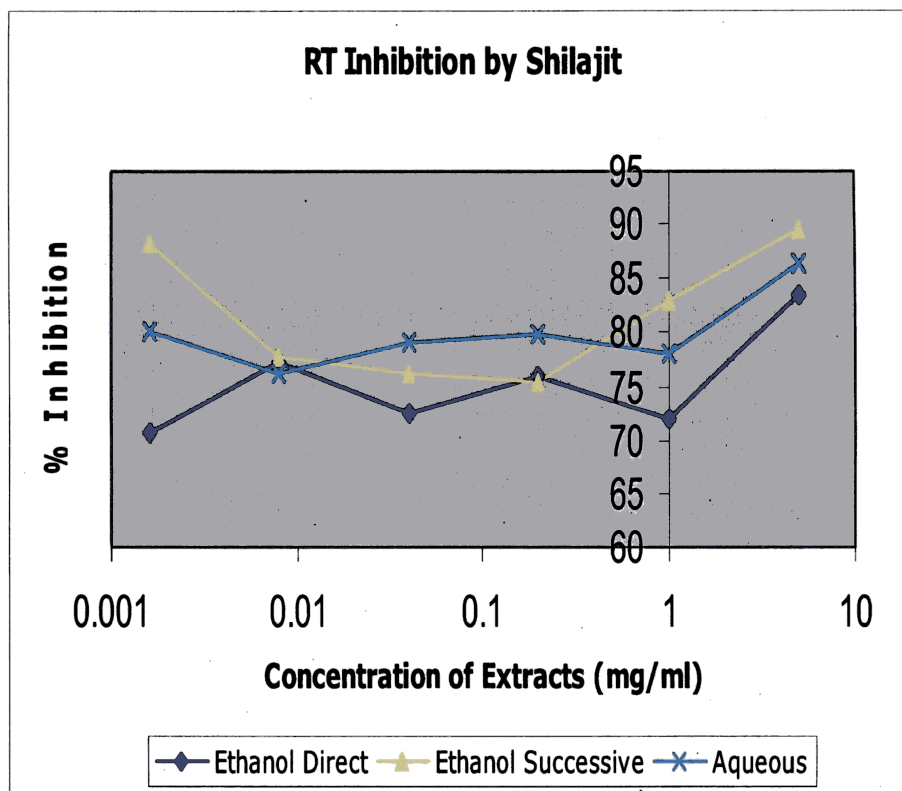
As per table-19, all the 3 extracts of Shilajit significantly ($\geq 50\%$ Inhibition) inhibited the RDDP function of recombinant HIV-RT at all given concentrations. Ethanol (Direct), Ethanol (Successive) and Aqueous extracts showed the highest inhibition at a concentration of 5 mg/ml with 83.55%, 89.45% and 86.35% respectively. Even at lower concentration of 0.0016 mg/ml, all the 3 extracts showed inhibition in the range of 70% to 88%.

Table-19: Effect of extracts of Shilajit on HIV-RT

Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
5	83.55 $\pm$ 1.04	NA	89.45 $\pm$ 1.08	86.35 $\pm$ 1.22
1	72.11 $\pm$ 0.53	NA	83.02 $\pm$ 0.99	78.13 $\pm$ 0.97
0.2	75.92 $\pm$ 0.63	NA	75.51 $\pm$ 0.47	79.80 $\pm$ 0.13
0.04	72.65 $\pm$ 1.20	NA	76.28 $\pm$ 1.42	79.14 $\pm$ 1.66
0.008	77.12 $\pm$ 2.46	NA	77.83 $\pm$ 0.13	76.10 $\pm$ 0.20
0.0016	70.68 $\pm$ 1.10	NA	88.08 $\pm$ 0.87	80.15 $\pm$ 0.04
AZT [control] (0.0016)	71.04 $\pm$ 1.94			

Note: Percent inhibition $\geq 50\%$ is considered as significant.

Graph 7 (a)-vi: Reverse Transcriptase Inhibition by Shilajit



7. b) Effect on clinical isolates:

As illustrated in table-20, Ethanol (Successive) extract of *O. sanctum* showed the highest inhibition of Non-ART isolate whereas Aqueous extract of *R. mucronata* showed the highest inhibition of ART isolate followed by Ethanol (Direct) extract of *A. officinalis*. In general, fourteen of twenty three extracts showed significant inhibition ($\geq 50\%$ inhibition) of Non-ART isolate and twelve of twenty three extracts showed significant inhibition of ART isolate. In general, Ethanol (Successive) extract of *O. sanctum* showed the highest inhibition (76.35%) of Non-ART isolate. Whereas, Aqueous extract of *R. mucronata* showed the highest inhibition (80%) of ART isolate. Only nine of twenty three extracts showed significant inhibition of both the clinical isolates. Especially, all the three extracts of Shilajit showed significant inhibition of both the clinical isolates.

Table-20: Effect of extracts on RT of Clinical Isolates

Plant / Herbomineral	Extracts	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]	
		Non-ART	ART
<i>O. sanctum</i>	Ethanol (Direct)	54.49 $\pm$ 2.27	31.53 $\pm$ 8.75
	Petroleum Ether	44.01 $\pm$ 0.67	24.32 $\pm$ 12.78
	Ethanol (Successive)	76.35 $\pm$ 0.57	63.24 $\pm$ 0.16
	Aqueous	57.49 $\pm$ 2.31	58.74 $\pm$ 5.71
<i>W. somnifera</i>	Ethanol (Direct)	37.28 $\pm$ 4.26	43.24 $\pm$ 0.80
	Petroleum Ether	46.41 $\pm$ 0.99	17.48 $\pm$ 4.50
	Ethanol (Successive)	46.26 $\pm$ 0.49	45.05 $\pm$ 8.58
	Aqueous	50.00 $\pm$ 3.07	16.22 $\pm$ 6.01
<i>T. cordifolia</i>	Ethanol (Direct)	57.19 $\pm$ 0.40	24.68 $\pm$ 5.20
	Petroleum Ether	51.80 $\pm$ 0.41	16.40 $\pm$ 10.34
	Ethanol (Successive)	64.52 $\pm$ 1.53	49.73 $\pm$ 0.62
	Aqueous	55.84 $\pm$ 2.37	65.05 $\pm$ 0.09
<i>A. officinalis</i>	Ethanol (Direct)	10.03 $\pm$ 3.92	79.46 $\pm$ 2.91
	Petroleum Ether	27.84 $\pm$ 1.51	31.89 $\pm$ 6.30
	Ethanol (Successive)	59.73 $\pm$ 1.05	65.59 $\pm$ 2.98
	Aqueous	43.86 $\pm$ 0.17	76.22 $\pm$ 2.72
<i>R. mucronata</i>	Ethanol (Direct)	49.10 $\pm$ 2.74	75.86 $\pm$ 0.38
	Petroleum Ether	30.69 $\pm$ 0.73	49.01 $\pm$ 6.08
	Ethanol (Successive)	58.23 $\pm$ 0.97	60.54 $\pm$ 1.85
	Aqueous	70.36 $\pm$ 1.06	80.00 $\pm$ 3.63
Shilajit	Ethanol (Direct)	65.12 $\pm$ 0.79	70.09 $\pm$ 3.13
	Petroleum Ether	NA	NA
	Ethanol (Successive)	56.89 $\pm$ 1.94	74.41 $\pm$ 0.63
	Aqueous	55.24 $\pm$ 2.49	67.03 $\pm$ 0.71
Control	AZT (0.0016 mg/ml)	26.50 $\pm$ 2.04	38.02 $\pm$ 10.42

Note: Percent inhibition $\geq 50\%$ is considered as significant.

Non-ART: Virus isolated from drug-naïve patient

ART: Virus isolated from drug-treated patient (IDV+d4T+NVP)

7. c) Time-coarse study:

When immobilized CD4 were pre-treated with the extracts, the 3 extracts of *R. mucronata* namely, Petroleum ether, Ethanol (Successive) and Aqueous showed the highest inhibition of gp120/CD4 interaction with 86.74%, 87.53% and 90.11% respectively. Among Ethanol (Direct) extracts, *R. mucronata* and *A. officinalis* showed same inhibition of 85.62%. Where as when the extracts were added after gp120/CD4 interaction in a separate experiment, Aqueous extract of *R. mucronata* blocked gp120/CD4 interaction with the highest inhibition of 94.61% by displacing pre-adsorbed gp120. Among other extracts, Ethanol (Direct) extract of *W. somnifera* (89.55%), Petroleum ether extract of *T. cordifolia* (87.42%) and Ethanol (Successive) extract of Shilajit (91.80%) showed the highest inhibition of gp120/CD4 interaction by displacing pre-adsorbed gp120. The positive control Heparin showed 87.64% and 58.09% inhibition when added before and after gp120/CD4 interaction respectively.

Table-21: Effect of extracts on gp120/CD4 interaction

Plant/ Herbomineral	Extracts	gp120/CD4 Interaction Inhibition (%) [Mean $\pm$ SD]	
		Addition of Extracts Before Interaction	Addition of Extracts After Interaction
<i>O. Sanctum</i>	Ethanol (Direct)	70.34 $\pm$ 0.45	84.94 $\pm$ 0.05
	Petroleum Ether	68.09 $\pm$ 0.63	7.75 $\pm$ 1.53
	Ethanol (Successive)	78.20 $\pm$ 0.23	74.27 $\pm$ 0.42
	Aqueous	78.43 $\pm$ 0.15	90.67 $\pm$ 0.07
<i>W. somnifera</i>	Ethanol (Direct)	21.57 $\pm$ 1.20	89.55 $\pm$ 0.08
	Petroleum Ether	75.73 $\pm$ 0.25	37.75 $\pm$ 0.52
	Ethanol (Successive)	67.30 $\pm$ 0.31	69.44 $\pm$ 0.21
	Aqueous	68.54 $\pm$ 0.38	87.53 $\pm$ 0.08
<i>T. cordifolia</i>	Ethanol (Direct)	61.69 $\pm$ 0.11	1.69 $\pm$ 1.50
	Petroleum Ether	76.18 $\pm$ 0.33	87.42 $\pm$ 0.17
	Ethanol (Successive)	64.04 $\pm$ 0.42	69.10 $\pm$ 0.30
	Aqueous	78.88 $\pm$ 0.39	35.62 $\pm$ 0.78
<i>A. officinalis</i>	Ethanol (Direct)	85.62 $\pm$ 0.18	66.97 $\pm$ 0.40
	Petroleum Ether	81.01 $\pm$ 0.37	83.71 $\pm$ 0.28
	Ethanol (Successive)	83.03 $\pm$ 0.28	91.35 $\pm$ 0.14
	Aqueous	55.28 $\pm$ 0.47	88.65 $\pm$ 0.16
<i>R. mucronata</i>	Ethanol (Direct)	85.62 $\pm$ 0.02	88.20 $\pm$ 0.16
	Petroleum Ether	86.74 $\pm$ 0.17	85.73 $\pm$ 0.10
	Ethanol (Successive)	87.53 $\pm$ 0.17	88.65 $\pm$ 0.33
	Aqueous	90.11 $\pm$ 0.07	94.61 $\pm$ 0.04
Shilajit	Ethanol (Direct)	73.60 $\pm$ 0.03	85.28 $\pm$ 0.18
	Petroleum Ether	NA	NA
	Ethanol (Successive)	47.19 $\pm$ 0.22	91.80 $\pm$ 0.10
	Aqueous	72.36 $\pm$ 0.20	94.38 $\pm$ 0.04
Control	Heparin (12.5 Units)	87.64 $\pm$ 0.17	58.09 $\pm$ 0.46

Note: Percent inhibition $\geq 50\%$ is considered as significant.

7. d) Combinatorial effect:

Different combinations of aqueous extracts of 5 plants and the herbomineral were prepared by mixing them in equal proportion and a mixture of all the 6 extracts was also prepared to study their combinatorial effect on HIV-RT. Non-ART (virus isolated from drug-naïve patient) clinical isolate was used as virus source for this study.

As illustrated in Table-22, combination of *A. officinalis* and Shilajit showed the highest inhibition (65.84%) of RDDP function of HIV-RT, followed by mixture of all the extracts that showed 65.61% inhibition. Whereas, combination of *W. somnifera* and *T. cordifolia* showed the lowest inhibition (21.32%) of the viral enzyme. In general, fourteen of sixteen combinations significantly ($\geq 50\%$ inhibition) inhibited the viral enzyme.

Table-22: Combinatorial effect of Extracts on HIV-RT

Combinations of Extracts	Reverse Transcriptase Inhibition (%) [Mean ± SD]
<i>O. sanctum</i> + <i>W. somnifera</i>	52.41 ± 1.46
<i>O. sanctum</i> + <i>T. cordifolia</i>	56.42 ± 2.07
<i>O. sanctum</i> + <i>A. officinalis</i>	57.44 ± 3.51
<i>O. sanctum</i> + <i>R. mucronata</i>	53.78 ± 0.93
<i>O. sanctum</i> + Shilajit	57.13 ± 2.09
<i>W. somnifera</i> + <i>T. cordifolia</i>	21.32 ± 4.83
<i>W. somnifera</i> + <i>A. officinalis</i>	60.24 ± 1.86
<i>W. somnifera</i> + <i>R. mucronata</i>	60.55 ± 1.70
<i>W. somnifera</i> + Shilajit	45.31 ± 2.22
<i>T. cordifolia</i> + <i>A. officinalis</i>	59.40 ± 1.74
<i>T. cordifolia</i> + <i>R. mucronata</i>	59.71 ± 4.76
<i>T. cordifolia</i> + Shilajit	65.49 ± 3.05
<i>A. officinalis</i> + <i>R. mucronata</i>	62.43 ± 2.06
<i>A. officinalis</i> + Shilajit	65.84 ± 1.03
<i>R. mucronata</i> + Shilajit	65.54 ± 3.27
Mixture (<i>O. sanctum</i> + <i>W. somnifera</i> + <i>T. cordifolia</i> + <i>A. officinalis</i> + <i>R. mucronata</i> + Shilajit)	65.61 ± 0.28
AZT [control] (0.0016mg/ml)	25.80 ± 1.06

Note: Percent inhibition ≥50% is considered as significant.

8. Preliminary Thin Layer Chromatography (TLC) Study:

In order to estimate number of components that can possibly be obtained from each crude extract, the extracts were analyzed on Thin Layer Chromatography using different solvents and their combinations.

As illustrated in Table-23, Petroleum ether extract of *A. officinalis* showed 2 spots with the R_f value of 0.18 and 0.36 respectively and Ethanol (Successive) extract of *A. officinalis* showed one spot with R_f value of 0.18 with Benzene and Chloroform solvent system in the ratio (40 : 60). Ethanol (Successive) extract of *R. mucronata* showed 2 spots with Benzene system with R_f value of 0.24 and 0.18 respectively. Whereas, Petroleum ether extract of *R. mucronata* and Ethanol (Successive) extract of Shilajit showed trailing (no good separation of spots) with any of the solvents or their combinations used.

Aqueous extracts of *A. officinalis*, *R. mucronata* and Shilajit were dissolved in Ethanol individually and spotted on TCL plates. But no movement of the spots was observed with any of the solvents or their combinations.

Table-23: TLC study of Plants and Herbomineral

Plant/ Herbomineral	Extracts	Prominent Solvent System	Spots	Rf Value
<i>A. officinalis</i>	Petroleum Ether	Benzene and Chloroform in the ratio (40:60)	2	0.18 0.36
	Ethanol (Successive)		1	0.18
	Aqueous	-	-	-
<i>R. mucronata</i>	Petroleum Ether	-	-	-
	Ethanol (Successive)	Benzene	2	0.24 0.18
	Aqueous			-
Shilajit	Ethanol (Successive)	-	-	-
	Aqueous	-	-	-

DISCUSSION

Human Immunodeficiency Virus (HIV) is the etiological agent of Acquired Immunodeficiency Syndrome (AIDS) that has created a major health care problem not only in India but also globally. The current strategy for the treatment of HIV infection is Highly Active Antiretroviral Therapy (HAART), which is based on combination of reverse transcriptase and protease inhibitors. A recent study of patients visiting a non-government provider in South India, estimated the median annual cost of treatment of AIDS patients to be of the order of rupees 6000 over a reference period of 6 months, with the treatment of patients on antiretroviral drugs averaging to rupees 18,150 over the same period. Another study from Delhi, suggests similar estimates of about rupees 30000 for antiretroviral treatment over a period of 1 year and another rupees 10000 annually for the monitory tests.⁷⁸ Although HAART has reduced death from AIDS-related diseases, it is still an expensive regime, often not well tolerated and leads to drug resistance. Moreover the vaccine development is also hampered due to high rate of spontaneous mutation of the virus. Most importantly, the ability of HIV to establish latent reservoirs early in the course of infection ensures the persistence of this pathogen even in the face of intensive drug therapy and vigorous antiviral immune responses. Besides, HIV/AIDS affects individuals in prime-working age group which has a large negative effect on domestic as well as national income. In Sri Lanka and India, lost lifetime earnings due to an AIDS death were estimated to be more than 10 times the annual treatment costs of AIDS. It also affects savings of government as owing to increased spending on treatment and prevention related to HIV/AIDS reduces budgets for road construction and maintenance and other infrastructure services.⁷⁸ Also,

Mother to child transmission is resulting in increasing number of orphans and children living with HIV that makes HIV/AIDS more of social, emotional problem than mere economical burden. Thus, there is a need for the discovery of novel (safe, effective and cheap) therapeutic strategy.

In the past decade, considerable attention has been paid to screening of plant extracts for possible anti-HIV activity with the aim of isolating bioactive compounds as an alternative source to chemical synthesis. Screening of plant extracts for antiretroviral activity has given encouraging results in that most plant-derived anti-HIV compounds inhibit replication of the virus by interfering with one or more of the ten steps of replicative cycle.

The therapeutic efficacy of a handful of these compounds, such as benzyloquinoline alkaloid, papaverine and the saponin glycyrrhizin has been studied in AIDS patients. It is evident, therefore, that plants could be useful sources or leads for the discovery of novel anti-HIV compounds.¹⁹⁴

Traditional knowledge will serve as a powerful search engine and most importantly, will greatly facilitate intentional, focused and safe natural product research to rediscover the drug discovery process. Also traditional knowledge-driven drug development can follow a reverse pharmacology path and reduce time and cost of development. Ayurveda remains one the most ancient and yet living traditions practiced widely in Indian sub-continent and has a sound philosophical, experiential and experimental basis. It aims at holistic management of health and disease. Plants form a

dominant part of Ayurvedic pharmacopoeia. *Charak Samhita* and *Sushrut Samhita* (100-500 B. C.) are main Ayurvedic classics, which describe over 700 plants along with their classification, pharmacological and therapeutic properties. *Charaka Samhita* consists of 8 sections divided into 150 chapters and lists 341 plants and plant products for use in medicine. Whereas, *Sushrut Samhita* has 6 sections covering 186 chapters and describes 395 medicinal plants, 57 drugs of animal origin and 64 minerals and metals as therapeutic agents. All these ancient texts on Ayurveda, divide medical knowledge into 8 branches. Rasayana therapy is one of the 8 branches and generally consists of nourishing and rejuvenating drugs with multiple applications for longevity and immunomodulation. *Withania somnifera* (Ashwagandha), *Ocimum sanctum* (Tulas), *Tinospora cordifolia* (Gulvel), *Asparagus racemosus* (Shatavari), *Tribulus terrestris* (Gokshura), *Piper longum* (Pippali), *Terminalia chebula* (Haritaki) and Shilajit are classified as *Rasayana* in Ayurveda. They strengthen the host's general resistance by stimulating the immune function, a concept similar to 'prohost therapy' of modern medicine. There is, thus, a remarkable similarity between the Ayurvedic concept of *rasayana* and the modern concept of a new class of plant-derived drugs. The Ayurvedic treatment is no longer different from the contemporary approach. Likewise, Ayurvedic description of a disease is also very much like the modern description. From this brief introduction, it is obvious that Ayurveda is a cogent, scientifically organized discipline.^{114,195,196,197}

Besides medicinal plants, mangrove plants are also used in folklore medicine for treatment of several diseases.¹²⁸ Mangroves are the highly productive ecosystem with various important economic and environmental

functions. Even though, majority of natural drugs are derived from plant origin, a few of them, obtained from mineral sources, such as Shilajit, are of paramount significance as pharmaceutical aids.¹³⁰

Thus for the present *in vitro* study, the plants were selected on citations in the literature on Indian Medicinal plants and also those commonly used in folk medicine. Root powder of *Withania somnifera* (Ashwagandha), stem powder of *Tinospora cordifolia* (Gulvel), leaves powder of *Ocimum sanctum* (Tulas) and the two mangrove species namely *Avicennia officinalis* and *Rhizophora mucronata* were used in the present investigation. Shilajit (Asphaltum), a herbomineral was also included for the *in vitro* study.

Due to financial constrains, lack of infrastructure and expertise, we had to restrict our work to virological aspect and that put limit on comprehensive phytochemical analysis. Thus for this preliminary study we just focused on the whole crude preparations and the emphasis was given to investigations of the distinctive antiviral activity.

Phytochemicals are the chemicals extracted from plants. These chemicals are classified as primary or secondary constituents, depending on their role in plant metabolism. Primary constituents include the common sugars, amino acids, proteins, purines and pyrimidines of nucleic acids, chlorophyll's etc that are needed for basic life functions. Secondary constituents are the remaining plant chemicals such as alkaloids (derived from amino acids), terpenes (a group of lipids) and phenolics (derived

from carbohydrates).¹⁹⁸ In many cases, these substances serve as plant defense mechanisms against predation by microorganisms, insects and herbivores. Some, such as terpenoids, give plants their odors, others (quinones and tannins) are responsible for plant pigment. Many compounds are responsible for plant flavor (eg. the terpenoid capsaicin from chilli peppers).¹⁴⁴

Before carrying out any phytochemical work, the botanical identity of the plant under investigation should be properly authenticated. Usually plants should be dried before extraction. Drying can be carried out by artificial heat or open air. Usually drying at low temperature is preferred to avoid any chemical change or loss of activity. Once thoroughly dried, plants can be stored before analysis in a suitable container for long periods of time.

The precise mode of extraction depends on the nature of the plant material and the components to be isolated. Any part of the plant may contain active components. For instance, the roots of ginseng plants contain the active saponins and essential oils, while eucalyptus leaves are harvested for their essential oils and tannins.¹⁴⁴ Dried plant materials are usually powdered before extraction whereas fresh plant material can be homogenized or macerated with a solvent such as alcohol.

Alcohol is a general solvent for many plant constituents. However, from the alcohol extract, subsequent elimination of non-polar components such as pigments, resins become difficult. Thus initially defatting (separation of lipids and terpenoids) of the plant material is usually carried out using non-polar solvent such as petroleum ether. Extraction may be performed by repeated maceration with frequent agitation, percolation or continuous

extraction. The classical procedure for obtaining organic constituents from the plant tissue is to continuously extract coarse powder of plant material in a Soxhlet extractor with a range of solvents, starting in turn with petroleum ether, ether (to separate essential and fixed oils, steroids), chloroform (alkaloids, quinones) and then with ethylacetate and alcohol (for more polar compounds). The extract obtained is clarified by filtration and is then concentrated *in vacuo* at low temperature.¹⁹⁹

Four different extracts were prepared from each plant and the herbomineral. The extraction has been carried out using Petroleum ether (60-80°C), Ethanol and Distilled water. Thus alkaloids, terpenoids, coumarins and fatty acids get extracted in Petroleum ether. Ethanol can be used to extract tannins, polyphenols, flavonol, terpenoids, alkaloids, polyacetylenes and sterols. Whereas water can be used to extract anthocyanins, tannins, saponins, terpenoids, starches, lectins and polypeptides.¹⁴⁴

Alkaloids are widely distributed and naturally occurring in plants. Most of the alkaloids from plants are used for medicinal purpose. Alkaloids are chemical constituents from plants that can work on the nervous system of the human body and used for analgesic, antispasmodic and bacterial effects. Tannins are antiseptic in nature; they have astringent properties and can hasten healing of wounds in an inflamed membrane. They are free from the attack of parasitic fungi and insects. Tannins can be used in this capacity to give the human body a resistance against parasites. Tannic acid has been found to have anti-bacterial, antiseptic, astringent, antiulcer and antiviral properties. Flavonoids, on the other hand, are

potent water-soluble antioxidants and free radical scavengers, which prevent oxidative cell damage and have strong anti-cancer activity. As antioxidants flavonoids from these plants provide anti-inflammatory activity. Saponin possesses specific physical, chemical and biological activities that make them useful as drugs. Some of these biological properties include antimicrobial and anti-inflammatory effects.²⁰⁰

All these phytochemicals are also capable of exhibiting anti-HIV activity. Alkaloids such as Michellamine B isolated from leaves of *Ancistroclaudus karupensis* acts by inhibiting HIV Reverse Transcriptase as well as by inhibiting cellular fusion and syncytium formation.¹⁵⁰ The mannose-specific plant lectins from *Cymbidium* hybrid, *Epipactis helleborine*, *Hippeastrum* hybrid and *Listeria ovata* and N-acetylglucosamine-specific plant lectins from *Urtica dioica* likely interfere with the virus-cell fusion process and it is assumed that they interact with specific glycosylation sites within the viral glycoproteins gp120 and/or gp41, particularly those sites that are rich in mannose or N-acetylglucose.^{134,135} Seed protein MAP-30 isolated from *Momordica charantia* and protein TAP-29 isolated from roots of *Trichosanthus kirilowii* have shown dose dependent inhibition of cell free HIV infection and replication with no cytotoxic effects.^{151,152,153} Terpenoids such as carnosolic acid isolated from *Rosmarinus officinalis* and ursolic acid isolated from *Geum japonicum* have exhibited anti-HIV activity by inhibiting HIV protease enzyme.^{201,202} Coumarin such as coriandrin isolated from *Coriandrum sativum* (coriander) showed anti-HIV activity by inhibiting HIV Reverse Transcriptase.¹⁴⁴ Escins, the saponin extracted from the seeds of *Aesculus chinensis* was found to show moderate anti-HIV-1 protease activity.²⁰³ Tannins, epicatechingallate and

epigallocatechingallate isolated from *Camellia sinensis* showed anti-HIV activity by inhibiting HIV-RT.²⁰⁴ Flavonoids are well-known inhibitors of enzymes that are essential for HIV replication. For instance, Swertifrancheside isolated from *Swertia franchetiana* have exhibited HIV-RT inhibitory activity,¹³⁸ while Quercetin isolated from *Quercus rubra* exhibited HIV integrase inhibitory activity.¹⁴⁰

A variety of extraction procedures and their comparative performance have been studied extensively and many studies reported Soxhlet extraction to be the most efficient among them.

There are studies that have compared the efficacy of extraction by applying different extraction techniques as well as by varying different physiological parameters. Also, studies have reported that the extraction temperature alters the phytochemical concentration.²⁰⁵

Comparison of yield, composition and antioxidant activity of turmeric (*Curcuma longa* L.) extracts obtained using various techniques carried out by Braga et al²⁰⁶ revealed that maximum amount of curcuminoids was obtained using Soxhlet extraction (ethanol/isopropyl alcohol) and exhibited the strongest antioxidant activities.

Room temperature, heat reflux, soxhlet, ultrasound-assisted and microwave-assisted extractions were employed to extract flavonoids from dry cell cultures of *Saussurea medusa* Maxim. It was observed that soxhlet extraction yielded more flavonoids as compared to room temperature.²⁰⁷

Another study carried out by Chukwumah et al²⁰⁸, has compared 4 methods of extraction (stirring, sonication, soxtec and microwave-assisted

sonication [MAS]) for runner peanuts. It has been reported that, MAS and soxtec methods extracted significantly higher amounts of the phytochemicals.

Consequently, for the preparation of crude extracts, the powders of selected plants and the herbomineral were subjected to Soxhlet extraction and extracted sequentially with AR grade solvents of increasing polarity viz. petroleum ether and ethanol. A separate extraction was carried out using ethanol alone. Aqueous extracts were prepared by simple decoction method. Thus 4 extracts namely, Ethanol (Direct), Petroleum ether, Ethanol (successive) and Aqueous were prepared from each plant and the herbomineral.

The powders of selected material were used in the range of 3-55 gm for sufficient preparation of Ethanol (Direct), Petroleum ether and Ethanol (Successive) extracts. Aqueous extracts were prepared using 20 gm of powder, except for *R. mucronata*. The quantity of *R. mucronata* was very less and hence approximately 3 gm was utilized for each extraction.

All the extracts were made free from solvents and percentage yield of individual extractives was calculated (Table-3). As per Table-3, percent yield of Petroleum ether extract of Shilajit was very low to pursue further work. Thus in the present study, total 23 extracts prepared from 5 plants and a herbomineral were subjected to *in vitro* anti-HIV activity.

For primary screening, the HIV used for infecting the cell culture was sourced from the cell culture supernatant after co-cultivation. It has been observed that, HIV is cultivable from PBMCs of most seropositive persons.

The sensitivity of HIV-1 detection by co-cultivation of subject's PBMCs with PHA-stimulated cells from sero negative donor is more than 90% in clinically asymptomatic patient and close to 100% in clinically symptomatic patients.²⁰⁹

Hence, virus has been grown by classical co-cultivation method by culturing Peripheral Blood Mononuclear Cells (PBMCs) of HIV positive individual with PHA-stimulated healthy donor's PBMCs. For this, the blood samples were collected in K3 EDTA bulbs. As there are reports that, heparinized blood samples resulted in poor recovery of virus. The collected blood samples were processed on the same day to assure optimal viral recovery from the cells. Also it has been reported that, use of frozen donor PBMCs remarkably reduce the recovery of virus. Hence during the cultivation every time fresh PBMCs were added.^{210,211}

Virus isolated from drug-naïve patient (Non-ART clinical isolate) was used for 4 assays namely, XTT formazan assay, p24 inhibition assay, to evaluate effect of extracts on reverse transcriptase of Non-ART clinical isolate and combinatorial effect of extracts. While, virus isolated from drug-treated patient (ART clinical isolate) was used only to evaluate effect of extracts on reverse transcriptase of ART clinical isolate. Hence the cultures were maintained for 4 weeks for Non-ART isolate and 2 weeks for ART isolate to prepare sufficient stock. The detection of HIV in culture fluid by the reverse transcriptase assay is considered to be the most sensitive and specific assay for the presence of virus in infected lymphocytes or in body fluids.^{212,213} Hence the titre of the culture supernatants was determined by reverse transcriptase assay (Table-4).

A number of laboratories reported PBMC-based culture systems that allow the evaluation of 80 to 95% of clinical HIV-1 isolates from patients receiving antiretroviral therapy.^{214,215,216,217}

Initial studies of zidovudine (ZDV) resistance used a syncytium-forming focus assay with a CD4-expressing HeLa cell line. However it is limited to HIV-1-positive individuals harboring clinical isolates that form syncytia.^{218,219} Recently, Several PBMC-based assays have been reported to distinguish consistently ZDV-susceptible from ZDV-resistant HIV-1.^{220,221,222,223}

Besides, an aqueous extract of the blue-green filamentous algae *Arthrospira platensis* (previously called *Spirulina platensis*) inhibited HIV-1 replication in PMBC.²²⁴ Also as shown by Kitamura et al²²⁵, the flavonoid baicalin markedly inhibited replication of HIV-1 in normal peripheral blood mononuclear cells (PBMC) stimulated with phytohemagglutinin (PHA) *in vitro*. Based on this background, PHA-stimulated PBMCs were used for the preliminary assays.

As all the extracts were crude extracts and not the fractionated or purified active moieties, it was preferred using not too high or not too low concentration of the extracts viz. 10 mg/ml at least at preliminary level. The preliminary screening of the extracts was carried out with the help of XTT formazan assay and HIV-p24 inhibition assay using six different concentrations of each extract. The microculture XTT antiviral assay provides a quantitative representation of HIV-induced cytopathic effects as modulated by test substances. This relatively simple assay facilitates the safe and rapid determination of *in vitro* antiviral activity as well as

direct cytotoxicity. During 1988, the microculture XTT antiviral assay system was applied as a primary screen to the evaluation of >20,000 samples.²²⁶ Vince et al²²⁷ described the first new synthetic anti-HIV-1 drug candidate (carbocyclic nucleoside analogue) identified by this screen.

In the present study, as most of the extracts were coloured, the extract controls were included to rule out the interference of the colour of the extracts. Moreover, colourless medium i.e. RPMI 1640 with out phenol red was used in order to reduce the error. Also, the care has been taken to consider the background OD count of extract controls while calculating CC₅₀ and EC₅₀ values. The result has been expressed as 50% Cytotoxic Concentration (CC₅₀), 50% Effective Concentration (EC₅₀) and ratio of CC₅₀/EC₅₀ as Selectivity Index (SI). The SI is an important parameter as it reflects both the antiviral activity and eventually toxicity of the extract. The high value of SI indicates low toxicity of the plant extract to the cells and high activity against the virus. Premnathan et al²²⁸ screened 69 Indian medicinal plants for anti-HIV activity, only 16 showed activity against the virus. The SI ranged from 1.39 in the root of *Glycyrrhiza glabra* to 6.73 in the bark of *Cinnamomum cassia*. Whereas the selectivity index of Olive Leaf Extract (OLE) has been reported as above 5000.¹⁸⁹

In the present study, Aqueous extract of *O. sanctum* showed the highest Selectivity Index (SI) of 125. Other three extracts of *O. sanctum* and aqueous extract of *T. cordifolia* showed SI of 25. Petroleum ether extract of *T. cordifolia* showed SI of 5 while, Ethanol (Direct) and Ethanol (Successive) extracts showed SI as 1. In case of *W. somnifera*, Ethanol (Direct), Ethanol (Successive) and Aqueous extracts showed SI of 1, whereas Petroleum ether extract showed SI as 5. Extracts of mangrove

plants namely, *A. officinalis* and *R. mucronata* showed SI of 1 and 5 respectively. The herbomineral Shilajit also showed SI as 5. However, it is possible that the active principle (s) and the toxic compound (s) of the extract are different from each other in which case fractionation of the total extract should help to determine the fraction with the highest selectivity index. The standard reference compound, AZT, showed SI as 25.

HIV mainly infects T-cells, hence effect of 23 extracts on HIV replication was evaluated by viral core protein p24 expression in primary PBMCs using commercial ELISA kit. This antigen capture assay appears to be the method of choice for detection of HIV in routine virus culturing as it can handle large number of samples, is relatively fast, does not require radioactive material and provides quantitative results. Gupta et al²²⁹ compared reverse transcriptase assay and the antigen capture assay for 962 samples and the 2 tests displayed comparable sensitivities (98.5% agreement) in detecting HIV antigen. In addition, these 2 tests displayed similar sensitivities when examined for the kinetics of HIV appearance following *in vitro* infection of normal lymphocytes.

As viral productions peaks at day 4, p24 expression was assayed in cell-free supernatants harvested at day 4. In this assay, the culture medium was incubated in microtiter wells precoated with anti-p24 antibody and assayed for p24 antigen by biotin-labeled anti-p24 antibody followed by incubation with streptavidine-peroxidase conjugate. The amount of captured p24 was determined by measuring the absorbance at 450 nm of tetramethylbenzidine (TMB) substrate.

Lee-Huang et al¹⁸⁹ showed inhibition of HIV replication by OLE as assayed by p24 expression in infected H9 cells. While Chen et al²³⁰ evaluated effect of Shikonin on replication of clinical HIV isolates as well of multi-drug resistant HIV isolate in PBMCs.

In the present study, in case of *O. sanctum*, Petroleum ether extract showed the highest inhibition of 88.24% at a concentration of 10 mg/ml. Where as, the lowest inhibition of 56.70% was noted with Aqueous extract at a concentration of 2 mg/ml. Among other extracts of *O. sanctum*, Ethanol (Direct) extract showed the highest inhibition (88.07%) at 0.08 mg/ml, Ethanol (Successive) extract at 2 mg/ml (87.25%) and Aqueous extract at 0.4 mg/ml (87.42%).

Among *W. somnifera* extracts, the highest HIV p24 antigen inhibition of 85.62% was seen with Ethanol (successive) extract at a concentration of 0.4 mg/ml. Ethanol (Direct) extract showed the highest inhibition (83.17%) at 10 mg/ml, Petroleum ether extract at 0.4 mg/ml (85.13%) and Aqueous extract at 0.08 mg/ml (83.50%).

In case of *T. cordifolia*, Ethanol (Direct) extract showed the highest inhibition of HIV p24 antigen with 91.67% at a concentration of 0.4 mg/ml. The lowest inhibition of 41.18% was noted with Aqueous extract at a concentration of 0.016 mg/ml. Among other extracts of *T. cordifolia*, Petroleum ether extract showed the highest inhibition (90.20%) at 2 mg/ml, Ethanol (Successive) extract at 0.016 mg/ml (88.24%) and Aqueous extract at 0.4 mg/ml (84.48%).

Among *A. officinalis* extracts, the highest HIV p24 antigen inhibition was seen with Ethanol (Successive) extract at a concentration of 0.016 mg/ml with 92.65%. Aqueous extract also showed similar activity (92.65%) at a

concentration of 0.4 mg/ml. Among other two extracts of *A. officinalis*, Ethanol (Direct) extract showed the highest inhibition (91.50%) at 0.0032mg/ml and Petroleum ether extract at 0.4 mg/ml (92.48%).

In case of *R. mucronata*, Petroleum ether extract showed the highest inhibition of HIV p24 antigen with 92.48% at a concentration of 0.4 mg/ml. Ethanol (Successive) extract showed the highest inhibition (92.16%) at 10 mg/ml and Aqueous extract at 0.4 mg/ml (91.83%). HIV p24 inhibition for Ethanol (Direct) extract was noted in the range of 91.50% to 91.99%.

Among Shilajit extracts, Ethanol (Successive) extract showed the highest inhibition of HIV p24 antigen with 90.20% at a concentration of 0.016 mg/ml, followed by Aqueous extract that showed 89.71% inhibition at a concentration of 10 mg/ml. While, the lowest inhibition of 11.44% was noted with Ethanol (Direct) extract at a concentration of 0.08 mg/ml. However it showed the highest inhibition of 83.50% at a concentration of 0.0032 mg/ml.

In this study, AZT was included as positive control that showed 76.31% inhibition.

As most of the extracts showed potent inhibitory activity in primary screening, they were further evaluated using specific and more sensitive assays and the concentration was reduced to half of the original i.e. 5 mg/ml. Antiviral research has been focused on compounds that interfere with various parts of the viral life-cycle such as proteins encoded by the virus itself, i.e. Reverse Transcriptase (RT), Protease and Integrase. Among these, Reverse Transcriptase is a popular therapeutic target. HIV-

RT performs 3 principle functions: firstly, the polymerase domain transcribes viral genomic RNA to viral DNA, a process referred to as the RNA-dependent-DNA-polymerase (RDDP) activity. Secondly, in the course of reverse transcription an intermediary RNA/DNA hybrid is formed. RT through its ribonuclease H (RNase H) domain degrades the RNA component of the hybrid. Thirdly, RT carries out DNA-dependent DNA polymerization activities, producing complementary DNA strands. The completion of each of these processes is required for the formation of a competent viral DNA capable of integrating into the genome of the infected cell. The function of RT is therefore essential for replication of HIV. Therefore this is a suitable target for chemotherapeutic intervention. Bessong et al²³¹ screened South African medicinal plants for activity against HIV-1 RT RDDP and RNase H activities. EL-Mekkawy et al¹⁴⁷ evaluated extracts of 41 medicinal plants used in Egyptian folk medicine for their HIV-1 RT inhibitory effects and identified putranjivain A from *P. emblica* as a potent inhibitory substance against HIV-RT.

In the present study, the assay was carried out using [3H]-methyl Thymidine triphosphate (dTTP) as the enzyme substrate and polyadenylic acid oligo deoxythymidylic acid [Poly(rA).p(dT)₁₂₋₁₈] as the template-primer dimmer. This assay was optimized and standardized with respect to various experimental parameters and then applied to test the HIV-RT inhibitory activity of the different extracts. To avoid additives present in conventional drugs, a pure compound AZT (standard drug) was included as positive control. Most studies considered inhibition $\geq 50\%$ as significant.^{232,233}

The assay was carried out at maximum concentration of 5 mg/ml. At this concentration, nineteen of twenty three extracts showed significant inhibition ($\geq 50\%$ Inhibition) of recombinant HIV-RT (Table-12). Among Ethanol (Direct), Ethanol (Successive) and Aqueous extracts, Shilajit showed the highest inhibition of RNA-Dependent-DNA-Polymerase (RDDP) function of recombinant HIV-RT. Whereas among Petroleum ether extracts *R. mucronata* showed the highest inhibition followed by *O. sanctum*. AZT was included as positive control that showed 71.04% inhibition.

This study was further extended to cover broad range of concentrations from microgram to milligram to evaluate the effect of different concentrations on recombinant HIV-RT. In case of *O. sanctum*, Aqueous extract showed the highest inhibition of 85.76% at a concentration of 0.0016 mg/ml. Ethanol (Direct) extract showed the highest inhibition of 82.96% at a concentration of 5 mg/ml, Petroleum ether at 0.04 mg/ml (82.42%) and Ethanol (Successive) extract at 0.2 mg/ml (78.07%). The lowest inhibition of 44.04% was noted with Ethanol (Direct) extract at a concentration of 0.04 mg/ml.

Among *W. somnifera* extracts, Petroleum ether extract showed the highest inhibition of 68.71% at a concentration of 5 mg/ml. But, it showed no inhibition at a concentration of 0.0016 mg/ml. In general, only four concentrations [0.2 mg/ml to 0.0016 mg/ml] of Ethanol (Direct), two concentrations each of Petroleum Ether [5 mg/ml and 1 mg/ml] and Ethanol (Successive) [1 mg/ml and 0.008 mg/ml] extracts significantly inhibited ($\geq 50\%$ Inhibition) recombinant HIV-RT. Aqueous extract showed no significant inhibition at any concentration tested.

In case of *T. cordifolia*, Ethanol (successive) extract showed the highest inhibition (85.88%) of the viral enzyme at a concentration of 0.04 mg/ml. Ethanol (Direct) and Aqueous extracts showed the highest inhibition at 0.008 mg/ml with 82.54% and 77% respectively. While Petroleum ether extract showed the highest inhibition of 77.53% at a concentration of 0.2 mg/ml.

Among *A. officinalis* extracts, the highest RT inhibition was noted with Ethanol (Direct) extract with 87.84% at a concentration of 1 mg/ml. Petroleum ether extract showed the highest inhibition of 81.59% at a concentration of 0.2 mg/ml. Ethanol (Successive) extract showed the highest inhibition at a concentration of 5 mg/ml with 82.00%. Whereas, Aqueous extract showed the highest inhibition of 85.10% at a concentration of 0.008 mg/ml.

In case of *R. mucronata*, Ethanol (Direct) and Aqueous extracts showed the highest inhibition of 87.31% and 84.51% respectively at a concentration of 0.0016 mg/ml. Whereas, Petroleum ether and Ethanol (Successive) extracts showed the highest inhibition at a concentration of 5 mg/ml with 87.07% and 84.74% respectively.

Among Shilajit extracts, Ethanol (Direct), Ethanol (Successive) and Aqueous extracts showed the highest inhibition at a concentration of 5 mg/ml with 83.55%, 89.45% and 86.35% respectively. All the 3 extracts of Shilajit significantly ($\geq 50\%$ Inhibition) inhibited the RDDP function of recombinant HIV-RT at all given concentrations.

Investigation of anti-HIV activity of medicinal plants against different strains of HIV is relevant in terms of promoting the traditional use of

plants as anti-HIV agents. Hence, effect of 23 extracts on reverse transcriptase of two different clinical isolates was evaluated by HIV RT inhibition assay. The virus isolated from drug-naïve patient was designated as Non-ART isolate, whereas, virus isolated from drug-treated patient was designated as ART isolate. As illustrated in Table-20, fourteen of twenty three extracts showed significant inhibition ($\geq 50\%$ Inhibition) of Non-ART isolate and twelve of twenty three extracts showed significant inhibition of ART isolate. Among *O. sanctum* extracts, Ethanol (Direct), Ethanol (Successive) and Aqueous extracts significantly inhibited RT of Non-ART isolate. While, Ethanol (successive) and Aqueous extracts significantly inhibited RT of ART isolate.

In case of *W. somnifera*, only Aqueous extract showed significant inhibition of Non-ART isolate. However none of the extracts showed significant inhibition of ART isolate.

In case of *T. cordifolia*, all the 4 extracts significantly inhibited RT of Non-ART isolate. While, only Aqueous extract significantly inhibited RT of ART isolate.

Among *A. officinalis* extracts, only Ethanol (successive) extract significantly inhibited RT of Non-ART isolate. Whereas, Ethanol (Direct), Ethanol (Successive) and Aqueous extracts significantly inhibited RT of ART isolate.

Among *R. mucronata* extracts, Ethanol (Successive) and Aqueous extracts significantly inhibited RT of Non-ART isolate. While, Ethanol (Direct), Ethanol (Successive) and Aqueous extracts significantly inhibited RT of ART isolate.

In general, Ethanol (Successive) extract of *O. sanctum* showed the highest inhibition (76.35%) of Non-ART isolate. Whereas, Aqueous extract of *R. mucronata* showed the highest inhibition (80%) of ART isolate. Only nine of twenty three extracts showed significant inhibition of both the isolates. Especially, all the 3 extracts of Shilajit showed significant inhibition of both the clinical isolates.

The development of resistant viruses is the major obstacle for sustained viral suppression and long-term clinical benefit of antiretroviral therapy. During early phase of HIV therapy, insufficient drug potency resulting from monotherapy was the major cause of drug failure and resistance. However the current therapy, "Highly active antiretroviral therapy" (HAART) refers to use of combination of antiretroviral agents for treatment of HIV infection. Among these the most important backbones are TDF+3TC, TDF+FTC and ABC+3TC. However some combinations such as AZT+d4T and FTC+3TC are not recommended as they are antagonistic.²⁶

Consequently, in the present study, different combinations of aqueous extracts of selected plants and the herbomineral were evaluated for their combinatorial effect on reverse transcriptase of Non-ART isolate using RT inhibition assay. The study revealed that, 14 of 16 combinations showed significant inhibition of RDDP function of HIV-RT (Table-22). Combination of *A. officinalis* and Shilajit showed the highest (65.84%) inhibition of viral enzyme followed by mixture of all the 6 extracts (65.61%). The study also marked the lowest inhibition (21.32%) with combination of *W. somnifera* and *T. cordifolia* extracts.

Apart from inhibition of viral proteins, binding of gp120 to CD4 is also a critical step in HIV infection, as the outer envelope glycoprotein gp120 of HIV mediates viral attachment to the cell-surface glycoprotein CD4 in the initial phase of HIV infection. Therefore, much interest has been focused on agents that block this interaction. Two agents that have been shown to potently inhibit the adsorption of HIV to CD4⁺ cells and inhibit HIV infection *in vitro* are the sulfated polysaccharides, dextran sulfate and heparin.²³⁴ Besides this, the purified extract of *Prunella vulgaris* antagonized HIV-1 infection by preventing viral attachment to the CD4 receptor.²³⁵

In the present study, we examined the effects of 23 extracts on gp120/CD4 interaction using a gp120 capture ELISA kit. The ability of extracts to inhibit this interaction by binding to gp120 was determined by pre-incubation of test compounds with the soluble gp120 before addition to immobilized CD4. The study revealed that only 9 of 23 extracts showed significant inhibition ($\geq 50\%$ inhibition) of gp120/CD4 interaction (Table-13). Among Ethanol (Direct), Petroleum ether and Aqueous extracts, *A. officinalis* showed the highest inhibition of 85.17%, 81.01% and 69.33% respectively. Where as among Ethanol (successive) extracts, *R. mucronata* (81.69%) showed the highest inhibition followed by *T. cordifolia* (80.22%). Ethanol (Direct) and Petroleum ether extracts of *O. sanctum* and Ethanol (successive) and Aqueous extracts of *W. somnifera* showed no inhibition. The extracts that inhibit this interaction after preincubation with gp120, may bind tightly to gp120 thereby inactivating them and preventing the interaction between gp120 and CD4. Boyd et al²³⁶ reported similar activity with Cyanovirin-N (CV-N), a novel virucidal

protein isolated from cultures of cyanobacterium (blue-green alga) *Nostoc ellipsosporum*.

The reciprocal experiment was then performed to measure the ability of 23 extracts to inhibit gp120/CD4 interaction by binding to immobilized CD4. In the present study, twenty one of twenty three extracts showed inhibition of this interaction by binding to immobilized CD4 (Table-21). These include all the 4 extracts of *O. sanctum*, *T. cordifolia*, *A. officinalis* and *R. mucronata*. Petroleum ether, Ethanol (Successive) and Aqueous extracts of *W. somnifera*, Ethanol (Direct) and Aqueous extracts of Shilajit. Ojwang et al¹⁹¹ demonstrated that oligonucleotides composed entirely of Guanosine and Thymidine (GTOs) could inhibit gp120/CD4 interaction by binding to both gp120 and CD4.

In a separate experiment, the test compounds were added after a 60 min preincubation of gp120 in CD4-coated plates. Nineteen of twenty three extracts significantly blocked the gp120/CD4 interaction (Table-21). These include all the 4 extract of *A. officinalis* and *R. mucronata*. All the 3 extracts of Shilajit. Ethanol (Direct), Ethanol (Successive) and Aqueous extracts of *O. sanctum* and *W. somnifera* respectively. Petroleum ether, Ethanol (Successive) and Aqueous extracts of *T. cordifolia*. These extracts competed with gp120 for binding to CD4 immobilized on a solid phase and displaced gp120 preadsorbed to CD4. Yang et al¹⁹² identified a group of small molecule Stibonic acid-containing compounds (NSC 13778) that competitively inhibited gp120 binding to CD4.

Heparin was included as positive control that showed highest inhibition of 87.64% when preincubated with immobilized CD4. It showed 75.96%

inhibition when preincubated with gp120 and 58.09% inhibition when added after gp120/CD4 interaction.

In general, mangrove plants namely, *A. officinalis* and *R. mucronata* showed effective inhibition of gp120/CD4 interaction by binding to both gp120 and to CD4 ligand. This binding resembles closely the binding of dextran sulfate/heparin that bind to CD4 and also to gp120 and inhibit the infection.^{237,238} Ethanol (Direct) extract of *R. mucronata* was the only exception that did not show significant inhibition of this interaction by binding to gp120. However all the 4 extracts of *A. officinalis* and *R. mucronata* also potently inhibited this interaction by displacing gp120 preadsorbed to immobilized CD4.

Besides, *O. sanctum*, *W. somnifera* and shilajit potently inhibited gp120/CD4 interaction by binding to CD4 (but not to gp120) except Ethanol (Direct) extract of *W. somnifera* and Ethanol (Successive) extract of Shilajit. O'keefe et al²³⁹ demonstrated similar activity with Niphatevirin, a novel anti-HIV protein isolated from aqueous extracts of the Caribbean sponge *Niphates erecta*. Niphatevirin bound to CD4 in a manner that prevented the binding of gp120, but did not directly bind gp120. Extracts of *O. sanctum*, *W. somnifera* and shilajit also competitively inhibited gp120 binding to CD4 except Petroleum ether extract of *O. sanctum* and *W. somnifera*. Among *T. cordifolia* extracts, only Petroleum ether and Ethanol (Successive) extracts significantly inhibited gp120/CD4 interaction by binding to gp120 and by competitive inhibition. All the 4 extracts of *T. cordifolia* also inhibited this interaction by binding to immobilized CD4.

The extensive literature search revealed that phytochemical data is available on the selected medicinal plants namely *O. sanctum*, *W. somnifera* and *T. cordifolia*.^{166,170,173,240,241} However, little data is available on mangrove plants namely *A. officinalis*, *R. mucronata* and the herbomineral Shilajit. Thin Layer Chromatography (TLC) is usually the first choice for separation of unknown sample mixture. Hence preliminary TLC study was carried out using different solvents and their combinations to estimate number of components that can possibly be obtained from each crude extract. As illustrated in Table-23, Petroleum ether extract of *A. officinalis* showed 2 spots with the R_f value of 0.18 and 0.36 respectively and Ethanol (Successive) extract of *A. officinalis* showed one spot with R_f value of 0.18 with Benzene and Chloroform solvent system in the ratio (40 : 60). Ethanol (Successive) extract of *R. mucronata* showed 2 spots with Benzene system with R_f value of 0.24 and 0.18 respectively. Whereas, Petroleum ether extract of *R. mucronata* and Ethanol (Successive) extract of Shilajit showed trailing (no good separation of spots) with any of the solvents or their combinations used. Also no movement of the spots was observed with any of the solvents or their combinations for aqueous extracts of *A. officinalis*, *R. mucronata* and Shilajit. Hence probably advance techniques such as High-Performance Thin Layer Chromatography (HPTLC), High-Performance Liquid Chromatography (HPLC), Column chromatography, Gas-Liquid chromatography (GLC), Spectral analysis such as Ultra Violet (UV), Infra Red (IR), Nuclear Magnetic Resonance (NMR) and Mass spectrometry are required to separate, identify and characterize the present compounds.

Among the plants and the herbomineral tested, *O. sanctum*, *T. cordifolia*, mangrove plants namely *A. officinalis* and *R. mucronata* and the herbomineral Shilajit showed substantial anti-HIV activity. The range and extent of the inhibitory activities displayed by the various extracts studied indicate that several different molecular species (phytochemicals) are responsible. In order to estimate number of compounds present in the selected plants and the herbomineral, they were subjected to preliminary phytochemical analysis. The result of the same is reported in (Table-2). This study of chemical characterization revealed presence of tannins, carbohydrates, alkaloids, saponins etc all of which are capable of exhibiting anti-HIV activity.²⁴² However, the most common phytochemicals from the active plants and the herbomineral are tannins and carbohydrates which could be the possible active constituents for the anti-HIV activity.

A number of polyphenolic compounds like tannins isolated from different botanicals have been reported to exhibit anti-HIV activity. A variety of tannins are capable of inhibiting HIV-RT. They may also inhibit HIV replication in cell culture by additional or other mechanisms, probably by interfering with HIV cell interactions.²⁴³ A red colored gallotannin, isolated from an active fraction of the methanol extract of stem bark and roots of *Peltophorum africanum* Sond., strongly inhibited HIV-RT functions.²⁴⁴ Similarly, Galloylquinates and Galloylshikimates (Tannin) isolated from *Castanopsis hystrix* and Caffeoylquinates (Tannin) isolated from *Lonicera japonica* showed HIV-RT inhibition activity.²⁴⁵ Also, Weaver et al²⁴⁶ showed that the tannins chebulic acid and punicalin were able to block the binding

of HIV rgp120 to CD4 and that these compounds were not toxic to the stimulated human peripheral blood lymphocytes.

Besides tannins, the antiviral activity of carbohydrates extracted from various natural sources has been known since long time. Polysaccharides rhamnansulfate isolated from *Monostroma talissimum* is reported to inhibit HIV replication while sulfonated polysaccharide isolated from *Viola yedoensis* are reported to exhibit anti-HIV activities.^{247,248} Similarly, polysaccharides extracted from the leaf of *Rhizophora apiculata* (RAP) and the bark of *Rhizophora mucronata* (RMP) were assessed by an *in vitro* cell culture system. Both RAP and RMP protected MT-4 cells from HIV-induced cytopathogenicity and blocked the expression of HIV p24 antigens, preventing the virus binding to the cell and the formation of syncytia upon co-cultivation of MOLT-4/HIV-1IIIB cells and MOLT-4 cells.^{249,250} The sulfated polysaccharides have consistently shown activity in anti-HIV assays. They are believed to function by destabilizing the glycoprotein complex.²⁵¹ They are also reported to inhibit the activity of reverse transcriptase and RNase H which are essential for retrovirus replication.²⁵² A polysaccharide fraction isolated from *Thuja occidentalis*, designated Thuja polysachharide g-fraction exhibited HIV-1 reverse transcriptase activity.²⁵³ Similarly, Nakashima et al²⁵⁴ reported a new HIV-RT inhibitor extracted and purified from red alga *Schizymenia pacifica*. The chromatographic behavior and chemical properties of this sea algal extract suggest that it is a sulfated polysaccharide composed of galactose, sulfonate and 3,6-anhydrogalactose.

Experimental results thus suggested that, most of the plant extracts exert their anti-HIV activity via multiple mechanisms of action, each of which contributes to the overall anti-viral profile of the selected plants and the herbomineral. For example inhibition of viral entry into cells could be accomplished by interference with the gp120 / CD4 interaction. Evidence is also presented that another mechanism, inhibition of viral RT, contributes to the overall anti-viral activity.

The results from the present study do seem to justify the traditional use of plants for the treatment of infectious diseases of viral origin. They further indicate that these plants might be of value as sources or leads for novel antiviral compounds which could be useful in the continuing fight against HIV. Any effective treatment of HIV infection should target as many aspects of the viral life cycle as possible. The potential targets of HIV have been described in detail elsewhere.¹⁵⁸ In order to assess the usefulness of these medicinal herbs and the herbomineral, it is recommended that several different screening assays be performed on each extract. It is also necessary to isolate from the crude extracts the active principle(s) responsible for these activities, identify them and study their mechanism of action. This will give wider choice of lead molecules for the drug design of anti-HIV agents, which will hopefully lead to the cure of this devastating human disease.

Phytomedicines usually have multiple effects on the body. Their actions often act beyond the symptomatic treatment of the disease. As AIDS is a syndrome comprising a dysfunction of the immune system, compounded

by opportunistic infections of bacterial, fungal, protozoan and viral etiology. The selected Indian medicinal plants and the herbomineral possess immunomodulatory activity apart from the other microbicidal activities. Besides, mangrove plants are also used in folklore medicine for treatment of several diseases. Hence they can be used to treat these opportunistic infections, neoplasm etc.

To end on an optimistic note, an integrated system of medicine can achieve the common goal of mankind that is to live a healthy and happy life!

SUMMARY

AND

CONCLUSION

The development of safe, effective and low-cost anti-HIV drugs is among the top global priorities of drug development, since the disease is not yet curable and the mortality is high. One of the strategies has been to identify anti-HIV compounds from natural sources, particularly plants.

In the present study, we analyzed twenty three extracts prepared from five plants namely *Ocimum sanctum*, *Withania somnifera*, *Tinospora cordifolia*, *Avicennia officinalis*, *Rhizophora mucronata* and Shilajit a herbomineral for their specific inhibitory activity against HIV *in vitro*.

- ❖ The preliminary screening of the plant extracts was carried out with the help of XTT formazan and HIV-p24 inhibition assays.
- ❖ In XTT formazan assay, Aqueous extract of *O. sanctum* showed the highest selectivity index (SI) of 125. Whereas for rest of the extracts SI ranged from 1 to 25.
- ❖ In HIV p24 inhibition assay, most of the plants and the herbomineral potently inhibited HIV p24 antigen, with mangrove plants namely, *A. officinalis* and *R. mucronata* showed the highest inhibitory activity.
- ❖ As most of the plants and the herbomineral showed potent inhibitory activity in preliminary screening, they were further evaluated using specific and more sensitive assays such as Reverse Transcriptase (RT) inhibition assay and gp120 inhibition assay.
- ❖ *O. sanctum*, *T. cordifolia*, *A. officinalis*, *R. mucronata* and Shilajit potently inhibited RDDP function of recombinant HIV-RT and the RT of clinical isolates. Especially, Shilajit showed significant inhibition of both the clinical isolates.

- ❖ The study of combinatorial effect of aqueous extracts on HIV-RT revealed the highest inhibition with combination of *A. officinalis* and Shilajit extracts and antagonistic nature of *W. somnifera* and *T. cordifolia* extracts.
- ❖ Mangrove plants namely, *A. officinalis* and *R. mucronata* showed effective inhibition of gp120/CD4 interaction by binding to both gp120 and to CD4 ligand and even by displacing gp120 preadsorbed to immobilized CD4.
- ❖ *O. sanctum*, *W. somnifera* and Shilajit potently inhibited gp120/CD4 interaction by binding to CD4 (but not to gp120) and also competitively inhibited gp120 binding to CD4. *T. cordifolia* inhibited gp120/CD4 interaction by binding to CD4 ligand mainly.

More or less most of the plants and the herbomineral showed anti-HIV potential by inhibiting the virus by 2 different mechanisms. Hence these promissory extracts open the possibility of finding new clinically effective anti-HIV compounds. However further research is necessary to isolate their active constituents and also to determine their full spectrum of efficacy by carrying out different assays.

The selected plants and the herbomineral also possess other medicinal values which will help minimizing the opportunistic infections associated with HIV. There is therefore, good reason to expect an appreciable clinical management of AIDS patients.

Thomas Jefferson wrote that "The greatest service which can be rendered by any country is to add a useful plant to its culture". The present *in vitro* study of anti-HIV evaluation of selected plants and the herbomineral forms a preliminary platform for further phytochemical and pharmacological studies.

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APPENDICES

1. Mayers Reagent:

Dissolve 1.36 gm of mercuric chloride (HgCl_2) in 60 ml distilled water. Five gm of potassium iodide is dissolved in 10 ml distilled water. Mix these 2 solutions and then dilute it to 100 ml volume using distilled water.

2. Benedict's Qualitative Reagent:

Put 17.3 gm sodium citrate and 10 gm sodium carbonate in conical flask. Add 90 ml distilled water and boil for 5 min. Add to this mixture 1.73 gm of copper sulfate and dissolve it completely. By using measuring cylinder make the final volume to 100 ml with distilled water.

3. 1% Aqueous HCl:

Dissolve 1 ml conc. HCl in 99 ml distilled water.

4. 5% Ferric chloride:

Dissolve 5 gm Ferric chloride in 100 ml distilled water.

5. 50 mM Tris:

Tris 0.3035 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

6. 150 mM KCl:

KCl 0.3728 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

7. 5 mM MgCl₂:

MgCl₂ 0.0077 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

8. 0.05% NP-40:

NP-40 83.5 µl

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

9. 0.05 mM EGTA:

EGTA 0.002 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

10. 5 mM DTT:

DTT 0.0386 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

11. 0.3 mM Glutathione:

Glutathione 0.0230 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

12. 20 μ M dTTP:

dTTP stock (100mM) 1.5 μ l

Protease-Nuclease free water 998.5 μ l

13. 2.5 μ g/ml BSA:

BSA 0.000625 gm

Protease-Nuclease free water 5 ml

The content was dissolved completely and filtered through bacteria proof filter.

LIST

OF

ABBREVIATIONS

ADCC: Antibody Dependent Cellular Cytotoxicity

AIDS: Acquired Immuno Deficiency Syndrome

ARC: AIDS Related Complex

ARV: Antiretroviral

ART: Antiretroviral Therapy

AZT: Azidothymidine / Zidovudine

BSA: Bovine Serum Albumin

CC₅₀: 50% Cytotoxic Concentration

CD: Cluster of Differentiation

CDC: Centre for Disease Control and Prevention

CDRI: Central Drug Research Institute

CMV: Cytomegalovirus

CNS: Central Nervous System

CPE: Cytopathic Effect

CSF: Cerebrospinal Fluid

CTL: Cytotoxic T Lymphocytes

CV-N: Cynovirin-N

dA: Deoxyadenosine

dC: Deoxycytidine

DCs: Dendritic Cells

DMSO: Dimethylsulphoxide

DNA: Deoxyribonucleic Acid

dT: Deoxythymidine

DTT: Dithiothritol

d4T: Stavudine

EC₅₀: 50% Effective Concentration

ECS: Elective Caesarian Section

EDTA: Ethylene Diamine Tetra Aceticacid

ELISA: Enzyme Linked Immuno Sorbent Assay

Env: Envelop

FDA: Food Drug Administration

FCS: Fetal Calf Serum

FIs: Fusion Inhibitors

Gag: Group antigen

gp41: Glycoprotein 41

gp120: Glycoprotein 120

HAART: Highly Active Antiretroviral Therapy

HCV: Hepatitis C Virus

HCW: Healthcare Worker

HIV: Human Immunodeficiency Virus

HLA: Human Leukocyte Antigen

HSR: Hypersensitivity Reaction

HTLV III: Human T Lymphocytotropic Virus Type III

IFN: Interferon

IDV: Indinavir

IL: Interleukin

IN: Integrase

LAV: Lymphadenopathy Associated Virus

LTNPs: Long Term Non Progressors

LTR: Long Terminal Repeat

MAC: *Mycobacterium avium* complex

MDR: Multidrug Resistant

MIP: Macrophage Inhibitory Protein

MTCT: Mother to Child Transmission

NF- κ B: NF kappa B

NCL: National Chemical Laboratory

NCR: Natural Cytotoxicity Receptors

Nef: Negative regulatory factor

NK cells: Natural Killer cells

NNRTIs: Non-Nucleoside Reverse Transcriptase Inhibitors

NPIL: Nicolas Piramal India Limited

NRTIs: Nucleoside Reverse Transcriptase Inhibitors

NSAID: Non-Steroidal Anti-Inflammatory Drug

NSI: Non Syncytia Inducing

NVP: Nevirapine

OD: Optical Density

OIs: Opportunistic Infections

OLE: Olive Leaf Extract

PBMCs: Peripheral Blood Mononuclear Cells

PDC: Plasmacytoid Dendritic Cells

PEP: Post Exposure Prophylaxis

PGL: Persistent Generalized Lymphadenopathy

PHA: Phytohemagglutinin

PIs: Protease Inhibitors

PMS: Phenazonium Methosulfate

Pol: Polymerase

PR: Protease

p24: Core protein

RANTES: Regulated upon Activation T cell Expressed and Secreted

RAP: *Rhizophora apiculata* polysaccharide

Rev: Regulator of late gene expression

RDDP: RNA-dependent-DNA-polymerase

RMP: *Rhizophora mucronata* polysachharide

RNA: Ribonucleic acid

RNase H: Ribonuclease H

RRE: Rev Response Element

RRL: Regional Research Laboratory

RT: Reverse Transcriptase

SDF-1: Stromal cell Derived Factor-1

SI: Selectivity Index

SI strains: Syncytia Inducing strains

SJS: Stevens-Johnson Syndrome

SOD: Superoxide Dismutase

START: Short Term Antiretroviral Therapy

Tat: Trans activator protein

TEN: Toxic Epidermal Necrolysis

TLC: Thin Layer Chromatography

TMB: Tetramethylbenzidine

TNF- α : Tumor Necrosis Factor- Alpha

UNAIDS: Joint United Nations program on HIV / AIDS

Vif: Viral infectivity factor

Vpr: Viral protein R

Vpu: Viral protein U

WHO: World Health Organization

SYNOPSIS

A SYNOPSIS OF THE THESIS
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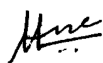
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***In-vitro* testing of the Anti-HIV properties of Mangrove and some other Medicinal plants**

Introduction

Acquired Immuno Deficiency Syndrome [AIDS] is devastating human disease since 1981 and is caused by Human Immuno Deficiency Virus [HIV]. AIDS is characterized by extensive immunosuppression that predisposes patients to life-threatening opportunistic infections and unusual forms of neoplasm.¹

According to a joint UNAIDS and WHO report the estimated number of persons living with HIV worldwide in 2007 was 33.2 million [30.6~36.1 million]. The deaths due to AIDS were 2.1 million [1.9~2.4 million] whereas the estimated number of new infections was 2.5 million [1.8~4.1million].² In India approximately 2.5 million [2 million~3.1 million] people are currently living with HIV, with national adult HIV prevalence of 0.36%.³ Since HIV belongs to a group of viruses called retroviruses, treatment to fight HIV infection is known as antiretroviral therapy (ART). It involves at least three drugs and hence it is also known as Highly Active AntiRetroviral Therapy (HAART), or drug cocktail or combination therapy.⁴ Currently, 25 single or combination preparations from four classes of drugs are licensed: nucleoside and nucleotide analogues reverse transcriptase (RT) inhibitors [NRTIs], non-nucleoside RT inhibitors [NNRTIs], Protease inhibitors and Fusion inhibitors.⁵

Although effective drugs are available, generation of novel drugs is the need, the main reasons being the adverse effects of current drugs, HIV drug-resistance and the expenditure involved in the therapy. The most common side-effects associated with anti-HIV drugs are those that affect the stomach and intestines such as abdominal cramps, nausea, vomiting and diarrhea. Certain anti-HIV drugs can cause skin rashes that are likely to occur in people taking

the non- nucleoside reverse transcriptase inhibitors. Some drugs may increase photosensitivity leading to rapid and severe sunburning. Several investigators have reported increased liver enzyme levels in patients taking anti-HIV drugs. More serious manifestations of liver toxicity include clinical hepatitis and long-term liver damage. There have been cases of severe drug-induced hepatitis and kidney damage in people taking protease inhibitors. Some drugs including AZT can cause bone marrow toxicity. Damage to bone marrow may lead to loss of ability to produce new blood cells. This can result in anemia, leukopenia, neutropenia and thrombocytopenia. Apart from this, various anti-HIV drugs have been associated with mental side-effects including confusion, anxiety, paranoia and depression.⁶

In addition, patients may receive other drugs for supportive care, treatment of opportunistic infections and immunomodulation. Hence drug interactions are often unavoidable in HIV-infected patients because of the drug classes involved and the number of drugs prescribed. These drug interactions alter the absorption, transport, distribution, metabolism or excretion of drug that may lead to viral resistance or serious toxic effects resulting in treatment failure.⁷

To date, several immune-based approaches have also been investigated to complement antiretroviral therapy, to name a few: Cytokines such as Interleukin IL-2, IL-12, IL-7 and IL-15, Immunosuppressive drugs such as corticosteroids, Hydroxyurea, Cyclosporin A and related compounds that selectively target HIV-specific immunity such as tucaresol and murabutide. However, no clear evidence has yet emerged on the clinical impact of immunotherapy in management of HIV/AIDS.⁸

Several approaches have been tried in the extensive effort to develop an effective AIDS vaccine. However, development has been hampered by extensive antigenic variation exhibited by HIV, the ability of HIV to exist as a provirus in host cells, where it is

inaccessible to the immune system and the lack of good animal model for AIDS.⁹

In view of magnitude of AIDS pandemic, absence of protective vaccine, paucity of non-toxic therapy and resistance developed by the virus to different anti-HIV therapeutic drugs, there is urgent need for the development of new, specific and non-toxic drugs. To obtain lead molecules for this drug development, a number of plant products are being screened all over the world by scientists.¹ Medicinal plants are important for pharmacological research and drug development not only as therapeutic agents, but also as starting materials for the synthesis of drugs or as models for pharmacologically active compounds. The process of synthetic drug discovery and development takes an average of 12 years, and any new drug requires the investment of an average of US\$ 230 million. It is seen that plant based drugs take a comparatively much less time and expenses than synthetic drugs. The worldwide market of herbal medicines is of the order of US\$ 60 billion to US\$ 80 billion and India is the second largest exporter of medicinal plants.¹⁰

Traditional medicine has served as a source of alternative medicine, new pharmaceuticals, and healthcare products. Ayurveda is a traditional Indian medicinal system being practiced for decades. Considerable research on pharmacognosy, phytochemistry, pharmacology and clinical therapeutics has been carried out on potential ayurvedic medicinal plants. Numerous molecules have come out of ayurvedic experimental base, examples include Rauwolfia alkaloids for hypertension, guggulsterons as hypolipidemic agents, mucuna pruriens for Parkinson's disease, picrosides in hepatic protection, phyllanthins as antivirals, curcumin in inflammation, withanolides and many other steroidal lactones and glycosides as immunomodulators.¹¹ It is documented that 80% of the world's population has faith in traditional medicine, particularly plant drugs for their affordable primary healthcare

because of better cultural acceptability, better compatibility with the human body and lesser side effects.¹²

Many compounds of plant origin such as alkaloids, carbohydrates, coumarins, flavonoids, tannins, lignins, lipids, proteins, terpenoids etc. have been identified that are reported to inhibit different stages in replicative cycle of HIV. Some of these plant-derived anti-HIV compounds like glycyrrhizin, papaverine, tricosanthin, castanospermin etc have been used in limited number of patients suffering from AIDS. Some more plant products are in clinical trials and many more hope to follow this to give new drugs and lead molecules for drug- development for treatment of HIV infection.¹

Besides medicinal plants, Mangrove plants are also used in folklore medicine for treatment of several diseases.¹³ The term mangrove is used to designate halophytic (salt loving) and salt resistant marine tidal forests comprising of trees, shrubs, palms and ground ferns.

Mangroves are highly productive ecosystem with various important economic and environmental functions. The uses of mangroves fall in two major categories: Firstly the indirect use of mangrove ecosystem in the form of vital ecological functions such as control of coastal erosion and protection of coastal land, stabilization of sediment, natural purification of coastal water from pollution. Secondly, the economic benefits which are many and varied. Apart from prawn fisheries, many other species of economic importance are associated with mangroves. These include crabs, shrimp, oysters, lobsters and fish. Traditionally, mangroves have been exploited for firewood and charcoal and their uses include construction of dwellings, furniture, boats and fishing gear around the Indian Ocean and everywhere in the Far East. They are the important source of tannins for dyeing, leather production and oil drilling.

Two basic factors justify the study of chemical constituents of mangrove plants. Firstly, mangroves are one of the easiest tropical

forest types to generate. They have the ability to grow where no other vascular plants can. They exist under stressful conditions such as violent environments, high concentration of moisture, high and low tides of water and abundant living microorganisms and insects. These have imposed several modifications in these plants to establish water and salt economy. There are modifications in other physiological processes such as carbohydrate metabolism or polyphenol synthesis and due to these reasons, they may have chemical compounds, which protect them from these destructive elements. The second reason is that recently extracts from mangroves have proven activity against human, animal and plant pathogens.¹⁴

Apart from this, numerous bioactive compounds are derived from mangroves. Skin disorders and sores, including leprosy, may be treated with ashes and bark infusions of certain mangrove species. Headaches, rheumatism, snakebites, boils, ulcers, diarrhea, hemorrhages and many more conditions are traditionally treated with mangrove plants.¹⁵ Also extracts from mangrove plants such as *Aegiceras corniculatum*, *Ceriops decandra*, *Excoecaria gallocha*, *Rhizophora apiculata* and *Rhizophora lamarckii* showed *in vitro* anti-HIV activity.¹⁶

For the present *in-vitro* study, the plants were selected on citations in the literature on Indian Medicinal plants and also those commonly used in folk medicine. Root powder of *Withania somnifera* (Ashwagandha), stem powder of *Tinospora cordifolia* (Gulvel), leaves powder of *Ocimum sanctum* (Tulas) and the two mangrove species namely *Rhizophora mucronata* and *Avicennia officinalis* were used for the analysis. Shilajit (Asphaltum), a herbo-mineral was also included for the *In-vitro* study.

Ocimum sanctum, called Tulsi in India, is a ubiquitous in Hindu tradition. Its therapeutic properties include Antimicrobial, Anti-malarial, anti-diabetic and anti-stress effect.¹⁷ *Withania somnifera* is

widely used in Ayurvedic medicine. Studies indicate ashwagandha possesses anti-tumor, anti-oxidant, anti-inflammatory, hemopoetic and rejuvenating properties.¹⁸ *Tinospora cordifolia* is also a widely used shrub in folk and ayurvedic systems of medicine. The notable medicinal properties reported are anti-diabetic, anti-spasmodic, anti-inflammatory, anti-arthritis, anti-oxidant, anti-leprotic, anti-malarial and anti-neoplastic activities. It has also exhibited *in vitro* inactivating property against Hepatitis B and E surface antigens.¹⁹ Additionally, all the three plants possess immunomodulatory activity.

In the present study, we analyzed twenty three extracts prepared from five plants namely *Ocimum sanctum*, *Withania somnifera*, *Tinospora cordifolia*, *Avicennia officinalis*, *Rhizophora mucronata* and Shilajit a herbomineral for their specific inhibitory activity against HIV *in vitro*.

Rationale:

The chemotherapy of AIDS is based on development of anti-HIV drugs. Generation of novel drugs is the need although effective drugs are available. The main reasons being the adverse effects of current drugs, HIV drug resistance, the expenditure involved in the therapy and the absence of protective vaccine. To obtain lead molecules for this drug development, a number of plant products are being screened all over the world. Many compounds of plant origin such as alkaloids, carbohydrates, coumarins, flavonoids, tannins, lignins, lipids, proteins, terpenoids etc. have been identified that are reported to inhibit different stages in replicative cycle of HIV. The present study has been carried out to study *in-vitro* Anti-HIV activity of mangrove and selected medicinal plants.

Aims and Objectives:

The aims and objectives of the present *In-vitro* study are as follows:

1. To collect the raw material of the five selected medicinal plants namely, root powder of *Withania somnifera*, Stem powder of *Tinospora cordifolia*, leaves powder of *Ocimum sanctum*. Also, leaves of two mangrove plants namely, *Aviccenia officinalis* and *Rhizophora mucronata* and a herbomineral Shilajit.
2. To prepare different extracts with the help of following methods and to calculate their percentage yields.
 - ❖ By standard Soxhlet extraction method using non-polar and polar solvents.
 - ❖ Aqueous extraction by Decoction method.
3. To prepare cells and virus stock for the *In-vitro* study.
4. To carry out preliminary screening of plant extracts using,
 - ❖ XTT Formazan Assay
 - ❖ p24 Inhibition Assay
5. To evaluate specific inhibition of viral target molecule in the presence or absence of plant extracts by performing,
 - ❖ Reverse Transcriptase (RT) Inhibition Assay
 - ❖ gp120 Binding Inhibition Assay
6. To evaluate the Antiviral effect of extracts by using following parameters,
 - ❖ Dose-response study
 - ❖ Effect on clinical isolates
 - ❖ Time-course study

Materials and Methods:

1. Collection of the material:

a) Procurement of Raw Plant Material:

Root powder of *Withania somnifera* and stem powder of *Tinospora cordifolia* were procured from Zandu Pharmaceuticals, Mumbai. Leaves powder of *Ocimum sanctum* was purchased from Atul Medical Stores, Vile Parle (E), Mumbai.

b) Collection of Mangrove plants:

Avicennia officinalis was collected from Mahim Creek, Mumbai whereas *Rhizophora mucronata* was collected from Vikroli highway, Mumbai. The leaves were washed, sun-dried and powdered in a grinder mixer.

c) Shilajit was purchased from Vaidya (Dr.) Rajeev K. Kanitkar, Vile Parle (E), Mumbai.

The plant material and the herbomineral was identified and authenticated by Dr. J. M. Pathak, Zandu Pharmaceutical, Mumbai.

2. Preparation of extracts:

a) Soxhlet extraction using AR organic solvents:

The material was first extracted in Soxhlet apparatus with Petroleum Ether (60-80⁰C) and then with Ethanol in succession. A separate extraction of plant material was carried out with Ethanol alone. All the extracts were made free from solvents and percentage yield of individual extractives was calculated.

b) Aqueous extraction:

Aqueous extracts were prepared by plain decoction method²⁰ and then the percent of water-soluble extractives with respect to the air-dried drug was calculated.

Thus there are four extracts of each plant namely, Ethanol (Direct), Petroleum Ether, Ethanol (Successive) and Aqueous.

c) Reconstitution of Extracts:

Petroleum Ether and Ethanol extracts were dissolved in DMSO (not more than 1%) and then diluted with RPMI 1640 medium, whereas aqueous extracts were reconstituted in RPMI 1640 medium alone.

Each extract was reconstituted to prepare stock of 25 mg/ml. The stock solutions were then filtered through 0.22 μ m membrane filter and stored at 4⁰ C until further use.

3. Preparation of cells and virus stock:

a) Cells:

Blood sample was collected from healthy donor. Peripheral Blood Mononuclear Cells (PBMCs) were separated using Ficoll Hypaque density gradient centrifugation.²¹ Preliminary screening assays for anti-HIV activity were accomplished with 3-day old phytohemagglutinin (PHA) stimulated PBMCs. The cells were cultured in RPMI 1640 containing 10% Fetal Bovine Serum, 2 mM L-glutamine, 10 U/ml Interleukine-2 and 50 μ g/ml gentamycine.

b) Virus stock:

Blood sample was collected from a known HIV seropositive patient after taking written consent. PBMCs were separated as described²¹ The cells were then co-cultivated with the Healthy PBMCs to prepare virus stock.²¹ The culture supernatant was collected at regular time period and stored at -70⁰C as a source

of HIV for the study. The virus titer was determined using reverse transcriptase assay.

4. Preliminary Screening by:

a) XTT Formazan Assay:

Antiviral and cytotoxic effects of the extracts were evaluated simultaneously with the XTT Formazan assay.²² Extracts were further compared on the basis of concentration that increases XTT formazan production in infected cultures to 50% of that in untreated, uninfected control cells, i.e. 50% Effective Concentration (EC₅₀) and the concentration that inhibits XTT formazan production in uninfected cultures to 50% of that in untreated, uninfected control cells i.e. 50% Cytotoxic Concentration (CC₅₀). The selectivity index was also calculated as ratio of CC₅₀/EC₅₀.

b) HIV p24 Inhibition Assay:

PBMCs were seeded in 24-well plates with six five fold dilutions of each extract, followed by addition of virus. The cells were harvested on day 4 post infection and cell free supernatants were collected for the determination of p24 production by commercially available HIV-1 p24 Antigen ELISA kit (ZeptoMetrix Corporation) according to the manufacturer's instructions.

5. Anti-HIV Assays:

a) Reverse Transcriptase (RT) Inhibition Assay:

The HIV reverse transcriptase (RT) enzyme inhibition due to each of the extract was determined by adding 25 µl of each extract (Final concentration 5 mg/ml) to the reaction mixture. The reaction mixture (Final volume: 100µl) contained the following: 50 mM Tris (pH 7.8), 150 mM KCl, 5 mM MgCl₂, 0.05% NP-40, 0.5 mM EGTA, 5 mM DTT, 20 µM dTTP, 0.3 M

Glutathione, 2.5 µg/ml BSA, 0.5 µCi of ^3H TPP, 2.5 µg/ml POLY(rA).p(dT)₁₂₋₁₈.

The reaction was started by addition of 0.5 Units of recombinant reverse transcriptase enzyme (Ambion). The mixture was then incubated for 3 hrs at 37°C. The reaction was terminated by addition of 25 µl of 0.1 M EGTA followed by chilling the mixture on ice. 100 µl of each reaction mixture was then spotted uniformly onto DE-81 Whatman filter and kept at ambient temperature for 15 min. The dried filters were washed four times with 5% aqueous Na₂HPO₄.7H₂O followed by two more washings with doubly distilled water. Finally, the filters were thoroughly dried and subjected to scintillation counting.

b) gp120 Binding Inhibition Assay:

Binding of gp120 to CD4 was analyzed using a commercially available gp120 Capture ELISA kit (ImmunoDiagnostics, Inc.). Each extract (Final concentration 5 mg/ml) was mixed with 25 ng of purified gp120 in a total volume of 100 µl. This mixture was then incubated at room temperature for 1 hr. After incubation, each mixture was added to microtiter plate wells coated with CD4 ligand and incubated at room temperature for 1 hr. The extent of gp120 binding was assessed by using detector reagent provided in the kit.

6. Assessment of Antiviral activity by:

a) Dose-response study:

Six five fold dilutions of each extract were prepared as described earlier. The HIV reverse transcriptase enzyme inhibition due to each of the extract was determined by analyzing 25 µl of each of the concentration by adding it to the reaction mixture containing 0.5 Units of recombinant reverse transcriptase enzyme (Ambion). A drug AZT was

included as positive control in the study. Extent of HIV RT inhibition was evaluated by scintillation counting.

b) Effect on Clinical Isolates:

The analysis was carried out to determine the activity of each extract against two different clinical samples. The reverse transcriptase of *in vitro* cultivated virus stock from blood samples of HIV seropositive drug naïve and drug treated individual was considered for this study. The reverse transcriptase inhibition assay was carried out as described earlier.

c) Time-course study:

The analysis of time-course study of each plant extract is being carried out. The detailed methodology and result of the same will be furnished in Thesis.

Results:

1. Selected Plants:

Indigenous plants and a herbomineral selected in the present study are listed in the following table.

Table-1 : Indigenous Plants and Herbomineral under study

Plant / Herbomineral	Family	Vernacular Name	Part Used
<i>Ocimum sanctum</i> Linn	Labiatae	Tulas	Leaves
<i>Withania somnifera</i> Dunal	Solanaceae	Ashwagandha	Root
<i>Tinospora cordifolia</i> Willd.	Menispermaceae	Gulvel	Stem
<i>Avicennia officinalis</i> Linn.	Verbenaceae	Tivar	Leaves
<i>Rhizophora mucronata</i> Lam.	Rhizophoraceae	Tivar	Leaves
Shilajit	-	Shilajit	Granules

2. Yield of crude extracts:

The selected plant parts were extracted with Distilled Water, Non-polar and Polar solvents. The percent yield of individual extractives was then calculated. (Table-2)

Table-2: Percentage yield of the extractives

Plant / Herbo-mineral	Yield (%)			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
<i>O. sanctum</i>	8.13	1.68	6.88	4.4
<i>W. somnifera</i>	9.44	0.47	7.08	7.0
<i>T. cordifolia</i>	5.55	1.15	4.63	5.8
<i>A. officinalis</i>	18.56	1.47	14.73	16.0
<i>R. mucronata</i>	22.40	5.98	19.60	24.77
Shilajit	20.86	0.07	17.44	66.0

As shown in Table-2, percent yield of Petroleum Ether extract of Shilajit was very low to pursue further work. Thus, total 23 extracts were subjected to *In-vitro* anti-HIV activity.

3. Preliminary Screening Assays:

a) XTT Formazan Assay:

As per the table-3, Aqueous extract of *O. sanctum* showed highest Selectivity Index (S.I.) of 125. Rest of the extracts showed S.I. between 1 to 25.

Table-3 : Comparative study of Cytotoxicity and Antiviral activity

Plant / Herbo-mineral	Extracts	CC ₅₀ (mg/ml)	EC ₅₀ (mg/ml)	Selectivity Index (SI)
<i>O. Sanctum</i>	Ethanol (Direct)	>0.08	<0.0032	25
	Petroleum Ether	>0.08	<0.0032	25
	Ethanol (Successive)	>0.08	<0.0032	25
	Aqueous	>0.4	<0.0032	125
<i>W. somnifera</i>	Ethanol (Direct)	>0.016	<0.016	1
	Petroleum Ether	>0.08	<0.016	5
	Ethanol (Successive)	>0.016	<0.016	1
	Aqueous	>0.08	<0.08	1
<i>T. cordifolia</i>	Ethanol (Direct)	>0.016	<0.016	1
	Petroleum Ether	>0.016	<0.0032	5
	Ethanol (Successive)	>0.016	<0.016	1
	Aqueous	>0.08	<0.0032	25
<i>A. officinalis</i>	Ethanol (Direct)	>0.0032	<0.0032	1
	Petroleum Ether	>0.0032	<0.0032	1
	Ethanol (Successive)	>0.0032	<0.0032	1
	Aqueous	>0.016	<0.016	1
<i>R. mucronata</i>	Ethanol (Direct)	>0.016	<0.0032	5
	Petroleum Ether	>0.016	<0.0032	5
	Ethanol (Successive)	>0.016	<0.0032	5
	Aqueous	>0.08	<0.016	5
Shilajit	Ethanol (Direct)	>0.016	<0.0032	5
	Petroleum Ether	NA	NA	NA
	Ethanol (Successive)	>0.016	<0.0032	5
	Aqueous	>0.016	<0.0032	5
Control	AZT	>0.008	<0.00032	25

Note: CC₅₀ : 50% Cytotoxic Concentration

EC₅₀ : 50% Effective Concentration

Selectivity Index (SI) : CC₅₀ / EC₅₀

NA : Not Applicable

b) p24 Inhibition Assay:

Plant / Herbo-mineral	Conc. (mg/ml)	% p24 Inhibition [Mean $\pm$ SD]			
		Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
O. sanctum	10	74.51 $\pm$ 1.80	88.24 $\pm$ 0.71	83.33 $\pm$ 1.05	77.78 $\pm$ 1.34
	2	87.58 $\pm$ 0.65	84.31 $\pm$ 0.99	87.25 $\pm$ 0.91	56.70 $\pm$ 2.94
	0.4	87.09 $\pm$ 0.69	74.35 $\pm$ 1.35	82.84 $\pm$ 1.09	87.42 $\pm$ 0.77
	0.08	88.07 $\pm$ 0.72	82.84 $\pm$ 0.98	86.11 $\pm$ 1.22	86.76 $\pm$ 0.81
	0.016	86.11 $\pm$ 1.10	82.35 $\pm$ 1.13	85.78 $\pm$ 0.77	82.68 $\pm$ 0.99
	0.0032	83.17 $\pm$ 1.07	86.44 $\pm$ 0.83	75.82 $\pm$ 1.48	83.99 $\pm$ 1.24
W. somnifera	10	83.17 $\pm$ 1.20	83.33 $\pm$ 1.05	81.21 $\pm$ 1.21	80.07 $\pm$ 1.41
	2	81.70 $\pm$ 1.41	79.25 $\pm$ 1.47	82.84 $\pm$ 1.32	82.84 $\pm$ 1.21
	0.4	78.10 $\pm$ 1.55	85.13 $\pm$ 1.16	85.62 $\pm$ 1.02	76.80 $\pm$ 1.87
	0.08	81.37 $\pm$ 1.43	80.88 $\pm$ 1.24	82.19 $\pm$ 1.26	83.50 $\pm$ 1.67
	0.016	80.07 $\pm$ 1.41	82.52 $\pm$ 1.24	78.76 $\pm$ 1.62	81.21 $\pm$ 1.44
	0.0032	79.74 $\pm$ 1.54	80.72 $\pm$ 1.36	80.72 $\pm$ 1.46	80.72 $\pm$ 1.48
T. cordifolia	10	67.48 $\pm$ 3.92	89.38 $\pm$ 2.50	80.88 $\pm$ 2.94	86.76 $\pm$ 0.35
	2	69.93 $\pm$ 2.36	90.20 $\pm$ 2.09	82.35 $\pm$ 1.43	63.73 $\pm$ 0.57
	0.4	91.67 $\pm$ 0.12	88.89 $\pm$ 2.81	87.75 $\pm$ 2.50	84.48 $\pm$ 2.38
	0.08	75.82 $\pm$ 0.61	87.42 $\pm$ 0.04	76.47 $\pm$ 2.28	81.86 $\pm$ 5.20
	0.016	85.46 $\pm$ 1.87	89.54 $\pm$ 0.04	88.24 $\pm$ 2.18	41.18 $\pm$ 0.13
	0.0032	84.97 $\pm$ 1.72	73.53 $\pm$ 2.88	87.75 $\pm$ 1.57	79.74 $\pm$ 3.20
A. officinalis	10	89.38 $\pm$ 0.28	92.32 $\pm$ 0.31	91.67 $\pm$ 0.23	90.52 $\pm$ 0.55
	2	91.18 $\pm$ 0.39	92.32 $\pm$ 0.31	90.52 $\pm$ 0.32	90.85 $\pm$ 0.42
	0.4	91.01 $\pm$ 0.40	92.48 $\pm$ 0.41	91.34 $\pm$ 0.26	92.65 $\pm$ 0.28
	0.08	89.22 $\pm$ 0.41	91.83 $\pm$ 0.35	92.48 $\pm$ 0.30	89.22 $\pm$ 0.41
	0.016	91.01 $\pm$ 0.17	91.01 $\pm$ 0.51	92.65 $\pm$ 0.40	90.52 $\pm$ 0.32
	0.0032	91.50 $\pm$ 0.37	87.58 $\pm$ 0.42	84.31 $\pm$ 0.76	92.32 $\pm$ 0.31
R. mucronata	10	91.83 $\pm$ 0.35	91.83 $\pm$ 0.23	92.16 $\pm$ 0.21	89.38 $\pm$ 0.06
	2	91.83 $\pm$ 0.45	71.24 $\pm$ 1.68	86.76 $\pm$ 0.47	90.52 $\pm$ 0.32
	0.4	91.67 $\pm$ 0.23	92.48 $\pm$ 0.41	81.54 $\pm$ 0.61	91.83 $\pm$ 0.11
	0.08	91.99 $\pm$ 0.33	91.67 $\pm$ 0.36	91.18 $\pm$ 0.39	91.50 $\pm$ 0.14
	0.016	91.50 $\pm$ 0.37	91.50 $\pm$ 0.48	91.50 $\pm$ 0.25	90.85 $\pm$ 0.18
	0.0032	91.67 $\pm$ 0.23	91.99 $\pm$ 0.33	91.99 $\pm$ 0.21	90.52 $\pm$ 0.08
Shilajit	10	67.48 $\pm$ 1.29	NA	81.21 $\pm$ 1.57	89.71 $\pm$ 0.15
	2	70.75 $\pm$ 0.79	NA	89.71 $\pm$ 1.25	78.59 $\pm$ 0.59
	0.4	70.10 $\pm$ 1.07	NA	89.71 $\pm$ 1.94	51.47 $\pm$ 2.16
	0.08	11.44 $\pm$ 3.59	NA	35.95 $\pm$ 2.79	86.60 $\pm$ 0.25
	0.016	46.73 $\pm$ 0.29	NA	90.20 $\pm$ 0.10	80.23 $\pm$ 0.59
	0.0032	83.50 $\pm$ 2.20	NA	32.68 $\pm$ 3.49	52.12 $\pm$ 2.34
Control	AZT (0.0032 mg/ml)	76.31 $\pm$ 3.54			

Table-4: Inhibition of HIV p24 antigen by Extracts

As illustrated in Table-4, all the plants potently inhibit p24 antigen production, with mangrove plants namely *A. officinalis* and *R. mucronata* showing highest activity.

4. Anti-HIV Assays:

a) Reverse Transcriptase (RT) Inhibition Assay:

As per Table-5, among Ethanol (Direct), Ethanol (Successive) and Aqueous extracts, Shilajit showed highest inhibition of RNA Dependent DNA Polymerase (RDDP) function of recombinant HIV-RT. Whereas among Petroleum Ether extracts *R. mucronata* showed highest inhibition followed by *O. sanctum*. AZT was included as positive control that showed 71.04% inhibition.

Table-5: Inhibition of HIV-RT by Extracts

Plant / Herbo- mineral	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
<i>O. sanctum</i>	82.96 $\pm$ 1.20	79.92 $\pm$ 1.22	76.22 $\pm$ 0.96	85.04 $\pm$ 0.04
<i>W. somnifera</i>	18.77 $\pm$ 1.84	68.71 $\pm$ 1.23	46.19 $\pm$ 1.77	32.30 $\pm$ 5.50
<i>T. cordifolia</i>	57.27 $\pm$ 1.57	64.48 $\pm$ 0.50	54.29 $\pm$ 1.24	66.03 $\pm$ 0.88
<i>A. officinalis</i>	43.92 $\pm$ 4.06	74.79 $\pm$ 3.47	82.00 $\pm$ 0.26	82.18 $\pm$ 1.72
<i>R. mucronata</i>	81.17 $\pm$ 0.02	87.07 $\pm$ 1.85	84.74 $\pm$ 0.05	82.48 $\pm$ 1.93
Shilajit	83.55 $\pm$ 1.04	NA	89.45 $\pm$ 1.08	86.35 $\pm$ 1.22
AZT [control] (0.0016 mg/ml)	71.04 $\pm$ 1.94			

b) gp120 Binding Inhibition Assay:

As illustrated in Table-6, among Ethanol (Direct), Petroleum Ether and Aqueous extracts, *A. officinalis* showed highest inhibition where as among Ethanol (successive) extracts, *R. mucronata* showed highest inhibition followed by *T. cordifolia*. Also Ethanol (Direct) and Petroleum Ether extracts of *O. sanctum* and Ethanol (successive) and Aqueous extracts of *W. somnifera* showed no inhibition. Heparin a known gp120/CD4 binding inhibitor was included as positive control.

Table-6: gp120 Binding Inhibition by Extracts

Plant / Herbo- mineral	gp120 Binding Inhibition (%) [Mean $\pm$ SD]			
	Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
<i>O. sanctum</i>	0	0	36.85 $\pm$ 0.29	21.24 $\pm$ 0.88
<i>W. somnifera</i>	23.15 $\pm$ 0.71	1.12 $\pm$ 0.63	0	0
<i>T. cordifolia</i>	39.44 $\pm$ 0.67	78.54 $\pm$ 0.16	80.22 $\pm$ 0.38	41.91 $\pm$ 0.57
<i>A. officinalis</i>	85.17 $\pm$ 0.19	81.01 $\pm$ 0.30	78.65 $\pm$ 0.23	69.33 $\pm$ 0.37
<i>R. mucronata</i>	49.10 $\pm$ 0.52	69.33 $\pm$ 0.22	81.69 $\pm$ 0.13	60.11 $\pm$ 0.69
Shilajit	35.62 $\pm$ 0.62	NA	28.88 $\pm$ 0.75	26.40 $\pm$ 0.92
Heparin [control] (12.5 Units)	75.96 $\pm$ 0.25			

5. a) Dose-Response Study:

Plant / Herbo-mineral	Conc. (mg/ml)	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]			
		Ethanol Extract (Direct)	Petroleum Ether Extract	Ethanol Extract (Successive)	Aqueous Extract
O. sanctum	5	82.96 $\pm$ 1.20	79.92 $\pm$ 1.22	76.22 $\pm$ 0.96	85.04 $\pm$ 0.04
	1	68.06 $\pm$ 0.66	80.81 $\pm$ 0.62	75.09 $\pm$ 1.48	77.89 $\pm$ 0.67
	0.2	60.73 $\pm$ 3.19	62.40 $\pm$ 1.90	78.07 $\pm$ 1.26	72.17 $\pm$ 1.28
	0.04	44.04 $\pm$ 6.92	82.42 $\pm$ 0.18	68.95 $\pm$ 0.34	70.50 $\pm$ 0.03
	0.008	78.90 $\pm$ 1.45	73.42 $\pm$ 0.17	65.91 $\pm$ 1.66	69.19 $\pm$ 1.34
	0.0016	65.61 $\pm$ 1.46	81.35 $\pm$ 0.31	60.55 $\pm$ 1.25	85.76 $\pm$ 0.86
W. somnifera	5	18.77 $\pm$ 1.84	68.71 $\pm$ 1.23	46.19 $\pm$ 1.77	32.30 $\pm$ 5.50
	1	46.78 $\pm$ 2.15	53.58 $\pm$ 1.46	59.00 $\pm$ 1.29	22.05 $\pm$ 3.16
	0.2	57.27 $\pm$ 1.23	33.37 $\pm$ 3.08	11.08 $\pm$ 3.88	41.24 $\pm$ 3.14
	0.04	60.43 $\pm$ 2.09	46.72 $\pm$ 0.42	44.40 $\pm$ 3.16	38.74 $\pm$ 2.09
	0.008	62.46 $\pm$ 1.10	38.68 $\pm$ 1.37	51.61 $\pm$ 0.33	30.15 $\pm$ 4.74
	0.0016	60.67 $\pm$ 1.36	0	43.21 $\pm$ 1.41	45.17 $\pm$ 2.85
T. cordifolia	5	57.27 $\pm$ 1.57	64.48 $\pm$ 0.50	54.29 $\pm$ 1.24	66.03 $\pm$ 0.88
	1	65.49 $\pm$ 1.04	60.79 $\pm$ 1.00	52.32 $\pm$ 2.72	64.30 $\pm$ 2.28
	0.2	32.66 $\pm$ 2.45	77.53 $\pm$ 2.31	44.64 $\pm$ 0.50	64.90 $\pm$ 0.58
	0.04	65.91 $\pm$ 0.27	65.97 $\pm$ 1.53	85.88 $\pm$ 0.47	68.71 $\pm$ 2.78
	0.008	82.54 $\pm$ 0.97	61.50 $\pm$ 0.45	69.73 $\pm$ 0.45	77.00 $\pm$ 1.83
	0.0016	74.67 $\pm$ 0.99	57.75 $\pm$ 2.48	62.28 $\pm$ 1.01	74.08 $\pm$ 0.70
A. officinalis	5	43.92 $\pm$ 4.06	74.79 $\pm$ 3.47	82.00 $\pm$ 0.26	82.18 $\pm$ 1.73
	1	87.84 $\pm$ 0.57	77.65 $\pm$ 0.08	73.36 $\pm$ 0.17	80.69 $\pm$ 1.55
	0.2	66.51 $\pm$ 2.23	81.59 $\pm$ 1.10	73.30 $\pm$ 2.06	78.84 $\pm$ 0.19
	0.04	71.69 $\pm$ 0.35	77.71 $\pm$ 0.46	78.90 $\pm$ 2.60	79.62 $\pm$ 0.68
	0.008	75.45 $\pm$ 0.59	77.41 $\pm$ 0.56	77.23 $\pm$ 0.81	85.10 $\pm$ 0.48
	0.0016	76.10 $\pm$ 1.68	77.83 $\pm$ 0.54	66.39 $\pm$ 1.77	79.32 $\pm$ 1.08
R. mucronata	5	81.17 $\pm$ 0.02	87.07 $\pm$ 1.85	84.74 $\pm$ 0.05	82.48 $\pm$ 1.93
	1	76.58 $\pm$ 0.61	83.97 $\pm$ 0.62	79.32 $\pm$ 1.48	65.67 $\pm$ 2.38
	0.2	79.50 $\pm$ 1.57	79.68 $\pm$ 0.37	82.72 $\pm$ 0.52	74.14 $\pm$ 0.66
	0.04	72.59 $\pm$ 1.28	87.01 $\pm$ 1.09	83.67 $\pm$ 1.15	71.51 $\pm$ 0.72
	0.008	85.16 $\pm$ 1.21	82.36 $\pm$ 0.84	68.18 $\pm$ 1.95	80.10 $\pm$ 0.68
	0.0016	87.31 $\pm$ 1.34	83.73 $\pm$ 0.28	70.92 $\pm$ 0.16	84.51 $\pm$ 1.98
Shilajit	5	83.55 $\pm$ 1.04	NA	89.45 $\pm$ 1.08	86.35 $\pm$ 1.22
	1	72.11 $\pm$ 0.53	NA	83.02 $\pm$ 0.99	78.13 $\pm$ 0.97
	0.2	75.92 $\pm$ 0.63	NA	75.51 $\pm$ 0.47	79.80 $\pm$ 0.13
	0.04	72.65 $\pm$ 1.20	NA	76.28 $\pm$ 1.42	79.14 $\pm$ 1.66
	0.008	77.12 $\pm$ 2.46	NA	77.83 $\pm$ 0.13	76.10 $\pm$ 0.20
	0.0016	70.68 $\pm$ 1.10	NA	88.08 $\pm$ 0.87	80.15 $\pm$ 0.04
Control	AZT (0.0016)	71.04 $\pm$ 1.94			

Table-7: Effect of different concentrations of Extracts on HIV-RT

As shown in table-7, in general Shilajit, *A. officinalis*, *R. mucronata* and *O. sanctum* potently inhibit RDDP function of recombinant HIV-RT. Apart from this, *T. cordifolia* showed promising activity whereas petroleum ether extract of *W. somnifera* showed no inhibition at lowest concentration (0.0016 mg/ml).

b) Effect on Clinical Isolates:

Plant / Herbo-mineral	Extracts	Reverse Transcriptase Inhibition (%) [Mean $\pm$ SD]	
		Non-ART	ART
<i>O. sanctum</i>	Ethanol (Direct)	54.49 $\pm$ 2.27	31.53 $\pm$ 8.75
	Petroleum Ether	44.01 $\pm$ 0.67	24.32 $\pm$ 12.78
	Ethanol (Successive)	76.35 $\pm$ 0.57	63.24 $\pm$ 0.16
	Aqueous	57.49 $\pm$ 2.31	58.74 $\pm$ 5.71
<i>W. somnifera</i>	Ethanol (Direct)	37.28 $\pm$ 4.26	43.24 $\pm$ 0.80
	Petroleum Ether	46.41 $\pm$ 0.99	17.48 $\pm$ 4.50
	Ethanol (Successive)	46.26 $\pm$ 0.49	45.05 $\pm$ 8.58
	Aqueous	50.00 $\pm$ 3.07	16.22 $\pm$ 6.01
<i>T. cordifolia</i>	Ethanol (Direct)	57.19 $\pm$ 0.40	24.68 $\pm$ 5.20
	Petroleum Ether	51.80 $\pm$ 0.41	16.40 $\pm$ 10.34
	Ethanol (Successive)	64.52 $\pm$ 1.53	49.73 $\pm$ 0.62
	Aqueous	55.84 $\pm$ 2.37	65.05 $\pm$ 0.09
<i>A. officinalis</i>	Ethanol (Direct)	10.03 $\pm$ 3.92	79.46 $\pm$ 2.91
	Petroleum Ether	27.84 $\pm$ 1.51	31.89 $\pm$ 6.30
	Ethanol (Successive)	59.73 $\pm$ 1.05	65.59 $\pm$ 2.98
	Aqueous	43.86 $\pm$ 0.17	76.22 $\pm$ 2.72
<i>R. mucronata</i>	Ethanol (Direct)	49.10 $\pm$ 2.74	75.86 $\pm$ 0.38
	Petroleum Ether	30.69 $\pm$ 0.73	49.01 $\pm$ 6.08
	Ethanol (Successive)	58.23 $\pm$ 0.97	60.54 $\pm$ 1.85
	Aqueous	70.36 $\pm$ 1.06	80.00 $\pm$ 3.63
Shilajit	Ethanol (Direct)	65.12 $\pm$ 0.79	70.09 $\pm$ 3.13
	Petroleum Ether	NA	NA
	Ethanol (Successive)	56.89 $\pm$ 1.94	74.41 $\pm$ 0.63
	Aqueous	55.24 $\pm$ 2.49	67.03 $\pm$ 0.71
Control	AZT (0.0016 mg/ml)	26.50 $\pm$ 2.04	38.02 $\pm$ 10.42

Table-8: Effect of extracts on different Clinical Isolates

As illustrated in table-8, Ethanol (Successive) extract of *O. sanctum* showed highest inhibition of Non-ART isolate whereas Aqueous extract of *R. mucronata* showed highest inhibition of ART isolate followed by Ethanol (Direct) extract of *A. officinalis*. In general, all the three extracts of Shilajit showed significant inhibition ($\geq 50\%$ inhibition) of both the clinical isolates.

An exploratory TLC study of the extracts of promising plants is initiated. Result of the same will be furnished in the thesis.

Discussion:

The development of safe, effective and low-cost anti-HIV drugs is among the top global priorities of drug development, since the disease is not yet curable and mortality is high.

In the past decade, considerable attention has been paid to screening of plant extracts for possible anti-HIV activity with the aim of isolating bioactive compounds as an alternative source to chemical synthesis. Screening of plant extracts for antiretroviral activity has given encouraging results in that most plant derived anti-HIV compounds inhibit replication of the virus by interfering with one or more of the ten steps of replicative cycle.

The therapeutic efficacy of a handful of these compounds, such as benzyloquinoline alkaloid, papaverine and the saponin glycyrrhizin has been studied in AIDS patients. It is evident therefore that plants can be useful sources or leads for the discovery of novel anti-HIV compounds.²³

In the present study, twenty three extracts prepared from five plants and a herbomineral used in traditional and folklore medicine were tested for their inhibitory effects on the *In-vitro* replication of HIV.

The preliminary screening of the plant extracts was carried out with the help of XTT formazan and HIV-p24 inhibition assays. XTT formazan assay allowed simultaneous estimation of the cytotoxicity of these extracts and the evaluation of their anti-HIV potential. In the present study, Aqueous extract of *O. sanctum* showed highest Selectivity Index (S.I.) of 125. Whereas rest of the extracts showed S.I. between 1 to 25. However, it is possible that the active principle (s) and the toxic compound (s) of the extract are different from each other in which case fractionation of the total extract should help determine the fraction with the highest selectivity index.

HIV mainly infects T-cells, hence anti-HIV activity of twenty three extracts was evaluated by measuring p24 expression in primary PBMCs. Lee-Huang et al²⁴ showed inhibition of HIV replication as assayed by p24 expression in infected H9 cells. While Chen et al²⁵ evaluated effect of shikonin on replication of clinical HIV isolates as well of multi-drug resistant HIV isolate in PBMCs. As per table-4, mangrove plants namely *A. officinalis* and *R. mucronata* showed substantial HIV p24 antigen inhibition.

As all extracts showed potent inhibitory activity in primary screening, they further evaluated using specific and more sensitive assays. Antiviral research has been focused on compounds that interfere with various parts of the viral life-cycle such as proteins encoded by the virus itself, i.e. Reverse Transcriptase (RT), Protease and Integrase. Among these, Reverse Transcriptase is a popular therapeutic target as RT plays a very important role in controlling the replication of HIV. Bessong et al²⁶ screened South African medicinal plants for activity against HIV-1 RT RDDP and RNase H activities. EL-Mekki et al²⁷ evaluated extracts of 41 medicinal plants used in Egyptian folk medicine for their HIV-1 RT inhibitory effects and identified putranjivain A from *P. emblica* as a potent inhibitory substance against HIV-RT. In the present study, Shilajit, *A. officinalis*, *R. mucronata* and *O. sanctum* potently inhibit RDDP function of recombinant HIV-RT. Investigation of anti-HIV activity of medicinal plants against different strains of HIV is relevant in terms of promoting the traditional use of plants as anti-HIV agents. Hence, effect of twenty three extracts on two different clinical isolates was evaluated by HIV RT inhibition assay. As illustrated in Table-8, fourteen of twenty three extracts showed significant inhibition ($\geq 50\%$ inhibition) of Non-ART isolate and twelve of twenty three extracts showed significant inhibition of ART isolate. Where as only nine of twenty three extracts showed significant inhibition of both the isolates.

Apart from inhibition of viral proteins, binding of gp120 to CD4 is also a critical step in HIV infection. Therefore, much interest has been focused on agents that block this interaction. Two agents that have been shown to potently inhibit the adsorption of HIV to CD4⁺ cells and inhibit HIV infection *in-vitro* are the sulfated polysaccharides, dextran sulfate and heparin.²⁸ Besides this, the purified extract of *Prunella vulgaris* antagonized HIV-1 infection by preventing viral attachment to the CD4 receptor.²⁹ Also, Ojwang et al³⁰ studied effects of oligonucleotides on gp120 / CD4 interaction using a gp120 capture ELISA kit. In the present study, a similar estimation revealed that only 9 of 23 extracts showed significant inhibition ($\geq 50\%$ inhibition) of gp120 / CD4 interaction. The extracts that inhibit this interaction after preincubation with gp120, may bind tightly to gp120 thereby inactivating them and preventing the interaction between gp120 and CD4.

Experimental results suggest that most of the plant extracts exert their anti-HIV activity via multiple mechanisms of action, each of which contributes to the overall anti-viral profile of the selected plants. For example inhibition of viral entry into cells could be accomplished by interference with the gp120 / CD4 interaction. Evidence is also presented that another mechanism, inhibition of viral RT, contributes to the overall anti-viral activity.

In conclusion, the results obtained from the present study do seem to justify the traditional use of plants for the treatment of infectious diseases of viral origin. They further indicate that these plants might be of value as sources or leads for novel antiviral compounds which can be useful in the continuing fight against HIV.

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