Evaluation of anti-HIV-1 potential in selected medicinal plants

Submitted By

Titas Mallick

4th Semester M.sc

Reg No. 621-1121-1230-14

Roll No. 91/BOT/1710008

2019

Under the guidance of

Prof. Binay Chaubey

Department of Botany

UNIVERSITY OF CALCUTTA

ACKNOWLEDGEMENT-

I express my deep sense of gratitude to our respected and learned guide, Prof. Binay Chaubey, Department of Botany, University of Calcutta for his valuable help and guidance, I am thankful to him for the encouragement he has given me in completing the project.

I sincerely record my thanks to Mr. Krishna Kumar Ganta and Ms. Sreetama Roy for their outstanding guidance during my dissertation project. I am deeply indebted to them for their advice, masterly guidance, and valuable suggestion and timely help, despite their busy schedule during my project work. I would like to extend my special thanks to all of the technical staff of the Department of Botany for their co-operation and help. I would like to express my deepest sense of gratitude towards my beloved parents who have supported me with their valuable suggestion and guidance. Last but not the least I would like to thank my lab mate Mr. Arijit Das Adhikary who was always with me with his support and critical reviews.

We are also grateful to respected Prof. Krishnendu Acharya HOD of our department for permitting us to utilize the necessary facilities of the institution. I am also thankful to all the other faculty & staff members of our department for their kind co-operation and help. Lastly, I would like to express my deep apperception towards my classmates and for providing me the moral support encouragement.

ABSTRACT

Acquired immunodeficiency syndrome (AIDS) is a global pandemic. Since its discovery >36.9 million people have lost their lives. Although highly active antiretroviral therapy (HAART) has been successful in significantly lowering viral titers as HIV-1 integrates into host genome it is not targeted by HAART and hence not eliminated. Therefore, the virus rebounds on discontinuation of drugs. Hence, patients are likely to take therapy for a lifetime and long-term drug treatment leads to severe drug-induced toxicity and also the emergence of multidrug-resistant viruses. It is pertinent to mention that the rate of emergence of resistant strains of HIV-1 is much greater than the rate of new drug development. Hence there is an urgent need to develop new strategies to tackle HIV-1 and emerging resistant strains.

Compounds from plant origin have enormous potential in terms of their incomparable structural diversity and plant kingdom needs to be explored for anti-HIV-1 molecules. In the present study medicinal plants namely *Catharanthus roseus, Ocimum gratissimum, Mangifera sp*., *Tinospora sp., Solanum sp*., *Swertia bimaculata* were evaluated for their anti-HIV-1 activity in cell culture. Crude extracts of the dried plants were prepared using solvents viz. hexane, chloroform, and methanol or Ethyl acetate. Extracts were dissolved in DMSO and filter sterilized and screened for anti-HIV-1 activity using pseudotyped HIV-1 virus with GFP (Green Fluorescent Protein) reporter system. Prior cytotoxic concentrations 50 (CC_{50}) of each extract was determined and all the extracts used in the present study were below their respective CC_{50}concentrations.

The significant anti-HIV-1 activity was observed in methanolic extracts of *Ocimum gratissimum*, ethyl acetate extracts of *Tinospora sp*. Further fractionation of *Ocimum gratissimum* showed anti-HIV-1 activity in- E3M7 1 fraction.

INTRODUCTION

Human immunodeficiency virus (HIV) is a (+) single-stranded, enveloped, RNA virus that causes acquired immunodeficiency syndrome (AIDS). It belongs to Lentivirus genus of Retroviridae family. AIDS is a global pandemic. Since its discovery in 1983, ~78 million people have been infected with HIV/AIDS and > 35 million people have lost their lives worldwide. However, the introduction of highly active antiretroviral therapy (HAART) which involves the administration of a combination of antiretroviral drugs, has led to a significant reduction in AIDS-related morbidity and mortality [41]. HAART has been successful in significantly lowering viral titers, however, viremia rebounds upon discontinuation of drugs. As patients are likely to take therapy for a lifetime, long-term drug treatment leads to severe toxicity and the emergence of multidrug-resistant viruses. Moreover, HAART is still out of reach of the majority of HIV-1 positive individuals and only ~18.2 million (50%) patients have access to the antiretroviral therapy (ART). Furthermore, there is a consistent emergence of resistant strains of HIV-1 which is much faster than the rate of new drug development. Last FDA approved drug Elvitegravir, an integrase inhibitor, was approved in 2014 after that no new drug has been approved.

In the absence of an effective vaccine, increasing demand of anti-HIV-1 drugs and emergence of resistant strains against present drugs, it is the need of the hour to develop new anti-viral drugs and identify novel drug targets. The new drug should be safe, effective, cheap and well tolerated upon long term administration.

Natural products such as compounds from plant origin have enormous potential in terms of their incomparable structural diversity [42-44, 48]. WHO has also declared the necessity to evaluate medicinal plants and other natural products in this context [47]. There is a need to evaluate traditional medicines, particularly medicinal plants and other natural products that might yield effective and affordable therapeutic agents against HIV. This will require a systematic approach. India is a megadiverse nation and harbors a vast reservoir of medicinal plants, however, only a fraction of plants have been evaluated for their anti- HIV-1 potential so far. Although there are many reports on the use of medicinal plants for the treatment of many ailments in our traditional literature reports on the use of medicinal plants for inhibition of HIV-1 are scanty. To address this issue, the project will focus on the preparation of crude extracts from medicinal plants and their screening for anti-HIV-1 potential. In the present study *Catharanthus roseus, Ocimum gratissimum, Mangifera sp., Tinospora sp., Solanum sp., Swertia bimaculata* were evaluated for their anti-HIV-1 potential. Extracts from these plants were prepared using different solvents based on polarity and first evaluated for their cytotoxic effects to determine CC_{50}. The extracts were then evaluated for anti- HIV-1 activity in a cell culture system using pseudotyped HIV-1 virus [45] with GFP (Green Fluorescent Protein) [46] reporter system. The concentrations used in this study were below their respective CC_{50} concentrations. Reduction in of expression of GFP reporter gene, visualized by a fluorescent microscope, indicated the anti-HIV-1 activity of the plant extract.

Literature Review

INTRODUCTION

AIDS is a global pandemic e, since the first report of the disease ~34.9 million people have died[1]. The number of infected people is still increasing and we still do not have any cure for the disease. In 1981 the disease was first reported and later the causal organism for the disease was identified to be the HIV-1 or Human Immunodeficiency Virus. Mostly the low and middle income countries have most of the infected people. ART or Anti-Retroviral therapy is the current mode of treatment of the disease. A report published by UNAIDS (2012) suggests 9.7 million people are currently having access to ART[1]. The human immunodeficiency virus (HIV) is grouped to the genus Lentivirus within the family of Retroviridae, subfamily Orthoretrovirinae.[2] On the basis of genetic characteristics and differences in the viral antigens, HIV-1 is classified into the types 1 and 2 (HIV-1, HIV-2).[3] The immunodeficiency viruses of non-human primates (simian immunodeficiency virus, SIV) are also placed in the genus Lentivirus. Epidemiologic and phylogenetic analyses imply that HIV-1 was introduced into the human population around 1920 to 1940. HIV-1 evolved from non-human primate immunodeficiency viruses from Central African chimpanzees and HIV-2 from West African sooty mangabeys[4]. The HIV-1 infection degrades the natural immune system, and the disease may undergo a very long period of asymptomatic stages, where influenza like infections are very common initially [4]. It follows a prolonged asymptomatic stage, and at later stages the person gets infected by common infections like tuberculosis and without having a working immune system patients die. The virus remains present and active along the all stages of the disease. The HIV-1 is transmitted by unprotected sex, blood transfer and any other ways of transfer of bodily fluid from infected people to healthy infection. The current treatment of HIV-1/AIDS is HAART or Highly active anti-retroviral therapy which include treatment with 7 classes of HIV-1 drugs[5]; non-nucleoside reverse transcriptase inhibitors (NNRTIs), nucleoside reverse transcriptase inhibitors (NRTIs), post-attachment inhibitors, protease inhibitors (PIs), CCR5 antagonists, integrase strand transfer inhibitors (INSTIs) and fusion inhibitors that prevent the different events in virus life cycyle[5]. HIV-1 undergoes a rapid replication cycle which starts with a reverse transcription of its RNA genome into DNA and multiplication of the DNA by reverse transcriptase, this process is a low fidelity process and therefore, error prone (~9 errors per replication cycle)[6]. This higher rate of mutation alter the HIV-1 genome, leading to drug resistance over time. Moreover, post reverse transcription the proviral DNA gets integrated into the nuclear DNA of the host Therefore, it is very difficult to be cure HIV-1 completely as the present therapy does not remove the integrated proviral DNA form the infected cells. Therefore, there is necessity for continuous new drug development against HIV. In this regard plants can be helpful.[7] Many plants show potent anti-viral properties. Plant produces thousands of natural phytochemicals in them. Some of them may have potential Anti-HIV-1 activity. Many reports have shown Anti-HIV-1 activity from natural compounds from different plants [8]. Therefore, proper evaluation of natural compounds from different plants is needed in order to develop these molecules into potential Anti-HIV-1 drugs.

HIV STATISTICS

AIDS is a burning problem in the under developed countries like Africa and even for the developing countries like India. India ranks 3rd in the Global HIV-1 pandemic indices. Currently 2.1 million People in India[9] are living with HIV. About 88,000 new HIV-1 infections and 69,000

HIV-1 related death were reported from India in 2017[9]. The HIV-1 infections in India are concentrated in some key population, mainly among sex workers. The national AIDS control program made particular effort to reach those particular niches and is somewhat successful in reducing the rate of new infection to half from 2014 to 2017 by providing free antiretroviral drugs. However, the social stigma and lack of general awareness makes the situation worse. Globally the figures are grim, at the end of 2016 there were ~36.9 million people living with HIV-1 (avert, 2018), 1.8 million new infections were reported in 2016 and 2.1 million of the globally infected patients are children[10]. Among them 70% people are aware of their HIV-1 status but still 30% of them need to get HIV-1 clinical testing and medications. Above 1 million people died of HIV-1 related illness in 2016[4]. About 53% of HIV-1 infections are concentrated in Eastern and Southern Africa, 17% in Western and Central Africa, 14% in Asia and pacific and 6% in the western countries[1]. Thus, HIV-1 as a global pandemic is a global sociological, economical problem even today. Understanding the HIV-1 biology, development of new drugs, spreading of general awareness among the people living in HIV-1 prone areas is needed to combat the disease.

HISTORY OF HIV PANDEMIC

Before 1981 there was no documented case of HIV/AIDS infection. In 1981 first cases of five people with Pneumocystis carinii pneumonia (PCP) and Kaposi 's sarcoma[11], a rare blood cancer was reported. Till the end of the year number of such infections reached 270 and 121 of them died[10],though the cause of disease was unknown. The first five people admitted with the disease were all gay and doctors assumed the disease has something to do with unprotected gay sex. It was referred as GRID or Gay Related Immune Deficiency[12]. The causal organism of the disease was still unknown, early reports of that time suggests scientists were confused if it is a disease caused by a pathogen or any toxin. Till the beginning of the next year the documented cases of the GRID infection raised to thousands, and not only Gay people, heterosexual men, women and also infants were getting infected. The disease took a figure of global pandemic. In 1982 CDC (US Centre for Disease Control) first proposed the term AIDS instead of GRID. . The emergency treatment began far earlier than the discovery of the lentivirus that was causing it. In 25th July of 1983 the first ever AIDS ward was opened in San Francisco Hospital. Luc Montagnier, a French molecular biologist first suggested that the disease may be caused by virus[13], Montagnier proposed Lymphadenopathy Associated Virus or LAV9[13] may be the causal organism of the disease. Around the same time Robert Gallo independently proposed HTLV-III as the causal organism of the disease[2],[14]. Later it was found that LAV and HTLV-III were [14] the same virus differently isolated and identified and this is might be the causal organism of AIDS, a retrovirus. In 1986 the virus causing AIDS was officially termed as HIV-1 or human immunodeficiency virus[2]. In 1996 the IAVI or International AIDS Vaccine Initiative was formed and research for anti-HIV-1 vaccination sky rocketed from then. In 2018 UNAIDS published a special analysis about the distribution of new HIV-1 infections among population groups. According to that report 3% are sex workers, 8% are people who used drugs, 18% were homosexual men, 1% are transgender women, 18% are clients of sex workers, and 52% are rest of the population[4] (avert, 2018). It also showed 8% decline in new HIV-1 infection in western Africa since 2010 to 2017, whereas 12% increase has been reported in northern part of Africa [6], 29% increase is reported in Eastern Europe and Central Asia[1]. Thus, it suggested the global pandemic of HIV-1 is not over yet. Higher

[1] Info: www.unaids.org/en/resources/fact-sheet

rate of mortality as well as increasing number of new infections in some regions is a big problem. The figure of 28.9 million in the year 2000 to 36.9 million people in 2017 with HIV-1 suggests severe state of global HIV-1 pandemic.

HIV BIOLOGY

There are multiple subtypes of HIV, HIV-1 and HIV-2, they differ very little in term of their sequence and protein structure. To develop drugs against HIV-1 it is important to understand the HIV-1 biology in details.

HIV SUBTYPES

Basis on phylogenetic analysis HIV-1 is subdivided into the groups M, N, O and P. Various chimpanzee viruses can be allocated in between groups N and O of humans and SIV of gorillas in group P. It is unresolved whether the group P chimpanzee virus was transmitted to human directly from chimpanzee or from gorilla[4],[3].

HIV-1 M viruses are subdivided into subtypes A to D, F to H, J and K. In evolutionary terms, groups A and D seem to be the oldest viruses. Subtypes B and D are very closely related, therefore, considered to be sub-subtypes.[3] Recombinant HIV-1 are derived from various subtypes and are named CRF (circulating recombinant form). Approximately 20% of group M viruses belong to these subtypes. For example, the previously described HIV-1 subtype E is only unique regarding the gene for the envelope protein (env), whereas all other parts of the genome are derived from HIV-1 M.Till now>70 different epidemiologically stable CRF have been described and the development of additional CRF is also expected[3] in future.

Recombination, i.e. the exchange of entire gene sequences at unselected positions, is observed when a target cell is infected with two different HIV-1 subtypes (REF). Statistically, ~1 in 400 newly produced virus is a recombinant virus which can have a selection advantage within the host. Recombinants between different HIV-1 groups have also been observed[3]. However, no recombination between HIV-1 and HIV-2 has been reported so far [3].

HIV-1 LIFE CYCLE AND MECHANISM OF INFECTION

To combat the global HIV-1 pandemic, it was necessary to understand the HIV-1 biology. Currently the molecular biology of the HIV-1 infection and its replication in the host cell is well known. HIV-1 is currently classified as a GROUP VI (+ strand ssRNA-RT) virus belonging to family Retroviridae, subfamily Orthoretrovirinae and Genus Lentivirus. HIV-1 carries two + strand RNA as its genome[16], protected by a capsid and a glycoprotein envelope on the outer side. In its genome it carries genes and code for 19 proteins, the genes are Gag, Pol, Env, Tat, Rev, Nef, Vif, Vpr, Vpu, Tev (10th gene in some case present)[16]. P24 is the main coat protein whereas p17 is the capsid protein. Gag, Pol and Env genes are mandatory for new virus production[17], Gag encodes for gp120 the envelope protein and gp41 the transmembrane protein both essential for the viral [16].Tat encodes p16 and p14 which are transcriptase transactivator[16], Tar element can process into miRNA and can regulate ERCC1, IER3; important apoptotic genes. Rev binds with preRNA and very important for the HIV-1 genome to get entry into the host nucleus. Vif prevents APOBEC3G[6], a cellular protein that identifies poly C and poly U in viral tract and deactivate RT activity[6]. Vpr induces cell cycle arrest at G2/M checkpoint. Nef down regulate CD4 as well as both class I and class II MHCs. Vpu helps in viral packaging. The 9719bp HIV-1 genome

possess 5’ and 3’ long terminal repeats (LTR), during packaging PSI element is necessary adjacent to the 3’ end of the LTR. SLIP element is required for Gag-Pol frameshift during the transcription for the generation of different length proteins[18].

HIV-1 infect different cells CD4+ T lymphocytes, macrophages and microglial cells. The CD4-CCR5[19]coreceptor mediated viral entry is the most common type of viral entry, though different other pathways like mannose specific C type lectin mediated receptor DC-SIGV associated viral infection pathways are also well studied[20]. The dual tropic strains of HIV-1 use both CCR5 and CXCR5 as co receptor for entry. The viral envelope protein gp120 first interact with the chemokine receptor CCR5 and/or CXCR5 on the CD4+ T cell[6], after the initial establishment, the gp120 undergoes a structural change and it allows prolonged and stable trimeric binding by gp41, HR1 and HR2[6]. After the initial establishment the gp41 collapses that brings the virus and cell membrane closer together, and the fusion of envelope and cell membrane take place, allowing the initial entry. The gp120 binds with the integrin alpha4beta7 activating the LFA-I, the central integrin involved in the establishment of virological synapses and cell to cell viral transmission takes place[20].

After the entry into the host cell the viral capsid unpacks, the + sense strand RNA is then reverse transcribed into a cDNA by the reverse transcriptase (RT) that the virus carries with it. The RT function as RNA dependent DNA polymerase as well as DNA dependent DNA polymerase[21]. The antisense cDNA is then converted into a dsDNA by the RT itself, and then it enters the nucleus of the cell through REV independent method. After the entry of the HIV-1 DNA that DNA get incorporated into the host genome. The integration is dependent on the INT or Integrase Protein, after integration the HIV-1 DNA get transcribed by the cellular polymerase, though presence of some cellular polymerases seems to be necessary for the transcription of viral RNA. Especially the presence of NF-kB (Nuclear factor kappa B) is supposed to be important for the transcription[16].

The reverse transcription process is well understood, a cellular tRNA acts as the primer; Lysyl tRNA[2], it binds with the PBS of HIV-1 RNA, the RT then elongates the U5 (Untranslated) and R region[2], RT has both the polymerase as well as RNase H domain, the RNase H degrades the RNA of the U5 and R region as the polymerization progresses. Then the ‘strand jump’ takes place17, the primer jumps to the 3’ end of the viral genome and the newly synthesized DNA strands hybridizes to the complementary R region of the viral RNA. The cDNA continues to get extended and the majority of the viral RNA get degraded by the RNase H leaving only the PP sequence [22]. Synthesis of the second strand of the DNA begins using the remaining viral RNA portion as the primer. Then the second ‘strand jump’ takes place. Where, the PBS from the second strand hybridizes with the PBS of the first strand. Both strands are then extended to form the complete dsDNA.

The sequence of reverse transcriptase (RT) is situated in the POL of the RNA genome. Three proteins are present under Pol polyprotein, they are PR (Protease), RT (Reverse Transcriptase) and IN (Integrase). PR is located between the RT (C terminus overlapping) and p6. Its role is to cleave Gag-Pol precursor to develop MA, CA, NC, P6 like proteins. The RT is asymmetric heterodimeric protein with two subunits p66 and p51, having 560 and 440 amino acid residues respectively. The larger subunit p66 have both the active site for polymerization and RNase H domain. P66 have a finger, thumb, palm and a connection domain, however the p51 lacks the RNase H domain[6].

After the integration the HIV-1 DNA is transcribed into RNAs, they are then spliced and carried to the cytoplasm and translated into different viral proteins that include the regulatory proteins TAT and the Rev protein[22]. Translation of the Rev activates the Rev dependent life cycle of the virus, where the Rev is shuttled into the nucleus and binds with the unspliced complete RNAs, the RNA with the Rev is then transported back to the cytoplasm for packaging[22].

Finally, the assembly occurs in the host cell's plasma membrane. The Env encoded polyprotein p160 goes through the endoplasmic reticulum to the Golgi apparatus where it is cleaved by fur in into p160 and p41 proteins (REF) which get transported to the plasma membrane of the infected cell and Gag and Gag-Pol polyprotein also assemble to the plasma membrane of the infected cell. There the virion starts to get packed, still the virion remains immature, because the Gag still remain uncleved, during the maturation of the virion, just before it buds out of the infected cell the gag-pol get cleaved by the viral PR into functional viral enzymes[2].

Two copies of the HIV-1 RNA get packed into the virus, which have a tendency for 2-20 recombination per genome (REF).

CURRENT THERAPY

Early drug trial begun much before the HIV-1 biology was understood, one of such initial drugs was Suramin, which was developed in '60s for the treatment of African sleeping sickness and some viral diseases. In 1986 another drug was AZT or Azidothymidine that was used to treat HIV[23], it was initially developed as an anti-cancer drug. AZT was the first anti-HIV-1 drug approved by FDA in 1987 to be clinically used in America. In 1995 FDA approved first Protease inhibitor. Some iPR came in market before 2001 includes: *amprenavir (Agenerase)*, *atazanavir (Reyataz)*, *darunavir (Prezista)*, *fosamprenavir (Telzir, Lexiva)*, *indinavir (Crixivan)*, *lopinavir/ritonavir (Kaletra, Aluvia)*, *nelfinavir (Viracept)*, *ritonavir (Norvir)*, *saquinavir (Invirase)*, *tipranavir (Aptivus)*[5].In 1996, HAART was introduced. After the success of HAART, PREP or Pre-Exposer Prophylaxis came in clinical trial[24]. Where people with higher rate of disease exposure were treated much before the actual disease is diagnosed, it was proved if on a daily basis RT inhibitors are administered to the person with higher risk of getting infected the HIV-1 infection reduces up to 90%[24].

FUTURE APPROACH

Present approach to fight HIV-1 is to develop a successful vaccine or modern molecular biology techniques such as CRISPR that can make a sterilizing cure of AIDS possible[25]. Development for HIV-1 vaccine started a long back, some vaccines developed and are currently under clinical trials. Also, the genome editing techniques like CRISPER maybe helpful for the treatment of HIV. The CRISPR and shRNA mediated HIV-1 knockdown studies had shown promising performance in HIV-1 treatment in in vivo researches[25], though for legal issues the genome editing tool cannot be used in human sooner or later as far it seems now.
Though vaccination seems more promising, some vaccines like VAX003 and VAX004 is currently under trial on non-human primates and had shown up to 36% of positive result[26]. RV144 had shown ~32% efficiency in drug trial from 2003-2009[27]. Attenuated and killed viral vaccination

are also under trial. Recombinant protein subunit vaccine rGP120 had also shown promising results but it also can't be used on human. HTVN505[28]; a DNA vaccine had shown greater efficiency[29], but human drug trial was not allowed30. Adeno associated virus with gene encoding anti-HIV-1 Bn-Ab also showed efficiency under lab trial[29]. VLP or modified viral protein as vaccination had also showed efficiency in in-vitro studies[26]. Other vaccines like RV305, 306, 282 was under trial from 2012-2015, HTVN702 is in trial from 2016-2020[27]. HPX2008/HTVN 705 all are currently in drug trial and showing promising results. From 2017 November Romidepsin is under German drug trial on 18 patients, and so far, it showing very promising results[30].

DRAWBACK OF CURRENT TREATMENTS

The drawback includes absence of any cure[31]. The error prone replication cycle of the virus makes it very complicated to fight with the disease. The mutant variety after a certain time doesn't repose to ART s. Plus, it has some nasty side effects, common side effects include nausea, vomiting, heart burn, liver damage, kidney problem and much more[23]. With that non-specific interaction with eukaryotic enzymes by drug makes the situation much worse.

PLANT MOLECULES AS POTENTIAL NEW DRUGS

Human are using plants for their potential medicinal properties since the beginning of the civilization. Some of the plants have active anti-viral properties too. So plants can be utilized for new drug research against HIV[8]. Plants produce huge variety of bioactive natural compounds some of them may interact with HIV-1 enzyme and in turn block them without any cytotoxic effect[32]. Bioactive molecules like those can be purified from plants and can be use as Anti-HIV-1 drug[7]. They can potentially supplement existing HAART drugs. In India thousands of medicinal plants are being used to treat different disease. Even these plants may potentially have anti-HIV-1 potential. The tropical countries are blessed with thousands of medicinal plants, and some of which have been screened for anti-HIV-1 role and some showed significant efficiency against the reverse transcriptase or protease. Some plants reported for anti-HIV-1 properties are *Azadirachta indica*(semi purified; stem extract), *Acacia catechu*(semi purified; stem extract), *Terminlia chebula*(semi purified; bark extract), *Allium sativum*(semi purified; leaf extract), *Qercus infectoria*(semi purified; stem& leaf extract), *Acacia nilotica*(purified; stem and leaf extract), *Rubia cordifolia*(purified; stem extract), *Tylophora indica*(crude; stem extract), *Pisidium guajava*(crude; stem and leaf extract), *Swertia bimaculate*(semi purified; methanolic extract), *Moringa oleifera*(semi purified; stem extract), *Ceratothecases amoides*(semi purified; stem extract), *Amaranthus viridis*(crude; leaf extract), *Senna occidentalis*(crude; leaf extract), *Cassia singuena*[33], *Solanum americanum*(crude; stem and leaf extract), *Momordica balsaminia*(crude), *Jatropha curcas*(crude non polar extracts), *Vernonia amygdalina, Leptademia hastate*[34](semi purified; stem extract), *Cassia tora*(crude; stem and leaf extract)[35]and many more[36],[32]. There are also some initial reports of HIV-1 RT inhibition by extracts *Catharanthus roseus*[37] and *Ocimum gratissimum*[35]. Natural products such as plant-originated compounds and plant extracts have enormous potential to become drug leads with anti-HIV-1 and neuroprotective activity. Accordingly, many research groups are exploring the biodiversity of the plant kingdom to find new and better anti-HIV-1 drugs with novel mechanisms of action and for HIV-associated neurocognitive disorders (HAND)[38]. Bioactive molecules in some cases semi-purified extracted with different solvents from different plants are screened and some shown much more effective result than the conventional HAART drugs. Though in some cases it seems difficult to pin point

one single molecule that bear Anti-HIV-1 property[39]. It is more likely many different bioactive compounds act together to hinder HIV-1 enzyme activity[40]. But it is safe to assume most bioactive compounds from plants are safer than the synthetic compounds. Thus, bioactive phytochemicals or natural compounds could produce Anti-HIV-1 drugs with less cytotoxic and harmful effects.

Though bioactive phytochemicals seem to have enough potential to produce new alternative Anti-HIV-1 drugs it also has its own flaws. The bioactive molecules are hard to purify and it is not easy to produce in bulk. Some of the molecules evaluated never came up to drug trials. Extracting plant phytochemicals is not much efficient, some bioactive component that have Anti-HIV-1 properties are only produced in some specific variety of a plant that are not globally found. In some cases, the reported bioactive natural compounds having Anti-HIV-1 properties are only produced in a particular season or in a particular weather condition thus limiting their potential in making commercial drugs. Still plant source is a promising way for new drug researches.

CONCLUSION

Medicinal plants maintain the health and vitality of individuals and also cure various diseases. Medicinal plants have provided numerous leads to fight diseases and can serve as an excellent source of novel drug candidates with Anti-HIV-1 effect. Therefore, extensive investigation is needed to exploit for discovery and development of anti- HIV-1 drugs and an extensive investigation of its bioactivity, pharmaco- therapeutic, toxicity, standardization, clinical trial and mechanism of action should be encouraged and sustained. A number of plants are unique source of various types of chemical compounds, which control various activities including suppressing HIV-1 replication and development.

RECOMMENDATIONS

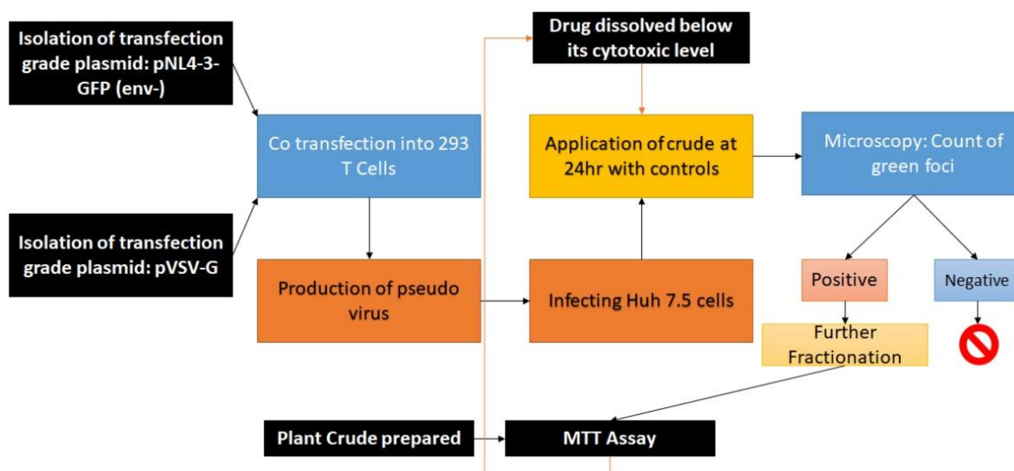
There is need to increase the evaluation of natural products based on bioactivity guided assay; this will quicken the search for novel anti-HIV-1 compounds. There is also an urgent need to fast-track HIV/AIDS clinical trials of candidate drugs developed from novel compounds isolated from plants. Post-genomics, phylogenetic analysis and other bioinformatics tools may shed light on other related plants that may contain similar active compounds.

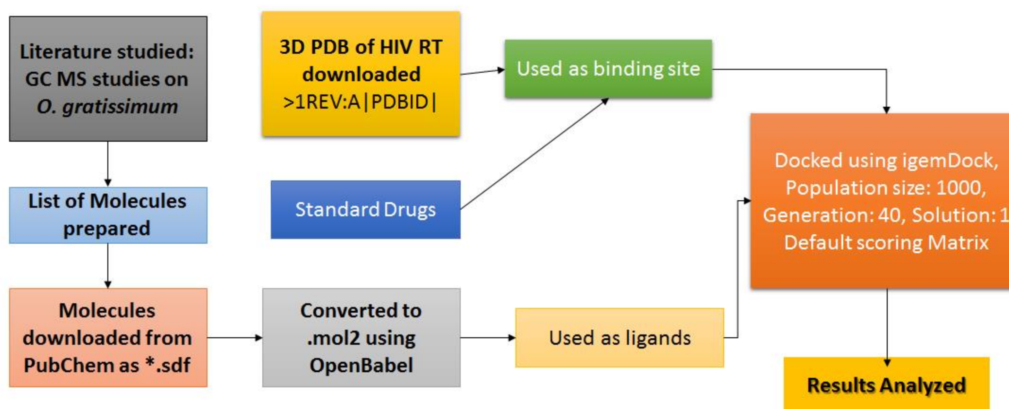
<u>OBJECTIVES</u>

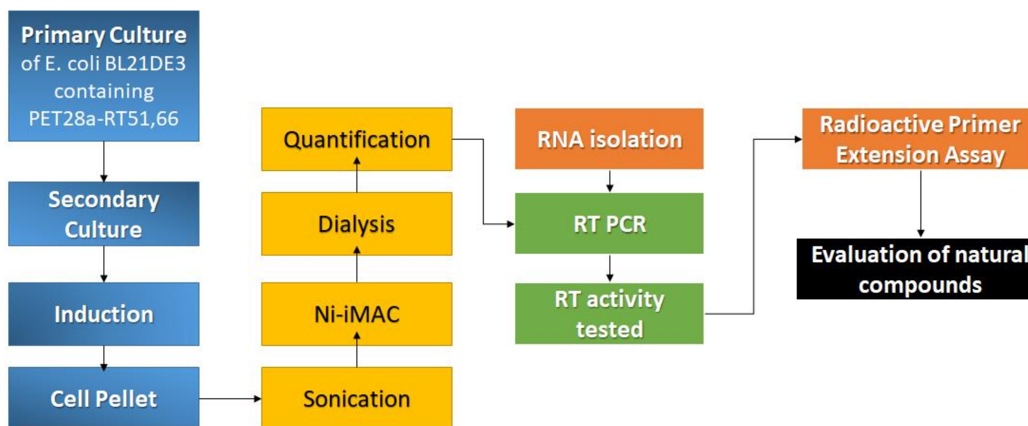
Objective 1: Bioactivity guided fractionations of selected medicinal plants.

Objective 2: Evaluation of anti-HIV-1 activity of plant extracts in cell culture.

PLAN OF WORK







Plans of works for this project are represented graphically here.

Material and methods

Preparation and purification of plant extracts:-

In present study 6 plants viz. *Catharanthus roseus, Ocimum gratissimum, Mangifera indica, Tinospora sp., Solanum sp., Swertia bimaculata* were included. Leaves or the stems of these plants were collected washed, air dried and powdered in grinder mixer. Powder was subsequently extracted with different solvents of low to high polarity viz. hexane, chloroform, methanol and Petroleum benzene, Ethyl acetate, methanol in cold extraction method. Each plant was extracted in at least three solvents. All the extracts were subjected to rotary vacuum evaporation and the dried extract was collected and weighed.

Table: Plants Used in Present Study

Name & Family	Parts used	Amount Taken	Extracted in	Selection Criteria
Catharanthus roseus	Stems and Leaves	1KG	Petroleum benzene, Ethyl acetate, Methanol	Known Medicinal plant
Ocimum gratissimum	Stems and Leaves	500gm	Petroleum benzene, Ethyl acetate, Methanol	Known medicinal plant with anti-viral activity
Mangifera indica	Stem	500gm	Hexane, Chloroform, Methanol	Common native plant with no medicinal property
Tinospora sp.	Stem	500gm	Hexane, Chloroform, Methanol	Medicinal plant with known anti-bacterial and anti-viral property
Solanum sp.	Stem	-	Hexane, Chloroform, Methanol	Some spp. Do have anti-viral and other medicinal activity.
Swertia bimaculata	Stem and Leaves	80gm	Hexane, Chloroform, Methanol	Known medicinal plant

- Plants material were first extracted in hexane (500ml), followed by chloroform (500ml) and methanol (500ml) or petroleum benzene (500ml), Ethyl acetate (500ml), methanol (500ml) and plant extract was collected.
- After extraction, extracts were concentrated and solvent was recovered by means of a rotary evaporator apparatus.
- Concentrated plant extracts were then placed in 2ml tubes and further dried under vacuum in a centrifugal evaporator.
- After complete removal of solvent, powdered plant extracts were dissolved in 50% DMSO to make 20 mg/ml stock.

- Now extracts were filtered using 0.22 µM membrane filter and stored at 4°Ctill further use.

Column Chromatography for further separation-

Fractions that showed positive Anti-HIV-1 activity in cell culture was again fractionized. Methanolic Fraction of *Ocimum gratissimum* showed positive result, thus this fraction was further separated using column chromatography. Slurry of the dried methanolic fraction was prepared using silica and dried. Column chromatography was used for this process. Column was first packed with silica beads in methanol. Then slurry was added on top of the bed. Different grades of polar to non-polar solvents then used for the chromatographic separation. Bands produced in different solvents were carefully collected and dried and evaluated in cell culture. Following fractions were recovered.

SOLVENT	NUMBER OF FRACTION
E (Ethyl acetate 100%)	3
E7M3 (Ethyl acetate: Methanol 70:30)	4
E5M5 (Ethyl acetate: Methanol 50:50)	0
E3M7 (Ethyl acetate: Methanol 30:70)	1
M (Methanol 100%)	4

- After extraction, extracts were concentrated and solvent was recovered by means of a rotary evaporator apparatus.
- Concentrated plant extracts were then placed in 2ml tubes and further dried under vacuum in a centrifugal evaporator.
- After complete removal of solvent, powdered plant extracts were dissolved in 50% DMSO to make 20 mg/ml stock.
- Now extracts were filtered using 0.22 µM membrane filter and stored at 4°Ctill further use.

Thin Layer Chromatography for further separation-

Thin Layer chromatography is a method for analyzing mixtures by separating the compound in the mixture. In this method, the number of the component can be determined. In TLC three steps were done, spotting, development and visualization. At first, a small amount of sample solution was spotted at the end of a TLC plate. Spotting solvent was quickly evaporated and left behind a small spot of the material. The TLC plate placed in a chamber containing solvent (methanol and water). Due to capillary action, the solvent and spotted material travel up. The outcome depends upon a balance among three polarities, that of the plate, the development solvent and the spot material. The spots can be directly observed after development. Rf value was calculated to quantify the movement of the materials along the plate. Rf is equal to the distance traveled by the substance divided by the distance traveled by the solvent.

Cell culture:

Experiments were performed on human hepatoma cell line Huh 7.5. Cells were grown in Dulbecco's modified Eagle's medium (DMEM) supplemented with 10% heat-inactivated fetal bovine serum (FBS), penicillin (100 U/ml), streptomycin (100 μg/ml), 0.1 mM non-essential amino acids and glutamax. Cells were incubated at 37°Cin a humidified 5% CO_2 incubator.

Cell cytotoxicity assay:

The cytotoxicity of each plant extract was evaluated by MTT assay on Huh 7.5 cells and 50% cytotoxic concentration (CC_{50}) i.e. extract concentration that reduced the cell viability by 50% when compared to untreated controls was calculated.

Huh 7.5 cells were seeded in a 96 well tissue culture plate (20,000 cells/well), after overnight incubation cells were treated with different concentrations of drugs in triplicate and incubated for 72 hours (six concentrations viz. 400 μg/ml, 200 μg/ml, 100 μg/ml, 50 μg/ml, 25 μg/ml and 12.5 μg/ml were taken). DMSO was used as vehicle control and its final concentration didn't exceed 1%. Control cells were grown in the same media without any plant extracts. After incubation, 20 μM 5 mg/ml MTT reagent was added to each well and incubated at 37°Cuntil dark purple formazan crystals visible in microscope were found. Media was carefully removed and 100 μl of solubilizing solution (4 mM HCl and 0.1% NP40 in isopropanol) was added in each well to dissolve formazan crystals. Plate was incubated for 15 min at room temperature and absorbance was measured in a microplate reader at 595 nm with reference wavelength of 695 nm. Absorbance values were normalized to that of untreated cells to get the percentage of surviving cells. CC_{50}values of the plant extracts were calculated using Excel with a dose response curve.

Anti-HIV-1 assay in Cell Culture System:

After evaluating plant extracts for their cytotoxic effects Anti-HIV-1 activity of extracts was evaluated by a high throughput Anti-HIV-1 assay in cell culture using different concentrations of extracts in a 96 well format. HIV-1 pseudo virus with EGFP reporter gene was prepared by co-transfecting pNL4-3-GFP (env-) and pVSV-G vectors in HEK293T cells using calcium phosphate transfection method. After 48h the pseudo virus was collected from the supernatant, purified by centrifugation and used for infecting Huh 7.5 cells.

Exponentially growing HEK293 T cells were plated on 100mm tissue culture dish, the previous day of transfection. Next day 4 hour prior to the transfection, DMEM media was replaced with fresh media. For the preparation of transfection reaction mix, Solution A (2.5M $CaCl_2$, 15μg pNL4-3 ΔEnv EGFP and 5μg pVSV-G, water) was drop wise added to Solution B (2X HEPES solution). The mixture was allowed to incubate for 15-20 minutes at room temperature. After incubation, reaction mixture was added drop wise to the cell culture dish with gentle swirling. Cells were incubatedovernight and next day fresh media was supplemented after washing the cells with PBS. The supernatant was collected after 48 hours containing the viral particles. The virus was collected and filtered through 0.45μm filter and was used to infect Huh7.5 cells.

In a 96 well tissue culture plate Huh 7.5 cells (15000 cells/well) were infected with HIV-1 pseudo virus containing GFP (Green Fluorescent Protein) reporter gene and incubated overnight. After 4h incubation cells were washed and incubated in fresh media containing different concentrations of plant extracts below their respective CC_{50} concentrations and incubated for 48 and 72 hours.

Post incubation, cells were visualized under fluorescent microscope. HIV-1 infected cells appeared green under fluorescent microscope due to expression of green fluorescent protein (GFP). Anti-HIV-1 activity of plant extracts were recorded as reduction of expression of GFP reporter gene as reduction in number of green foci.

As control one set was not treated with any extract or drugs, one set treated with only DMSO as solvent control and one set treated with efavirenz; an established drug as positive control.

Molecular Docking

Molecular docking is a well-established computational technique which can be used to predict the interaction between ligand and protein (receptor) and to find the best orientation of ligand which would form a complex with protein. Docking studies were executed for natural compounds (ligands) obtained from the literature of GC-MS analysis of the plants with HIV-1 RT polymerase (p51+p66), Protease and Integrase using igemdock software.

Step 1- Building of receptor- In this step three-dimensional structure of selected HIV-1 RT (PDB ID: 3HVT), HIV-1 PR (PDB ID: 1DMP), HIV-1 INT (PDB ID: 3IPT) was retrieved from protein data bank as 2.6A RMSD resolution in PDB format.

Step 2- Ligand preparation- The list of active compounds, as identified by GC-MS from the selected medicinal was obtained from literature[23]. The 3D structure of those compounds were retrieved from PubChem as SDF format and converted to MOL2 using open babel.

Step 3- docking- The compounds (ligands) were docked onto the HIV-1RT, PR and INT receptor and the interactions were checked the best fit of ligand and protein. The ligands showing best fit was considered potential Anti-HIV1-RT inhibitor subjected to verification by RT-inhibition assay activity in the next phase.

Isolation of HIV-1 RT protein (p66 and p51 subunit)

Cell culturing- HIV-1 RT was expressed in E. coli BL21DE3stain having clones of p66 or p51 sub unit of RT. For primary culture, 3% (v/v) bacterial culture was added in 10 ml LB media with kanamycin and incubated on shaker at 37°C. After overnight incubation 3% of culture was taken

[2] Joshi, Dr. R. K.. (2016). GC-MS Analysis of the Essential Oil of Ocimum gratissimum L. Growing Desolately in South India. Acta Chromatographica. 29. 1-9. 10.1556/1326.2017.29.1.10.

[3] C.R. Unnithan and Undrala Sushen. Chemical composition of ocimum gratissimum l by gc-ms analysis. ejpmr, 2017,4(06), 410-412. ISSN 2394-3211

to inoculate 50 ml LB media containing 50 µl kanamycin and incubated for 3-4h till the OD reached 0.6

Induction of protein with IPTG- Once the OD reached IPTG was added at final concentration of 1mM in the culture media and kept for overnight incubation at 28°C for HIV-1 RT protein expression. Protein induction is checked through SDS-PAGE [APPENDIX] and HIV-1 RT protein is identified with known concentration of BSA protein.

Cell Harvesting- After overnight incubation the bacterial biomass is collected by centrifugation (10000 rpm, 8 min) and mixed with binding buffer and vortexed to mix it well. This was followed by sonication with non-ionic detergent like NP40 and lysozyme at 75Hz for 15sec with 15sec gap on the ice for 10 cycles. The cell lysate was centrifuged at 13000 rpm for 15 minutes at 4oC.

The supernatant (containing all the proteins) was collected and subjected to Ni-iMAC column chromatography to separate the His tagged HIV-1 RT protein.

Protein purification through iMAC- The HIV-1 RT expressed was tagged with 6x Histidine, Sepharose beads were washed with different grades of alcohol (20% and 75%) and double distilled water. After that it was washed with binding buffer containing 100 mM EDTA until the color of the column turns white. To remove the EDTA the beads were washed with several rounds of double distilled water and binding buffer and finally 0.1 $NiSO_4$ solution was added in the column containing the beads. Until the full bed turned blue the recharging process was continued. The free Ni ions were washed with binding buffer. The supernatant was then loaded on the beads in the column and passed through it. The eluent was collected and reloaded on the column several times. Column was then washed with wash buffer several times till no free protein was detected in the flow through (as detected by Bradford reagent). The HIV-1RT proteins were then eluted in elution buffer. Fractions of flow through 1 ml each were collected and elution was continued till no free protein was detected in the flow through.

Confirmation of the presence of HIV-1 RT through SDS-PAGE after elution- The purified proteins were analyzed through SDS-PAGE and detected through negative staining (zinc imidazole) method and coomassie staining. SDS-PAGE separates proteins primarily by mass because the ionic detergent SDS denatures and binds to protein to make them uniformly negative charged. Thus, HIV-1RT protein were detected by using BSA as a marker. The eluted fractions showing good yield of RT and low contaminating proteins were pooled together and subjected to dialysis.

Dialysis of protein- The pooled proteins fractions were poured into the dialysis bag and kept in dialysis buffer 1 (APPENDIX) for overnight at 4°C on a magnetic stirrer. After overnight dialysis the buffer was changed to Buffer 2 (APPENDIX) and further dialyzed for 4 hours. The dialyzed protein was aliquot, checked by SDS-PAGE, quantified by Bradford reagent and stored at -20°C till further use.

In vitro assay of anti- HIV-1 activity of selected plants

RNA insolation and quantification- Huh 7.5 cells infected with pseudo-virus were taken by using trypsin and centrifuged with addition of TRIZOL and Chloroform to lysis the pellets. Supernatant is taken and kept it for overnight. Precipitate is centrifuged by adding with 70% Ethanol at 13000

rpm for 15 minute. Then 20 µl DEPC treated water is added in dry tube. Finally RNA quantification is done using nanodropspectrophotometer.

Preparation of cDNA-cDNA is prepared using isolated RNA as a template (2.5 µl). After adding 2 µl reverse primer and 1 µl dNTPS into template, it is heated for 65 degree Celsius for 5 minute followed by cooling at -20 degree Celsius for 10 minutes for annealing. Then HIV-1 RT enzyme buffer and DEPC water are added into the previous mixture followed by incubation for 1 hour at 42 degree Celsius.

RT PCR- RT PCR is performed to check the activity of the cDNA. 2 µl template cDNA 0.6 µl Forward Primer and Reverse primer 1.5 µl 10X Taq Buffer 0.6 µl dNTPs, 8.6 µl DEPC treated water 0.5 µl Taq pol 0.6 µl MgCl2 are used to set the PCR reaction.

Pico green Assay-HIV-1 RT is used in this reaction with prepared template RNA. In control condition without the presence of any inhibitor the RT produces cDNA and results in high emission peaks. In presence of inhibitor the activity of RT hinders resulting in quenching of the fluorescence.

The template DNA in different amounts (2800, 280, 28, 2.8, 0.28, 0.028 pico mole) with primer was taken in a tube and incubated at room temperature for one hour for annealing. Then the template/primer solution is diluted using polymerization buffer. For each samples to be assayed, 20 µl of this reaction mixture is aliquot in dry tubes. 5 µl of dilute enzyme (HIV-1 RT) is added to the tubes containing reaction mixture. The reaction mixture is then incubated at 25 degree Celsius for 10-60 minutes. The reaction is stopped with 2 µl EDTA. Multiple reaction stopped at 2.5, 5, 7.5, 10 minutes. The polymerizing activity of HIV-1 RT is measured using a fluorometric assay by adding chilled 2000 fold dilution of Pico green in TE buffer into the reaction mixture and incubating it on ice in the dark for 10 minutes. The readings are supposed to be taken in a fluorimeter.

RESULTS

Under objective 1, 6 plants were selected. *Catharanthus roseus, Ocimum gratissimum*, and *Tinospora sp*. were selected because they are well known medicinal plants. *Mangifera indica* and *Solanum sp*. were selected they are very common plants of this region and *Swertia bimaculata* was selected because anti-HIV-1 activity from it was previously reported. *Catharanthus roseus* was collected from Konnagar, *Ocimum gratissimum, Mangifera indica* was collected from the experimental garden of Ballygaunge Science College campus. Other plants were available as dried leaves or stem in the lab. The collected plants were first washed well with distilled water and dried for 1 to 2 week. Then the dried plant materials were ground using a mixture grinder. The dried powder was used for the extraction procedure. For the extraction process, a cold extraction procedure was used to reduce any alteration of the phytochemicals in heat. For the extraction process, the dried ground plant materials were packed into a large column and then different solvents were used for the extraction. Non-polar to Polar solvents were used for the extraction procedure. Two solvent combinations were used in this project, either Petroleum benzene, Ethyl acetate, Methanol or Hexane, Chloroform, Methanol. The solvents were run multiple times through the column for proper extraction. The extracts were then collected and the dried using a rotary evaporator where the temperature was kept below 42®C. The concentrated extracts were then left for drying in Petri dishes for 1 week. The dried samples were then prepared for further experiments. The crude extracts were collected in Eppendorf tube, weighed and dissolved in 50% DMSO solution to make the concentration 20mg/ml. Under objective 2, the dissolved extract was then subjected to filter sterilization and then used for cytotoxicity assay and evaluation in cell culture assay with GFP reporter system.

For cytotoxicity assay, MTT assay was used and six concentrations of the serially diluted extract were used for MTT assay. The procedure of MTT assay is stated in the material and method section of this project. The reading of the MTT assay was taken in a plate reader from the 96 well plates and the results were plotted in an excel graph and the CC_{50} value was calculated from the graph.

The crude was then used for GFP based cytotoxicity assay where the green foci indicate HIV-1 infection and the reduction in the number of green foci indicates the activity of a drug as an anti-HIV-1 agent. The procedure of the cell culture assay is mentioned in details in the materials and methods section of this project and the results of different plants in the cell culture assay are mentioned in this section of the project.

The crude extracts that showed positive results were subjected to further separation through column chromatographic method. Dried crude extracts were used to prepare the slurry. Silica bed was packed into a column and slurry was applied on top of the bed. Then different grade of polar to non-polar solvents were used for the fractionations of the crude extract. The fractions were again dried, dissolved and filter sterilized and evaluated in the cell culture assay as it was done for the crude extracts.

The fraction that showed anti-HIV-1 activity was then again subjected to TLC for further separation through the separated fractions with TLC was not further evaluated in the cell culture system and it was not analyzed for its individual component.

Then molecular docking was done with the GC-MS compounds of the plant that showed the positive anti-HIV-1 effect to check for any potential anti-HIV-1 compounds.

Then for in-vitro assay, HIV-1 RT was chosen as the target because an RT expression system and fluorescence-based assay were available. The RT clone was harvested, induced sonicated for RT production and isolation. The RT was produced, purified and quantified through the assay was not done because of some unexpected incidents.

Anti-HIV-1 properties of *Catharanthus roseus*

First the plants were collected from mentioned location. Dried and then then grinded. Then cold extraction method was performed for preparation of the extracts. Dried plant materials were loaded in a column then different solvents were added. The extract was carefully collected. For proper extraction this step was repeated several times. The collected extracts were then subjected to rotary evaporation for drying. After the extracts were dried they were dissolved in 50% DMSO and 50% double distilled water. The extracts were dissolved in a concentration of 20mg/ml. The dissolved crude drug was then subjected to filter sterilization and the sterilized crude extracts were used for cytotoxicity assay and evaluation in cell culture system.

The crude drugs were serially diluted to make 12.5 µg/ml, 25µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml. Cell cytotoxicity was evaluated using MTT assay as mentioned in the materials and methods.

Three extracts viz. petroleum benzene, ethyl acetate, methanol were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50} values were calculated. CC_{50} values for Ethyl acetate, Chloroform and Methanol were 426.81 µg/ml, 449.64 µg/ml and 451.95 µg/ml respectively.

Cell cytotoxicity assay- *Catharanthus roseus* ethyl acetate extract

Cell cytotoxicity assays were performed for all three crude extracts using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. All the next steps of the experiments are done using drug concentrations below their cytotoxicity level. Result of cell cytotoxicity assay of this crude extract is represented in Figure 1 in next page.

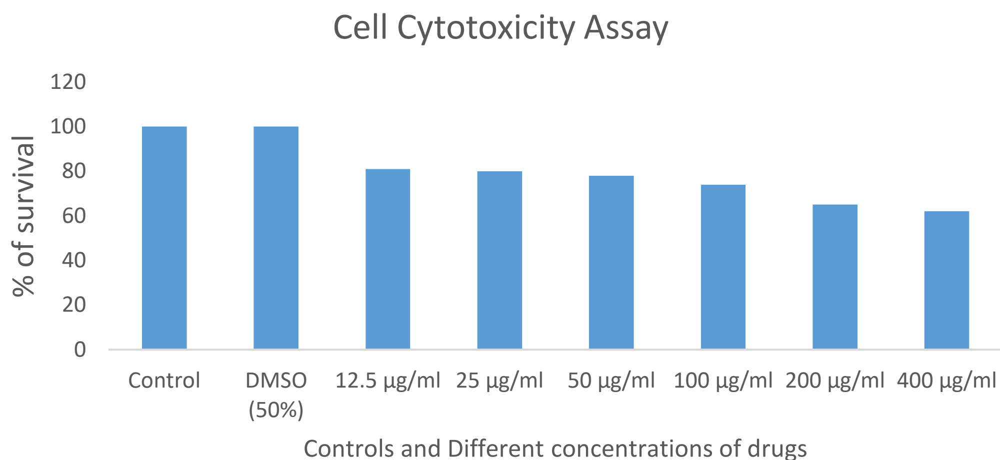


Figure 1: Effect of *Catharanthus roseus* ethyl acetate extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Catharanthus roseus* ethyl acetate extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 426.81 µg/ml.

After cytotoxicity of the crude extract was evaluated then the crude extracts were evaluated using cell culture-based assay where GFP expression indicates the HIV-1 infections in the cell and the reduction in GFP expression indicates the inhibition of HIV-1 by the crude drug.

Anti-HIV-1 activity of *Catharanthus roseus* ethyl acetate extract

Crude plant extracts were evaluated using cell culture based assay. First Huh7.5 cell plating were done. Then the cells were subjected to viral infections. Then crude drugs were applied to the cells and GFP fluorescence were observed after 60 hours. The image captured from fluorescence microscope is given below. Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Images captured through fluorescence microscope of this fraction is represented in figure 2.

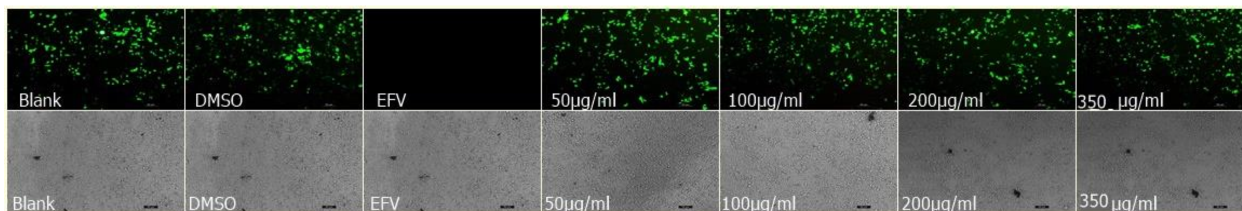


Figure 2- Anti-HIV-1 activity of *Catharanthus roseus* ethyl acetate extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Catharanthus roseus* ethyl acetate extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Ethyl acetate extract, indicating *Catharanthus roseus* ethyl acetate extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- Catharanthus roseus methanolic extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Figure 3 represents results of cytotoxicity assay of this fraction.

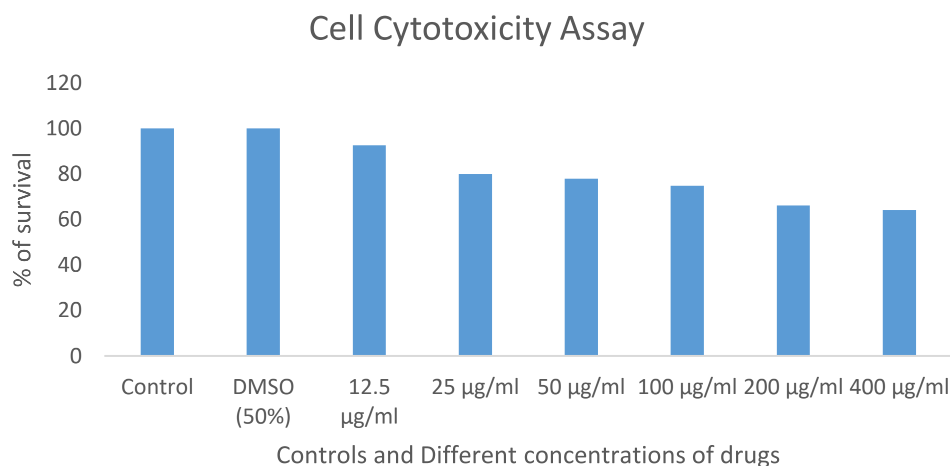


Figure 3: Effect of *Catharanthus roseus* methanolic extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Catharanthus roseus methanolic extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours And found to be 449.64 µg/ml.

Anti-HIV-1 activity of *Catharanthus roseus* methanolic extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Cell culture assay was performed as mentioned earlier and Figure 4 represents the observation through fluorescence microscope after treating the cells with crude methanolic extracts.

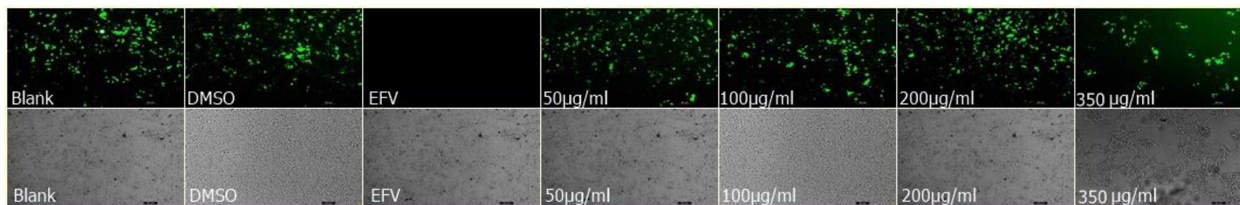


Figure 4- Anti-HIV-1 activity of *Catharanthus roseus* methanolic extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Catharanthus roseus* methanolic extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of methanolic extract, indicating *Catharanthus roseus* methanolic extract have no anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- Catharanthus roseus petroleum benzene extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. In figure 5 results of cell cytotoxicity assay of this fraction is represented.

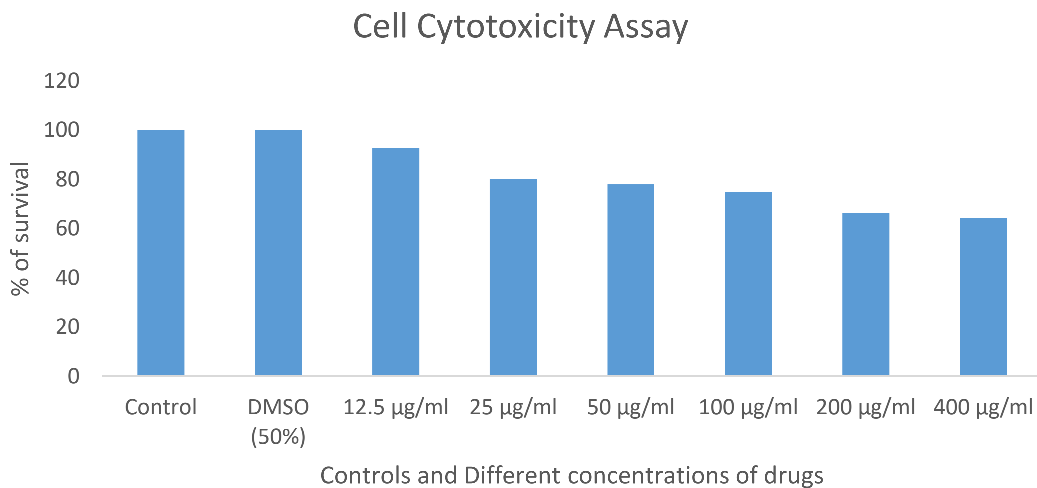


Figure 5: Effect of *Catharanthus roseus* Petroleum benzene extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Catharanthus roseus Petroleum benzene extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours And found to be 451.95 µg/ml.

Anti-HIV-1 activity of *Catharanthus roseus* petroleum benzene extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. In figure 6 the images taken through fluorescence microscope is represented.

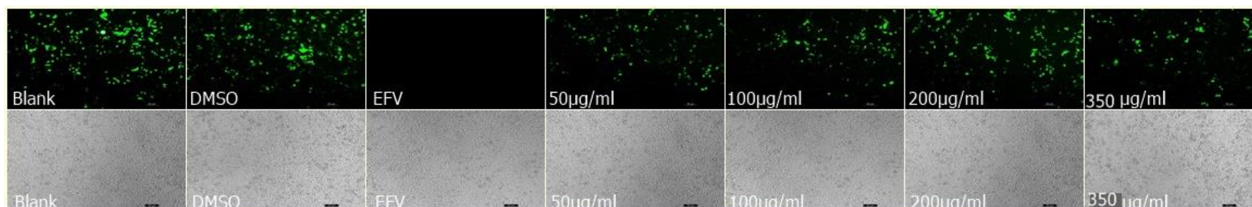


Figure 6- Anti-HIV-1 activity of *Catharanthus roseus* petroleum benzene extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Catharanthus roseus* petroleum benzene extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Petroleum benzene extract, indicating *Catharanthus roseus* petroleum benzene extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All three fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400µg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these particular sample, anti-HIV-1 activity was not detected in any of the extract and at any of its concentrations. None of the crude extracts in none of its concentrations do not showed any significant apparent reduction in number of green foci in the field.

Hence, anti-HIV-1 activity in any of the crude extract at any concentrations in this plant is not recorded in this study.

Anti-HIV-1 properties of *Ocimum gratissimum*

First the plants were collected from mentioned location. Dried and then then grinded. Then cold extraction method was performed for preparation of the extracts. Dried plant materials were loaded in a column then different solvents were added. The extract was carefully collected. For proper extraction this step was repeated several times. The collected extracts were then subjected to rotary evaporation for drying. After the extracts were dried they were dissolved in 50% DMSO and 50% double distilled water. The extracts were dissolved in a concentration of 20mg/ml. The dissolved crude drug was then subjected to filter sterilization and the sterilized crude extracts were used for cytotoxicity assay and evaluation in cell culture system.

The crude drugs were serially diluted to make 12.5 µg/ml, 25µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml. Cell cytotoxicity was evaluated using MTT assay as mentioned in the materials and methods.

Three extracts viz. Ethyl acetate, Chloroform, Methanol were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50} values were calculated. CC_{50} values for Ethyl acetate, Chloroform and Methanol were 411.1 µg/ml, 448.5 µg/ml and 441.24 µg/ml respectively.

Cell cytotoxicity assay- *Ocimum gratissimum* ethyl acetate extract

Cell cytotoxicity assays were performed for all three crude extracts using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. All the next steps of the experiments are done using drug concentrations below their cytotoxicity level. As expected earlier the data from the MTT assay is plotted in a excel graph and the CC_{50} value is measured from the graph. Figure 7 represents the results of cytotoxicity assay of this fraction.

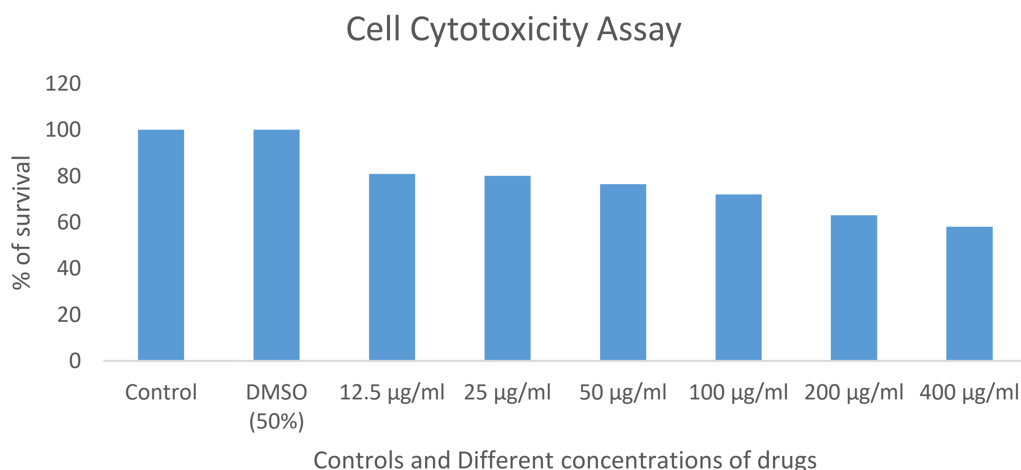


Figure 7: Effect of *Ocimum gratissimum* Ethyl acetate extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Ocimum gratissimum Ethyl acetate extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours And found to be 411.1 µg/ml.

Anti-HIV-1 activity of *Ocimum gratissimum* Ethyl acetate extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Figure 8 represents the images taken through fluorescence microscope.

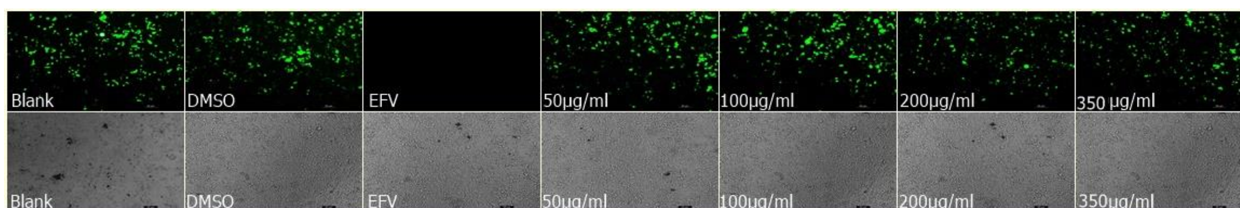


Figure 8- Anti-HIV-1 activity of *Ocimum gratissimum* ethyl acetate extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Ocimum gratissimum* ethyl acetate extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Ethyl acetate extract, indicating *Ocimum gratissimum* ethyl acetate extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Ocimum gratissimum* methanolic extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay of this particular fraction is represented in figure 9 below.

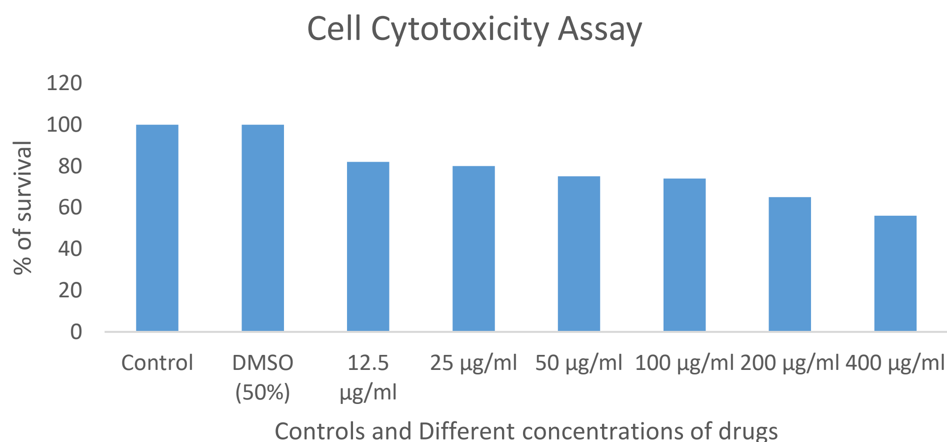


Figure 9: Effect of *Ocimum gratissimum* methanolic extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Ocimum gratissimum methanolic extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50}

values were calculated after 72 hours and found to be 441.24 μg/ml.

Anti-HIV-1 activity of *Ocimum gratissimum* Methanolic extract

Cell culture assay was performed as mentioned earlier. Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Figure 10 represents the images captured through the fluorescence microscope.

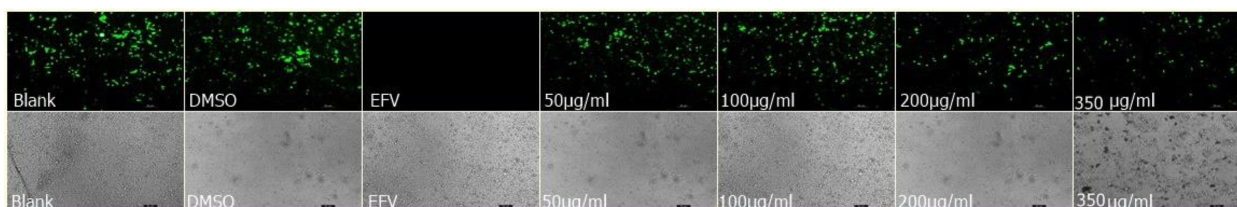


Figure 10- Anti-HIV-1 activity of *Ocimum gratissimum* methanolic extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Ocimum gratissimum* methanolic extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above **there is distinct reduction in GFP positive foci in cells treated with increasing concentration of methanolic extract, indicating *Ocimum gratissimum* methanolic extract have Anti-HIV-1 activity**. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Ocimum gratissimum* petroleum benzene extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay of this fraction is represented in Figure 11.

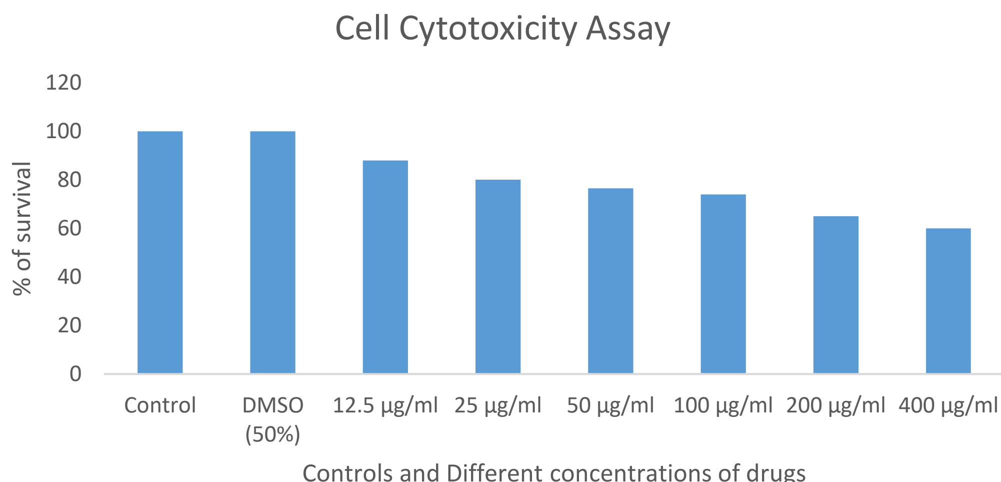


Figure 11: Effect of *Ocimum gratissimum* petroleum benzene extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Ocimum gratissimum* petroleum benzene extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 448.5 μg/ml.

Anti-HIV-1 activity of *Ocimum gratissimum* petroleum benzene extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. In figure 12 images captured through the fluorescence microscope is represented. The images show no prominent reduction in number of green foci in higher concentrations.

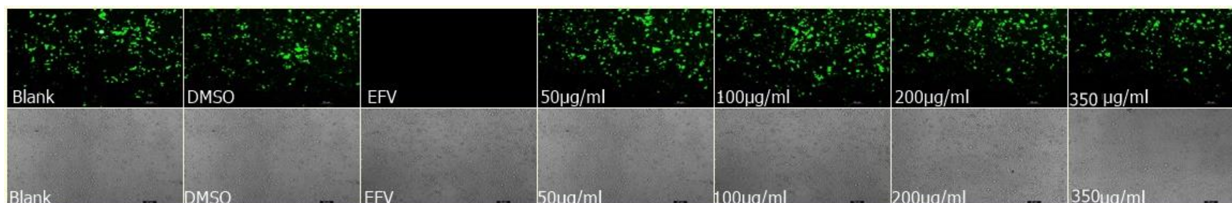


Figure 12- Anti-HIV-1 activity of *Ocimum gratissimum* Petroleum benzene extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Ocimum gratissimum* Petroleum benzene extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Petroleum benzene extract, indicating *Ocimum gratissimum* Petroleum benzene extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All three fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400μg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these particular sample, **anti-HIV-1 activity was detected in methanolic extract at 200μg/ml and 350μg/ml of its concentrations**. The methanolic crude extracts in above mentioned concentrations showed significant decrease in the number of green foci whereas the bright filed images of them shown the cells were intact. It indicates the drug at those concentrations does interfere with the HIV-1 and blocks the expression of GFP.

Hence, anti-HIV-1 activity in methanolic crude extract at **200μg/ml and 350μg/ml** concentrations in this plant is recorded in this study.

So methanolic crude extract of this plant was subjected to further separation by column chromatography and each sub-fractions were again subjected to cytotoxicity assay and cell culture based assays. The

results of the cell culture based anti-HIV-1 assays for those fractions are mentioned later in the result section of this project.

Anti-HIV-1 properties of *Tinospora sp.*

First the plants were collected from mentioned location. Dried and then then grinded. Then cold extraction method was performed for preparation of the extracts. Dried plant materials were loaded in a column then different solvents were added. The extract was carefully collected. For proper extraction this step was repeated several times. The collected extracts were then subjected to rotary evaporation for drying. After the extracts were dried they were dissolved in 50% DMSO and 50% double distilled water. The extracts were dissolved in a concentration of 20mg/ml. The dissolved crude drug was then subjected to filter sterilization and the sterilized crude extracts were used for cytotoxicity assay and evaluation in cell culture system.

The crude drugs were serially diluted to make 12.5 µg/ml, 25µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml. Cell cytotoxicity was evaluated using MTT assay as mentioned in the materials and methods.

Three extracts viz. petroleum benzene, ethyl acetate, methanol were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50}values were calculated. CC_{50} values for Ethyl acetate, Chloroform and Methanol were 411.1 µg/ml, 384.5 µg/ml and 441.24 µg/ml respectively.

Cell cytotoxicity assay- *Tinospora sp.* ethyl acetate extract

Cell cytotoxicity assays were performed for all three crude extracts using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. All the next steps of the experiments are done using drug concentrations below their cytotoxicity level. In figure 13 results of MTT assay of this fraction is represented graphically.

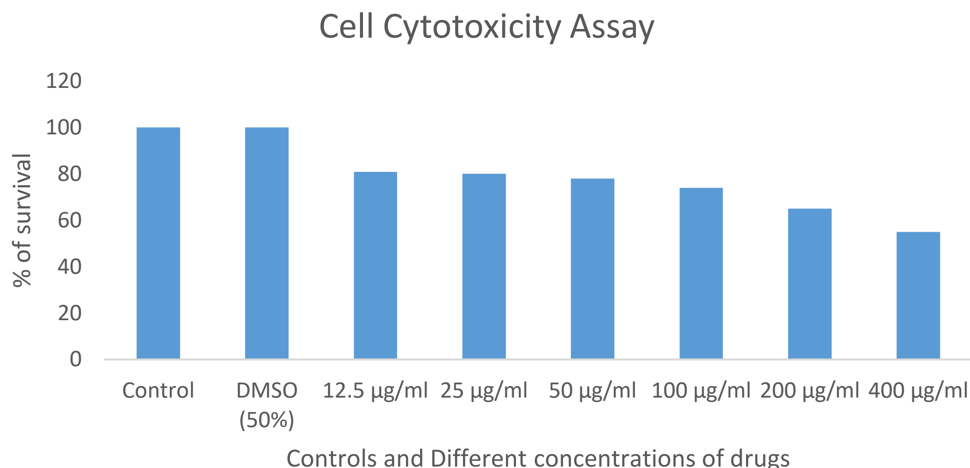


Figure 13: Effect of *Tinospora sp.* ethyl acetate extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Tinospora sp*. ethyl acetate extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 411.1 µg/ml.

Anti-HIV-1 activity of *Tinospora sp.* ethyl acetate extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The observation of green foci of cells treated with this fraction through a fluorescence microscope is represented in figure 14.

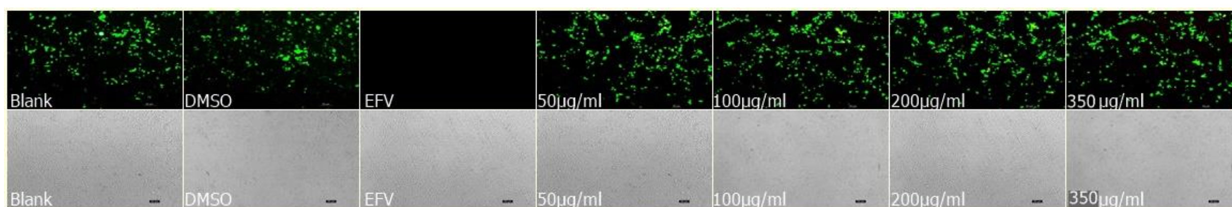


Figure 14- Anti-HIV-1 activity of *Tinospora sp.* ethyl acetate extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Tinospora sp.* Ethyl acetate extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Ethyl acetate extract, indicating *Tinospora sp.* Ethyl acetate extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- Tinospora sp. methanolic extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. The results of MTT assay is represented below graphically in figure 15

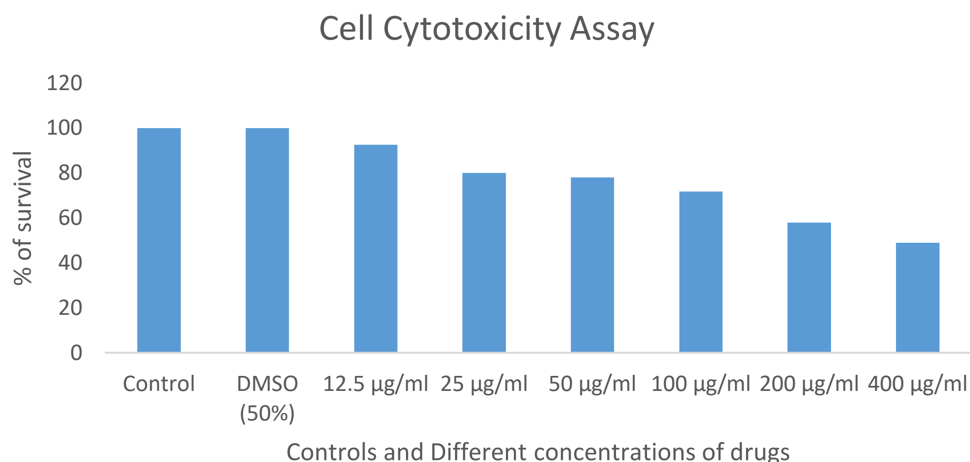


Figure 15: Effect of *Tinospora sp.* methanolic extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Tinospora sp. methanolic extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 384.5 µg/ml.

Anti-HIV-1 activity of *Tinospora sp.* methanolic extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Cell culture assay was performed as it is explained earlier. In figure 16 the observations of green foci expression are represented.

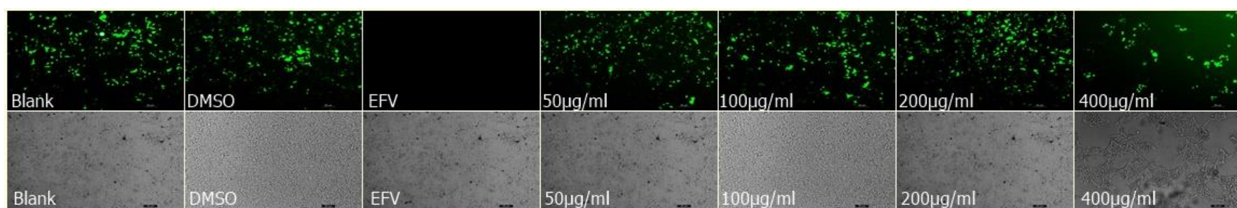


Figure 16- Anti-HIV-1 activity of *Tinospora sp.* methanolic extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Tinospora sp.* methanolic extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of methanolic extract, indicating *Tinospora sp.* methanolic extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- Tinopora sp. petroleum benzene extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. The results of MTT assay of this fraction is represented in figure 17.

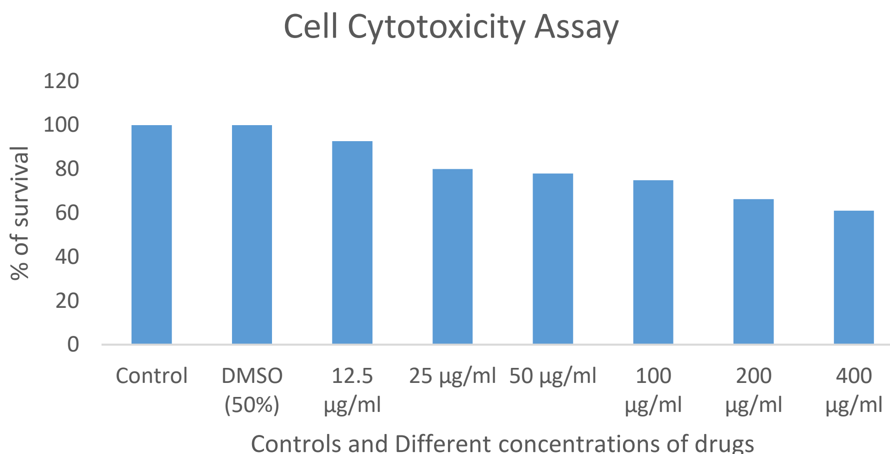


Figure 17: Effect of *Tinopora sp.* petroleum benzene extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Tinopora sp. petroleum benzene extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 441.24 µg/ml.

Anti-HIV-1 activity of *Tinopora sp.* petroleum benzene extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Results of fluorescence microscopy for this crude extract is represented in Figure 18.

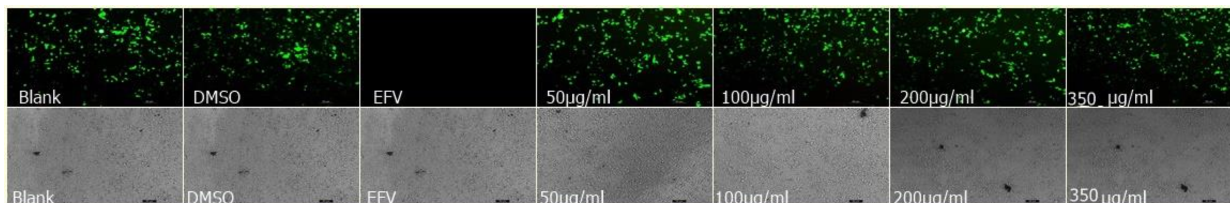


Figure 18- Anti-HIV-1 activity of *Tinopora sp.* Petroleum benzene extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Tinopora sp.* Petroleum benzene extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is **distinct reduction** in GFP positive foci in cells treated with increasing concentration of Petroleum benzene extract, indicating *Tinopora sp.* **petroleum benzene extract have significant anti-HIV-1 activity at 200µg/ml and 350µg/ml concentrations.** Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All three fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400µg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these particular sample, **anti-HIV-1 activity was detected in petroleum benzene extract at 200µg/ml and 350µg/ml of its concentrations**. The Petroleum benzene crude extracts in above mentioned concentrations showed significant decrease in the number of green foci whereas the bright filed images of them shown the cells were intact. It indicates the drug at those concentrations does interfere with the HIV-1 and blocks the expression of GFP.

Hence, anti-HIV-1 activity in Petroleum benzene crude extract at **200µg/ml and 350µg/ml** concentrations in this plant is recorded in this study.

No further fractionations and evaluation was done on the crude extract that showed positive anti-HIV-1 activity in cell culture assay mostly because of lack of sufficient time. So, the fractionation and evaluation of the individual fractions of the petroleum benzene crude extract of *Tinospora sp.* remain undone in this project.

Anti-HIV-1 properties of *Mangifera indica*

First the plants were collected from mentioned location. Dried and then then grinded. Then cold extraction method was performed for preparation of the extracts. Dried plant materials were loaded in a column then different solvents were added. The extract was carefully collected. For proper extraction this step was repeated several times. The collected extracts were then subjected to rotary evaporation for drying. After the extracts were dried they were dissolved in 50% DMSO and 50% double distilled water. The extracts were dissolved in a concentration of 20mg/ml. The dissolved crude drug was then subjected to filter sterilization and the sterilized crude extracts were used for cytotoxicity assay and evaluation in cell culture system.

The crude drugs were serially diluted to make 12.5 µg/ml, 25µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml. Cell cytotoxicity was evaluated using MTT assay as mentioned in the materials and methods.

Three extracts viz. Hexane, Chloroform, Methanol were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50} values were calculated. CC_{50} values for hexane, chloroform, methanol were 418.5 µg/ml, 435.25 µg/ml and 389.56 µg/ml respectively.

Cell cytotoxicity assay- *Mangifera indica* hexane extract

Cell cytotoxicity assays were performed for all three crude extracts using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. All the next steps of the experiments are done using drug concentrations below their cytotoxicity level. Results of MTT assay of this fraction is represented below in figure 19.

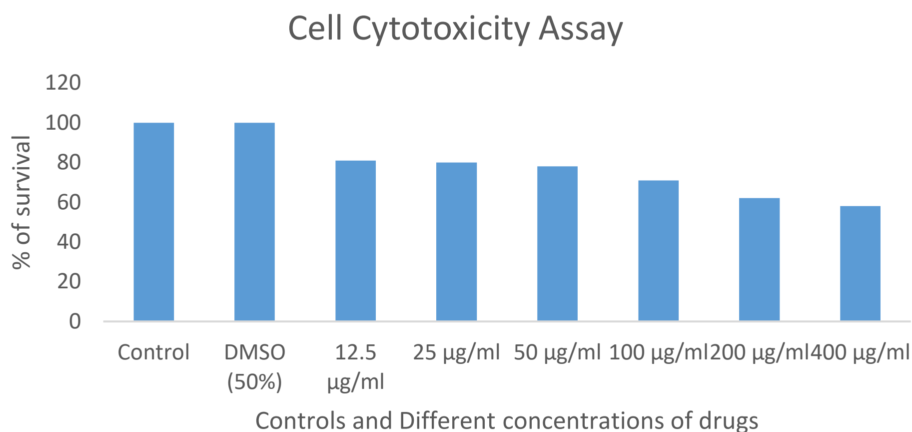


Figure 19: Effect of *Mangifera indica* hexane extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Mangifera indica* hexane extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 418.5 µg/ml.

Anti-HIV-1 activity of *Mangifera indica* hexane extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Cell culture assay was performed as stated earlier the results are represented for this fraction in figure 20.

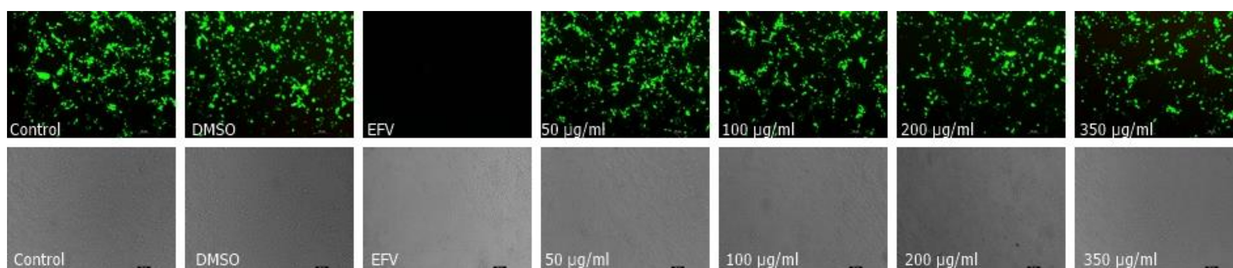


Figure 20- Anti-HIV-1 activity of *Mangifera indica* hexane extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Mangifera indica* hexane extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of hexane extract, indicating *Mangifera indica* hexane extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Mangifera indica* Chloroform extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation

in cell culture assay was done using drug concentrations below their cytotoxicity level. The results of MTT assay of this fraction is represented in figure 21.

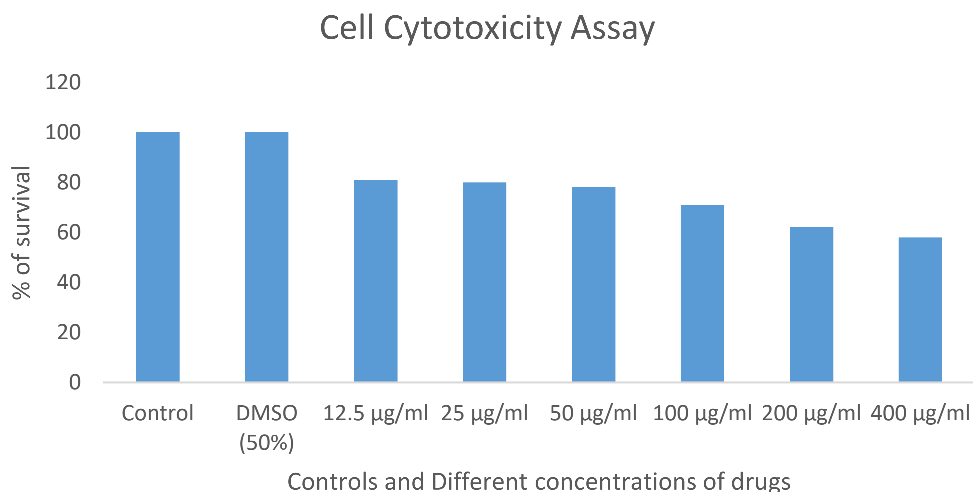


Figure 21: Effect of *Mangifera indica* Chloroform extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Mangifera indica* chloroform extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 435.25 µg/ml.

Anti-HIV-1 activity of *Mangifera indica* chloroform extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. As stated earlier the cell culture assay was performed and observation for this fraction is stated in figure 22.

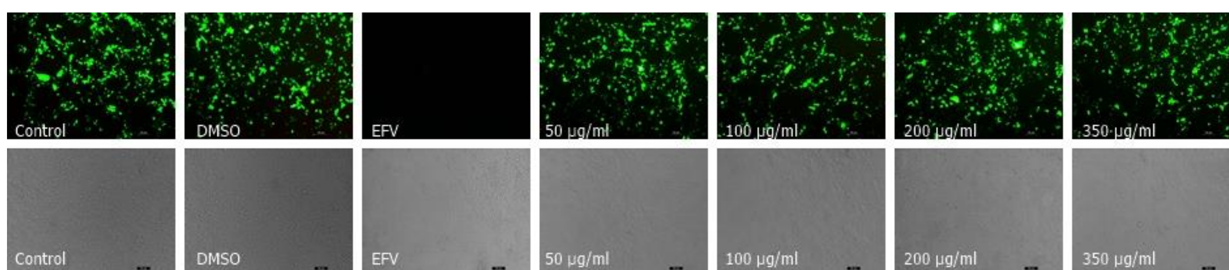


Figure 22- Anti-HIV-1 activity of *Mangifera indica* chloroform extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Mangifera indica* chloroform extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Chloroform extract, indicating *Mangifera indica* chloroform extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Mangifera indica* methanolic extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation

in cell culture assay was done using drug concentrations below their cytotoxicity level. Figure 23 represents the results of MTT assay of this fraction.

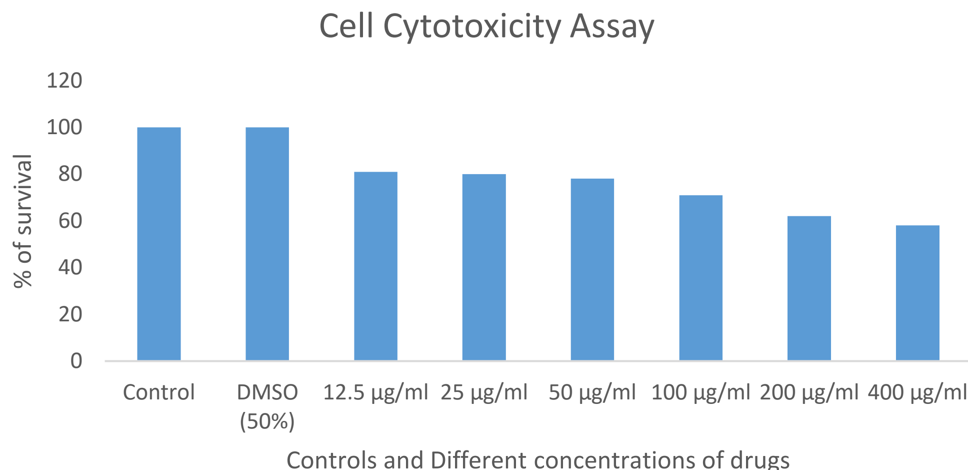


Figure 23: Effect of *Mangifera indica* methanolic extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration Mangifera indica methanolic extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 389.56 µg/ml.

Anti-HIV-1 activity of *Mangifera indica* methanolic extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The observation of fluorescence microscopy is represented in figure 24.

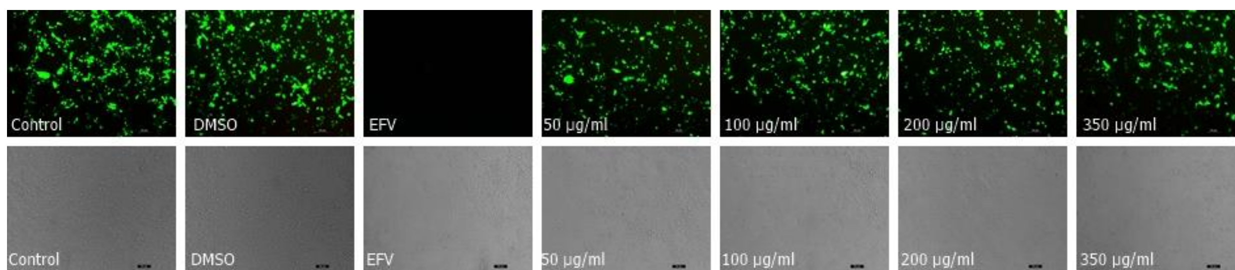


Figure 24- Anti-HIV-1 activity of *Mangifera indica* methanolic extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Mangifera indica* methanolic extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of methanolic extract, indicating *Mangifera indica* methanolic extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All three fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or

naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400μg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these particular sample, anti-HIV-1 activity was not detected in any of the extract and at any of its concentrations. None of the crude extracts in none of its concentrations do not showed any significant apparent reduction in number of green foci in the field.

Hence, anti-HIV-1 activity in any of the crude extract at any concentrations in this plant is not recorded in this study.

Anti-HIV-1 properties of *Swertia bimaculata*

First the plants were collected from mentioned location. Dried and then then grinded. Then cold extraction method was performed for preparation of the extracts. Dried plant materials were loaded in a column then different solvents were added. The extract was carefully collected. For proper extraction this step was repeated several times. The collected extracts were then subjected to rotary evaporation for drying. After the extracts were dried they were dissolved in 50% DMSO and 50% double distilled water. The extracts were dissolved in a concentration of 20mg/ml. The dissolved crude drug was then subjected to filter sterilization and the sterilized crude extracts were used for cytotoxicity assay and evaluation in cell culture system.

The crude drugs were serially diluted to make 12.5 μg/ml, 25μg/ml, 50 μg/ml, 100 μg/ml, 200 μg/ml, 400 μg/ml. Cell cytotoxicity was evaluated using MTT assay as mentioned in the materials and methods.

Three extracts viz. Hexane, Chloroform, Methanol were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50} values were calculated. CC_{50} values for hexane, chloroform, methanol were 411.1 μg/ml, 348.5 μg/ml and 441.24 μg/ml respectively.

Cell cytotoxicity assay- *Swertia bimaculata* hexane extract

Cell cytotoxicity assays were performed for all three crude extracts using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. All the next steps of the experiments are done using drug concentrations below their cytotoxicity level. Figure 25 represents results of MTT assay of this fraction.

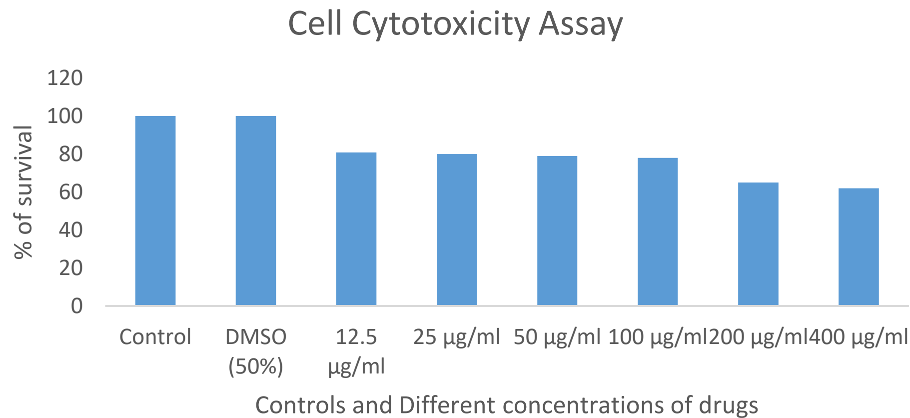


Figure 25: Effect of *Swertia bimaculata* hexane extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Swertia bimaculata* hexane extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 411.1 µg/ml.

Anti-HIV-1 activity of *Swertia bimaculata* hexane extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Results of fluorescence microscopy is represented for this fraction in figure 26.

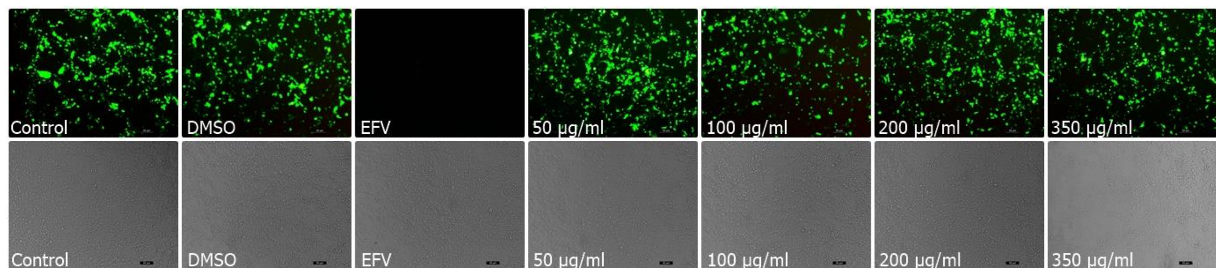


Figure 26- Anti-HIV-1 activity of *Swertia bimaculata* hexane extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Swertia bimaculata* hexane extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Hexane extract, indicating *Swertia bimaculata* Hexane extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Swertia bimaculata* chloroform extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay of this fraction is represented in figure 27.

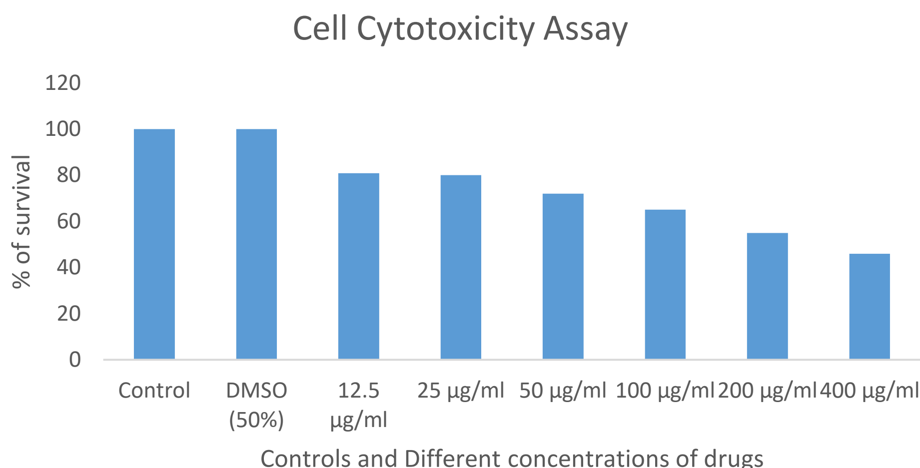


Figure 27: Effect of *Swertia bimaculata* chloroform extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Swertia bimaculata* chloroform extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing conc. of extract. The CC_{50} values were calculated after 72 hours and found to be 348.5 µg/ml.

Anti-HIV-1 activity of *Swertia bimaculata* chloroform extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Figure 28 represents results of cell culture assay of this fraction.

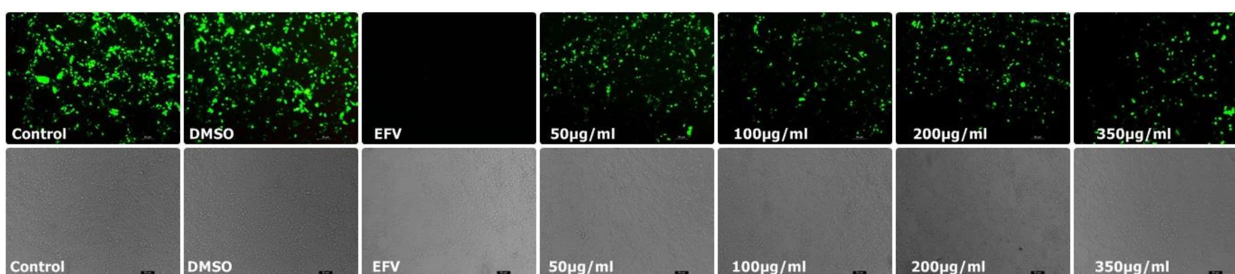


Figure 28- Anti-HIV-1 activity of *Swertia bimaculata* chloroform extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Swertia bimaculata* chloroform extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of chloroform extract, indicating *Swertia bimaculata* chloroform extract have no anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Swertia bimaculata* methanolic extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Figure 29 below represents results of MTT assay of this fraction.

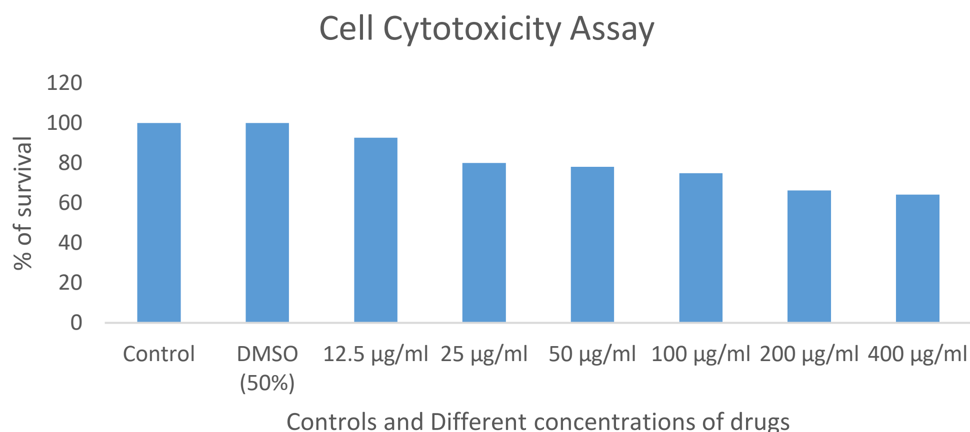


Figure 29: Effect of *Swertia bimaculata* methanolic extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Swertia bimaculata* methanolic extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 441.24 µg/ml

Anti-HIV-1 activity of *Swertia bimaculata* methanolic extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Cell culture assay was performed as stated earlier. The changes in number of green foci was observed under a fluorescence microscope and results are represented in figure 30.

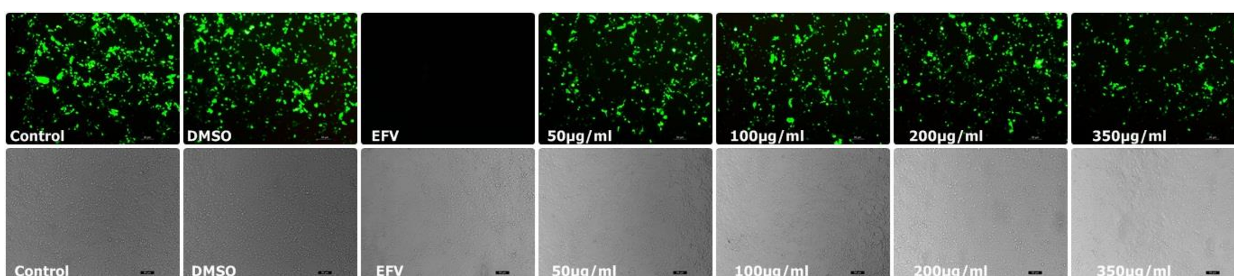


Figure 30- Anti-HIV-1 activity of *Swertia bimaculata* methanolic extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Swertia bimaculata* methanolic extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is **distinct reduction** in GFP positive foci in cells treated with increasing concentration of methanolic extract, indicating *Swertia bimaculata* methanolic extract **have Anti-HIV-1 activity** at 200µg/ml and 300 µg/ml concentration. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All three fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400µg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these particular sample, **anti-HIV-1 activity was detected in methanolic extract and at 200µg/ml and 350µg/ml of its concentrations**. The methanolic crude extracts in above mentioned concentrations showed significant decrease in the number of green foci whereas the bright filed images of them shown the cells were intact. It indicates the drug at those concentrations does interfere with the HIV-1 and blocks the expression of GFP.

Hence, anti-HIV-1 activity in methanolic crude extract at **200µg/ml and 350µg/ml** concentrations in this plant is recorded in this study.

This activity was previously reported by Mr. Vivek Gairola in the same lab this project confirms his reporting. No further fractionations and evaluation of fractions were done for this plant in this project.

Anti-HIV-1 properties of *Solanum sp.*

First the plants were collected from mentioned location. Dried and then then grinded. Then cold extraction method was performed for preparation of the extracts. Dried plant materials were loaded in a column then different solvents were added. The extract was carefully collected. For proper extraction this step was repeated several times. The collected extracts were then subjected to rotary evaporation for drying. After the extracts were dried they were dissolved in 50% DMSO and 50% double distilled water. The extracts were dissolved in a concentration of 20mg/ml. The dissolved crude drug was then subjected to filter sterilization and the sterilized crude extracts were used for cytotoxicity assay and evaluation in cell culture system.

The crude drugs were serially diluted to make 12.5 µg/ml, 25µg/ml, 50 µg/ml, 100 µg/ml, 200 µg/ml, 400 µg/ml. Cell cytotoxicity was evaluated using MTT assay as mentioned in the materials and methods.

Three extracts viz. Hexane, Chloroform, Methanol were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50} values were calculated. CC_{50} values for hexane, chloroform, methanol were 390.5 µg/ml, 357.3 µg/ml and 398 µg/ml respectively.

Cell cytotoxicity assay- *solanum sp.* hexane extract

Cell cytotoxicity assays were performed for all three crude extracts using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. All the next steps of the experiments are done using drug concentrations below their cytotoxicity level. Figure 31 represents results of MTT assay of this fraction.

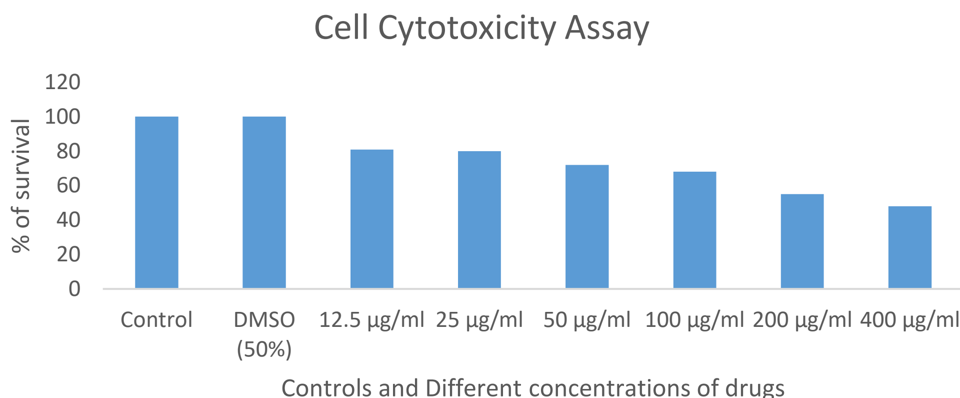


Figure 31: Effect of *Solanum sp.* hexane extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Solanum sp.* hexane extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 418.5 µg/ml.

Anti-HIV-1 activity of *solanum sp.* hexane extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Results of fluorescence microscopy of this fraction is represented in figure 32.

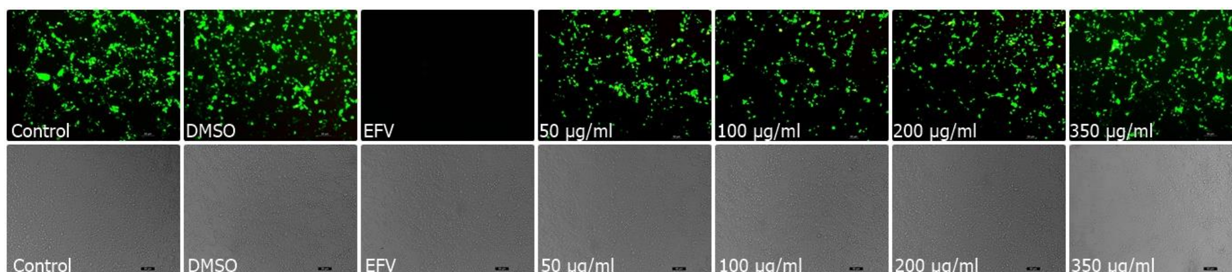


Figure 32- Anti-HIV-1 activity of *Solanum sp.* hexane extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Solanum sp.* hexane extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of Hexane extract, indicating *Solanum sp.* Hexane extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *solanum sp.* chloroform extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is represented in figure 33.

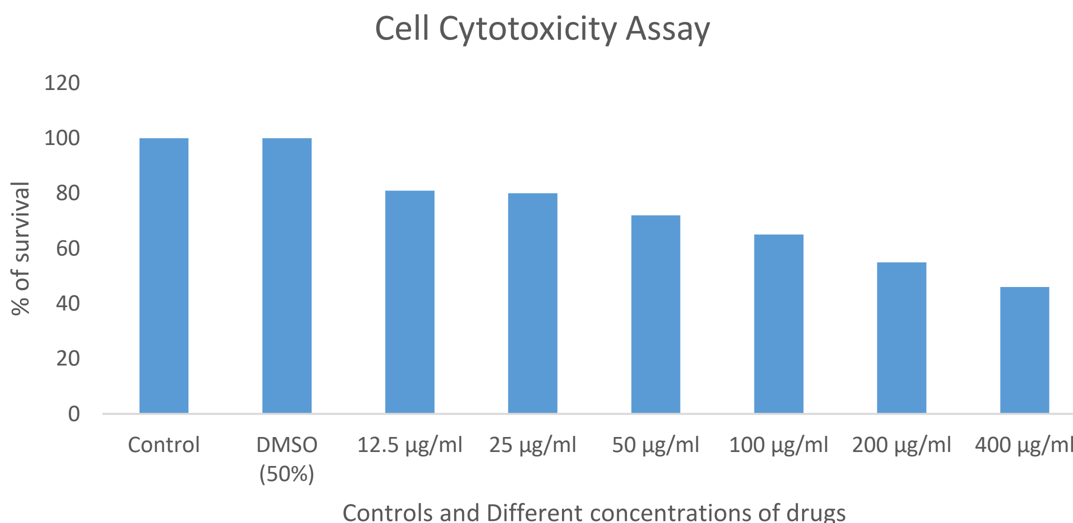


Figure 33: Effect of *Solanum sp.* chloroform extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Solanum sp.* chloroform extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing conc. of extract. The CC_{50} values were calculated after 72 hours and found to be 357.3 µg/ml.

Anti-HIV-1 activity of *Solanum sp.* chloroform extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Cell culture assay was performed as mentioned earlier and results were obtained using a fluorescent microscope and for this crude extract the results are represented in figure 34.

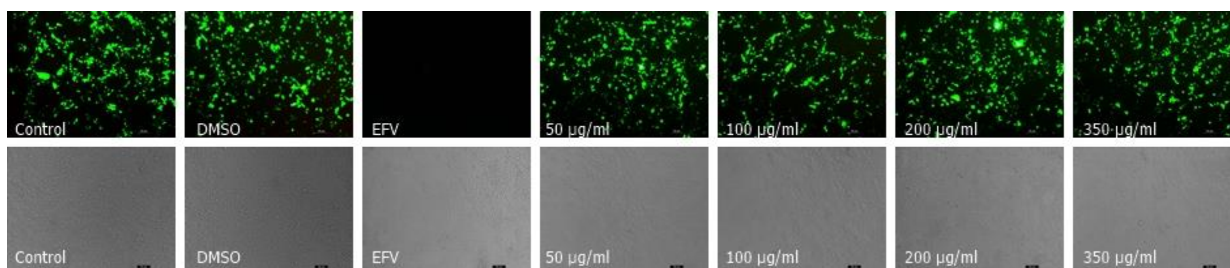


Figure 34- Anti-HIV-1 activity of *Solanum sp.* chloroform extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Solanum sp.* chloroform extract. After 48h and 72h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of chloroform extract, indicating *Solanum sp.* chloroform extract have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- *Solanum sp.* methanolic extract

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is represented in figure 35.

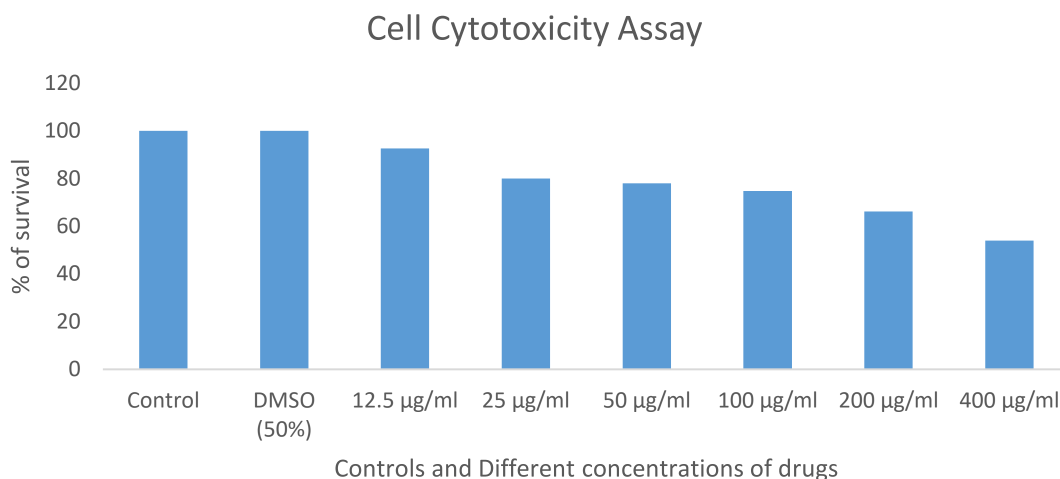


Figure 35: Effect of *Solanum sp.* methanolic extract on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration *Solanum sp.* methanolic extract in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentrations of extract. The CC_{50} values were calculated after 72 hours and found to be 398 µg/ml.

Anti-HIV-1 activity of *Solanum sp.* methanolic extract

Crude plant extracts were evaluated using cell culture-based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. Images were captured through the fluorescence microscope after cell culture assay was performed and is represented here in figure 36.

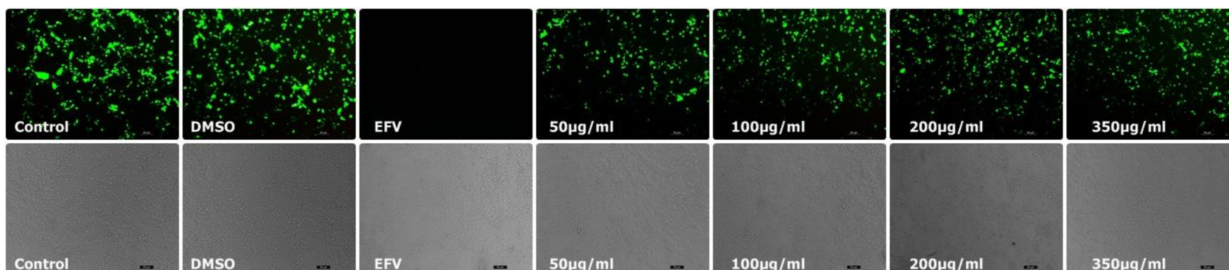


Figure 36- Anti-HIV-1 activity of *Solanum sp.* methanolic extract: In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of *Solanum sp.* methanolic extract. After 48h of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of methanolic extract, indicating *Solanum sp.* methanolic extract do not have Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All three fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400µg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these particular sample, anti-HIV-1 activity was not detected in any of the extract and at any of its concentrations. None of the crude extracts in none of its concentrations do not showed any significant apparent reduction in number of green foci in the field.

Hence, anti-HIV-1 activity in any of the crude extract at any concentrations in this plant is not recorded in this study.

Anti-HIV-1 properties of Different fractions of *Ocimum gratissimum* methanolic extract

The methanolic crude extracts of the *Ocimum gratissimum* showed positive anti-HIV-1 activity in cell culture-based assay as mentioned above. So further fractionations were performed using column chromatography to reach up to the individual component that interacts with the HIV-1 and blocks the expression of GFP. The dried methanolic crude extract was mixed with silica in methanol and they were left couple of days for air drying. The dried powdery slurry was used for further fractionation. First the column was packed with silica. After packing of the column with silica and methanol the slurry was put on top of the silica bed. Different solvents were prepared depending on their polarity. And the column was then allowed to slowly run. The visible fractions were collected dried, dissolved in 50% DMSO and filter sterilized. Then the samples were serially diluted as it was done for the crude extracts previously mentioned. Cytotoxicity for each of the fractions were evaluated and then the fractions were evaluated for their anti-HIV-1 activity in cell culture assay.

Fractionations made with 100% Ethyl acetate yielded 3 fractions, 30% Ethyl acetate and 70% methanol yielded only one visible fraction, 50% Ethyl acetate and 50% Methanol yielded no fractions, and 70% Ethyl acetate 30% Methanol yielded 4 fractions and 100% Methanol yielded 4 fractions.

These twelve fractions were subjected to cytotoxicity assay and then then evaluated in cell culture assay. The drugs were used under its cytotoxic level. After 60 hours the reading was taken with a florescent microscope. The number of green foci was observed. Then drugs were applied to the cells and GFP fluorescence were observed after 60 hours. The image captured from fluorescence microscope is given below. Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug

Twelve extracts viz. E1, E2A, E2B, E3M7, E7M3-1, E7M3-2, E7M3-3, E7M3-4, M1, M2, M3 and M4 were evaluated for their Anti-HIV-1 activity at 48h. Cell cytotoxicity assay was performed prior to Anti-HIV-1 assay and CC_{50} values were calculated. CC_{50} values for them were 392.5 μg/ml, 398 μg/ml, 384 μg/ml, 395.6 393 μg/ml, 440.25 μg/ml, 385.6 μg/ml, 411.2 μg/ml, 393 μg/ml, 421.25 μg/ml, 389.6 μg/ml, 396.25 μg/ml, respectively.

Cell cytotoxicity assay- E1 (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. The results of MTT assay is represented in figure 37.

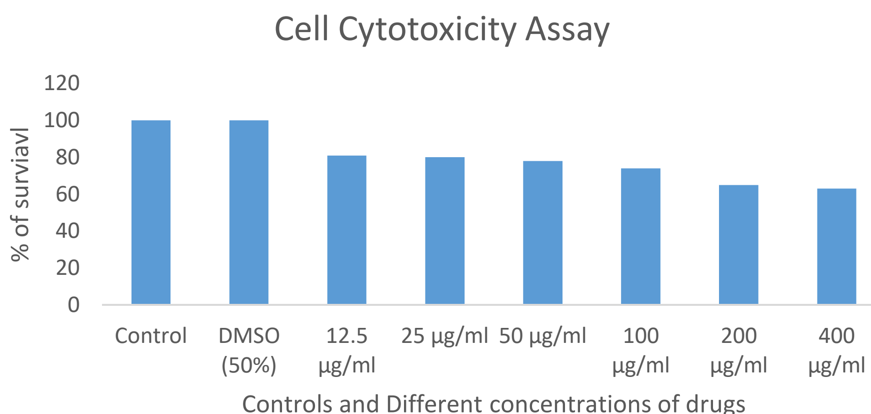


Figure 37: Effect of E1 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E1 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 392.5 μg/ml.

Anti-HIV-1 activity of E1 (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. Cell culture assay was performed in Huh 7.5 cells. The drug treated cells along with the controls were observed under a fluorescence microscope and the changes in the green foci were observed and captured images for this fraction is represented in figure 38.

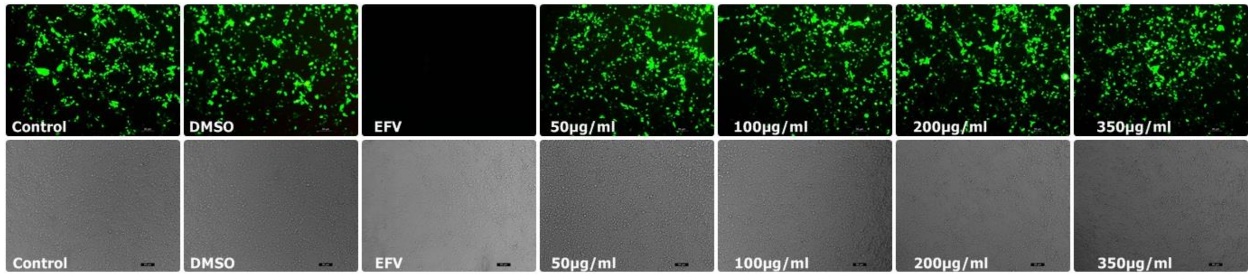


Figure 38- Anti-HIV-1 activity of E1 (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E1 (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E1 (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*), indicating E1 (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E2A (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay of this fraction is represented in figure 39.

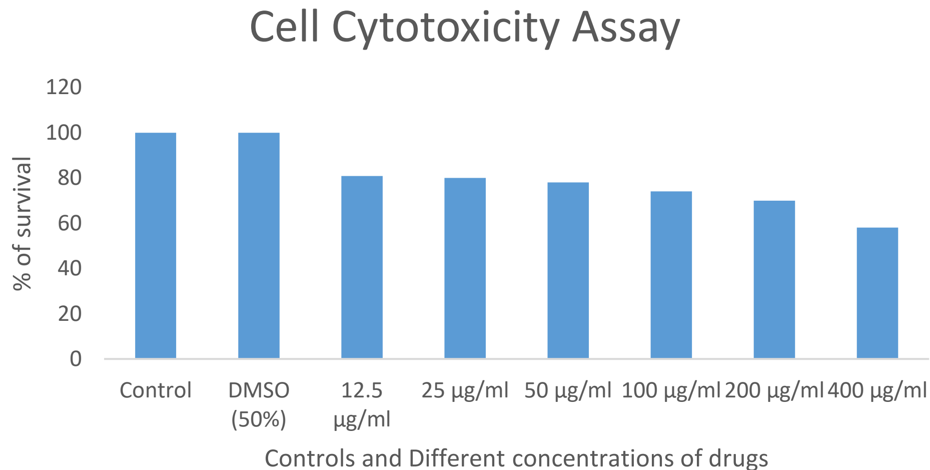


Figure 39: Effect of E2A on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E2A in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 398 μg/ml.

Anti-HIV-1 activity of E2A (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 40.

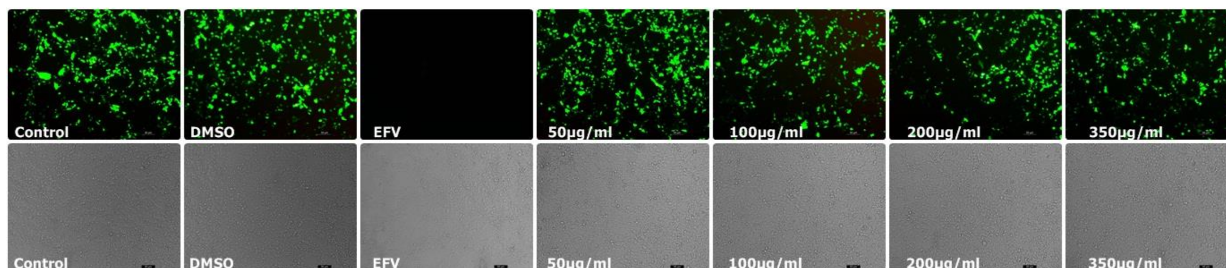


Figure 40- Anti-HIV-1 activity of E2A (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E2A (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E2A (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*), indicating E2A (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E2B (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay for this fraction is mention in figure 41.

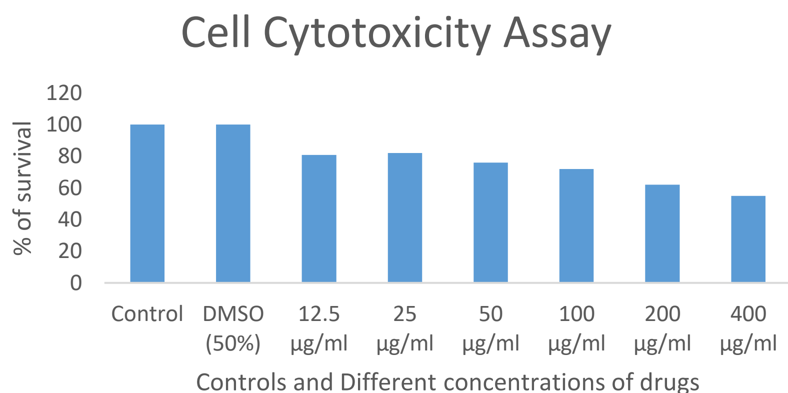


Figure 41: Effect of E2B on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E2B in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 384 µg/ml.

Anti-HIV-1 activity of E2B (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 42.

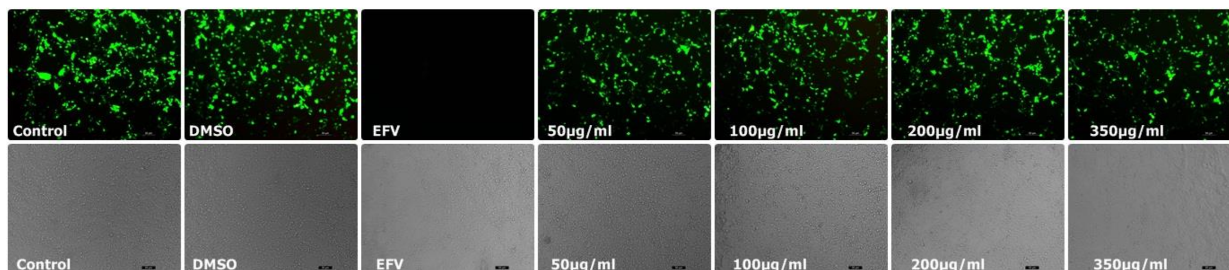


Figure 42- Anti-HIV-1 activity of E2B (ethyl acetate 100% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E2B (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E2B (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*), indicating E2B (Ethyl acetate 100% - methanolic Extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E3M7 (ethyl acetate 30% + methanol 70% - methanolic extract of Ocimum gratissimum)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay of this fraction is represented in figure 43.

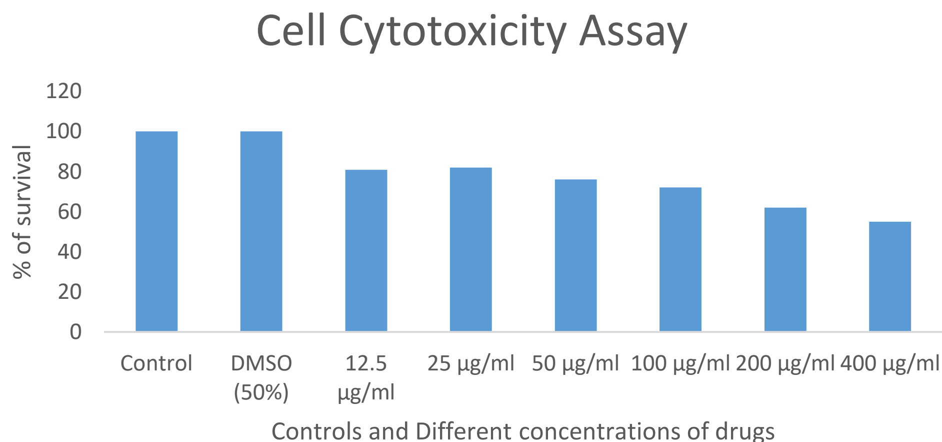


Figure 43: Effect of E3M7 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E3M7 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 395.6 µg/ml.

Anti-HIV-1 activity of E3M7 (ethyl acetate 30% + methanol 70% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 44.

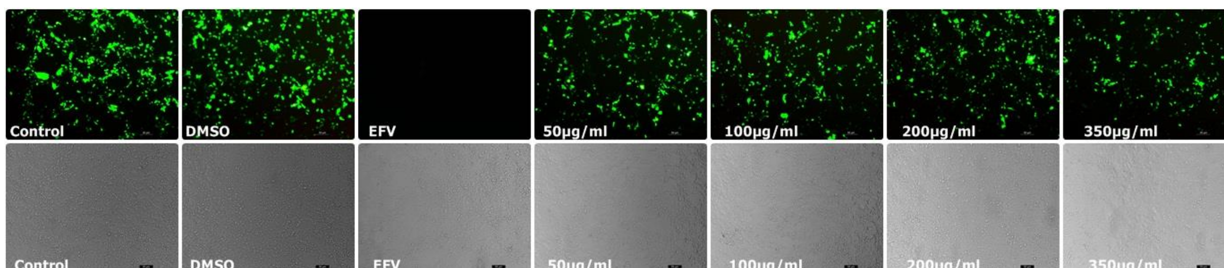


Figure 44- Anti-HIV-1 activity of E3M7 (ethyl acetate 30%+ methanol 70% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E3M7 (ethyl acetate 30%+ methanol 70% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above **there is a distinct reduction in GFP positive foci in cells treated with increasing concentration of E3M7** (ethyl acetate 30%+ methanol 70% - methanolic extract of *Ocimum gratissimum*), indicating **E3M7 (ethyl acetate 30%+ methanol 70% - methanolic extract of *Ocimum gratissimum*) have Anti-HIV-1 activity at 200 and 350 µg/ml**. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E7M3-1 (ethyl acetate 70% + methanol 30% - methanolic extract of Ocimum gratissimum)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay of this fraction is represented below in figure 45.

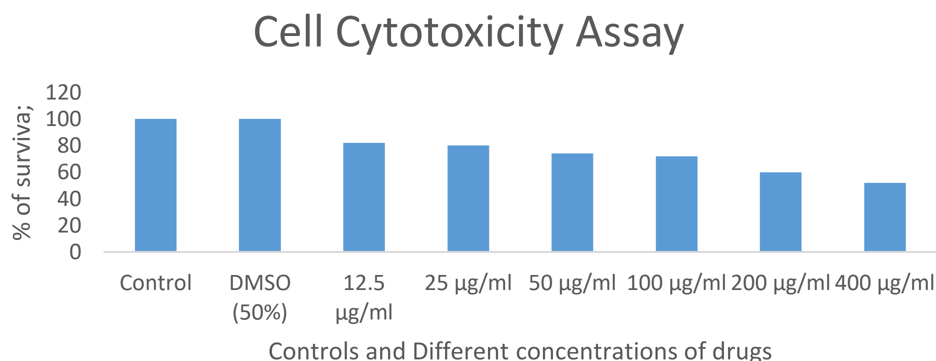


Figure 45: Effect of E7M3-1 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E7M3-1 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 393 µg/ml.

Anti-HIV-1 activity of E7M3-1 (ethyl acetate 70% + methanol 30% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 46.

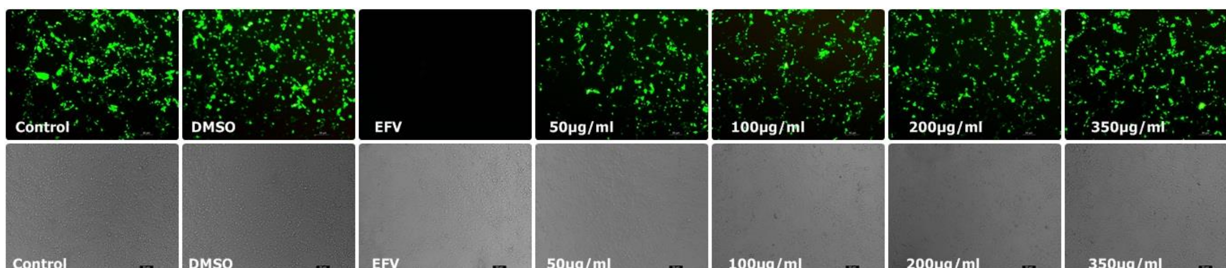


Figure 46- Anti-HIV-1 activity of E7M3-1 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E7M3-1 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E7M3-1 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*), indicating E7M3-1 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E7M3-2 (ethyl acetate 70% + methanol 30% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is performed in figure 47.

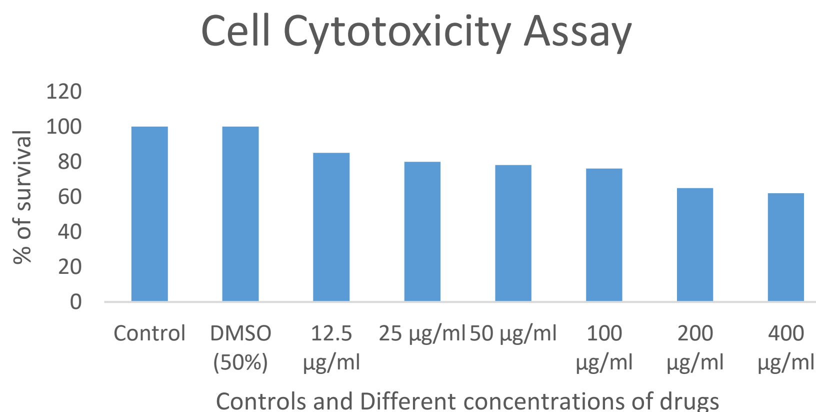


Figure 47: Effect of E7M3-2 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E7M3-2 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 440.25 μg/ml.

Anti-HIV-1 activity of E7M3-2 (ethyl acetate 70% + methanol 30% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 48.

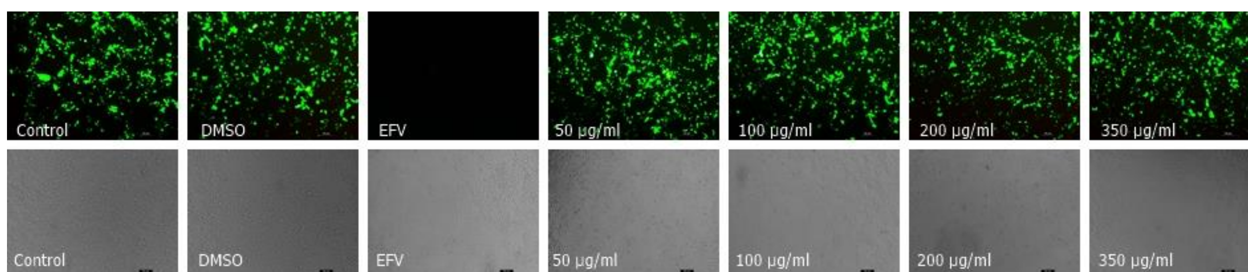


Figure 48- Anti-HIV-1 activity of E7M3-2 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E7M3-2 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E7M3-2 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*), indicating E7M3-2 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E7M3-3 (ethyl acetate 70% + methanol 30% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is graphically represented in figure 49.

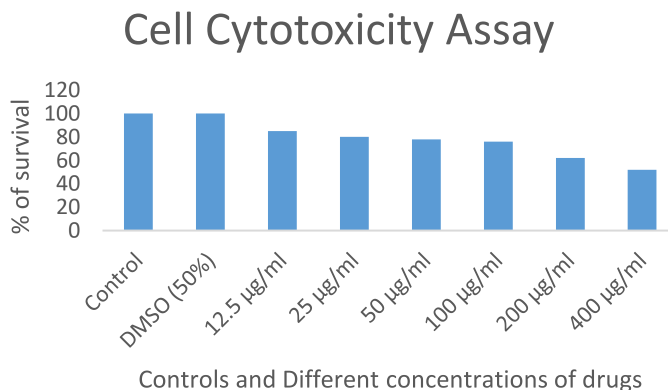


Figure 49: Effect of E7M3-3 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E7M3-3 in triplicate for 72 hours Post incubation MTT assay was

performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 385.6 µg/ml.

Anti-HIV-1 activity of E7M3-3 (ethyl acetate 70% + methanol 30% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 50.

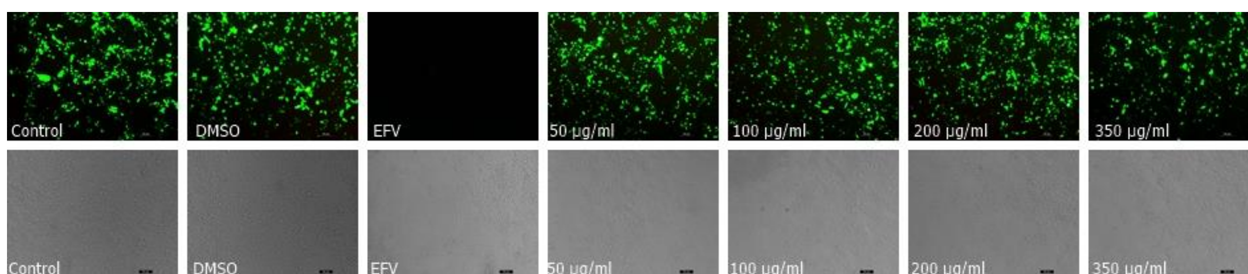


Figure 50- Anti-HIV-1 activity of E7M3-3 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E7M3-3 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E7M3-3 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*), indicating E7M3-3 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- E7M3-4 (ethyl acetate 70% + methanol 30% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is represented in figure 51.

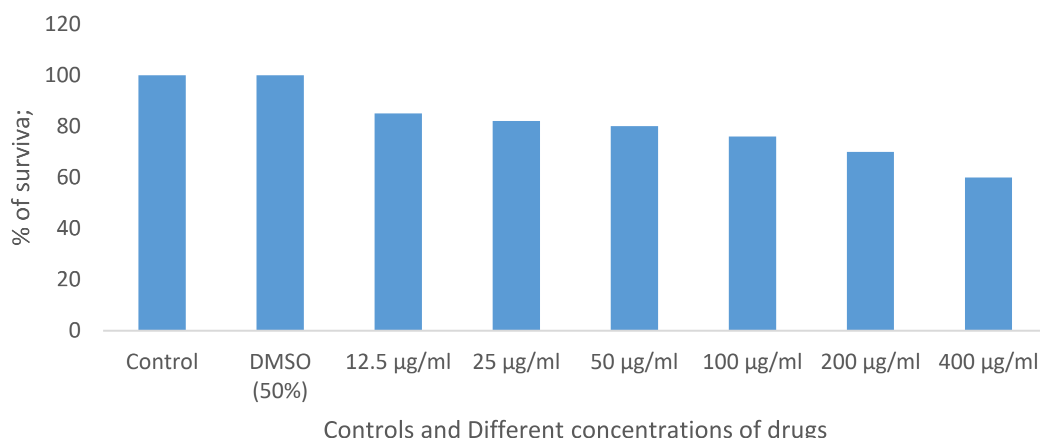


Figure 51: Effect of E7M3-4 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration E7M3-4 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 411.2 µg/ml.

Anti-HIV-1 activity of E7M3-4 (ethyl acetate 70% + methanol 30% - methanolic Extract of Ocimum gratissimum)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 52.

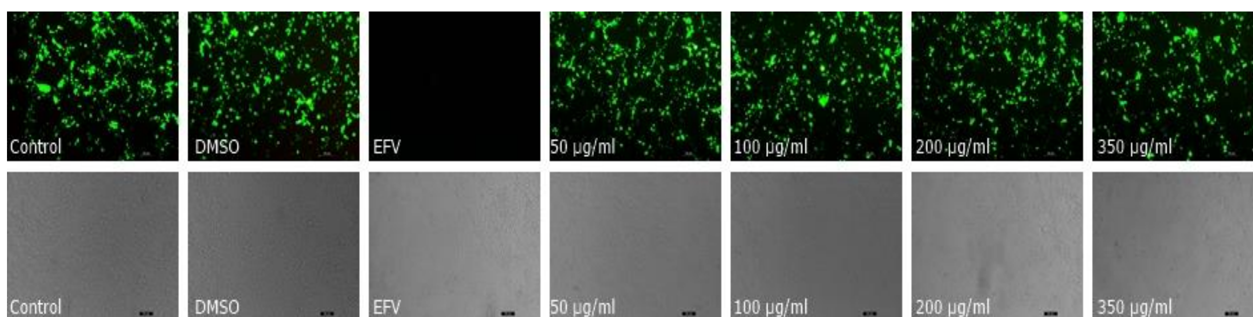


Figure 52- Anti-HIV-1 activity of E7M3-4 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of E7M3-4 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of E7M3-4 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*), indicating E7M3-4 (ethyl acetate 70%+ methanol 30% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- M1 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay for this fraction is represented in figure 53.

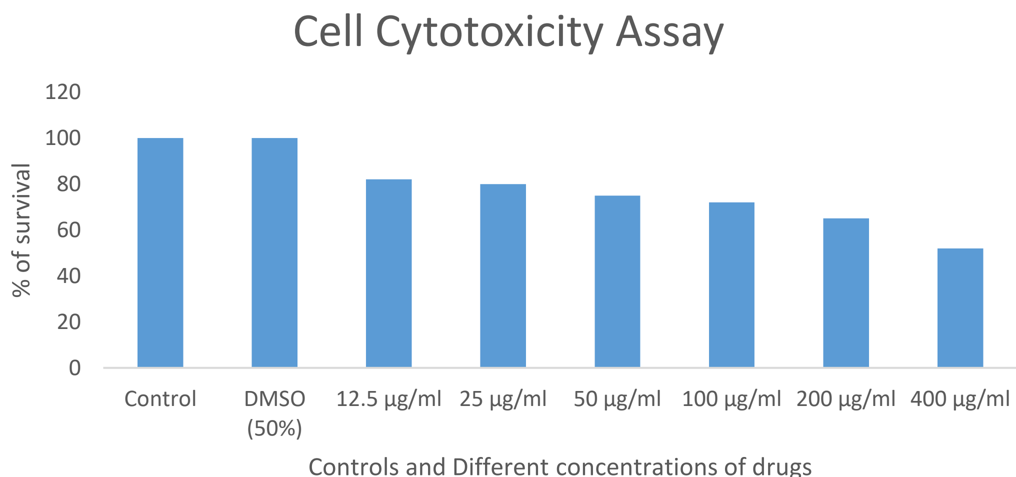


Figure53: Effect of M1 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration M1 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 393 µg/ml.

Anti-HIV-1 activity of M1 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 54.

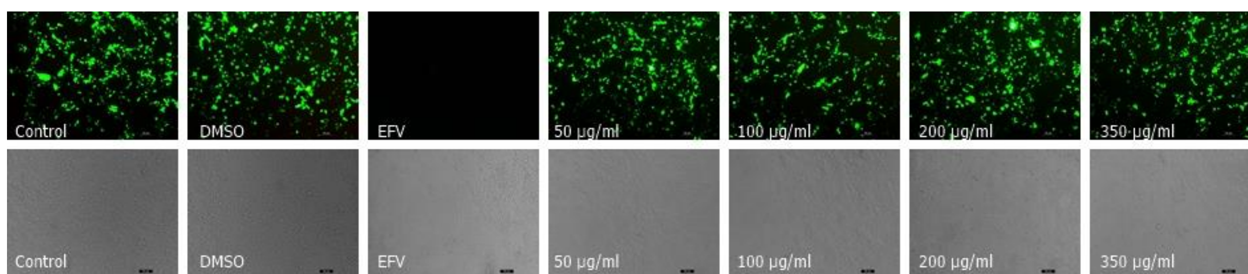


Figure 54- Anti-HIV-1 activity of M1 (methanol 100% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of M1 (methanol 100% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of M1 (methanol 100% - methanolic extract of *Ocimum gratissimum*), indicating M1

(methanol 100% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- M2 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is represented in figure 55.

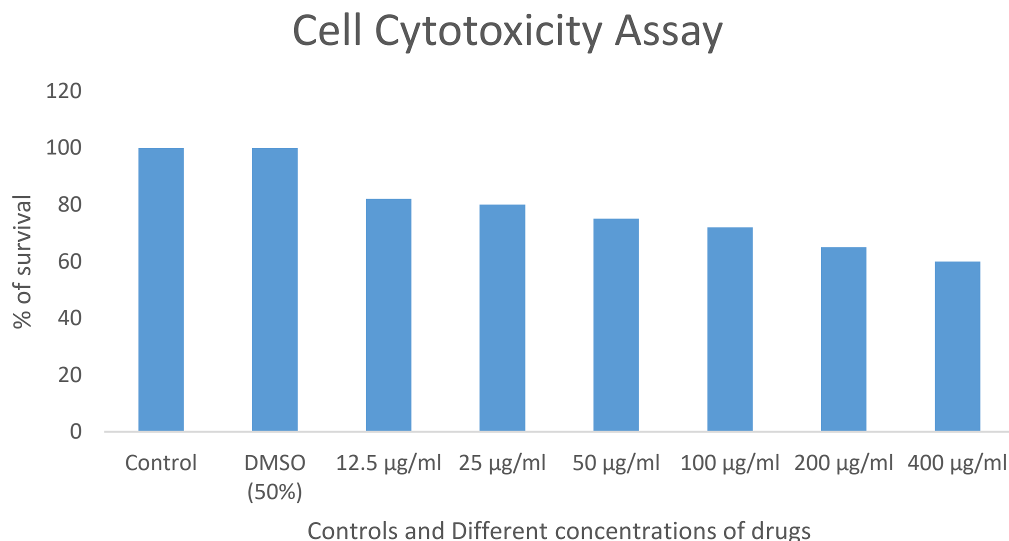


Figure55: Effect of M2 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration M2 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 421.25 µg/ml.

Anti-HIV-1 activity of M2 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 56.

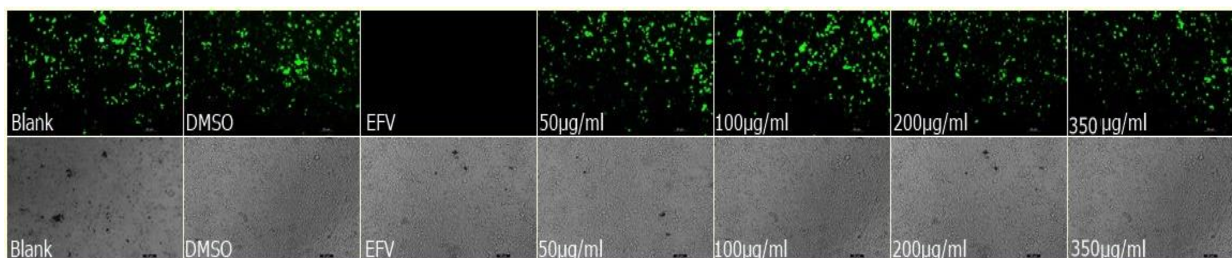


Figure 56- Anti-HIV-1 activity of M2 (methanol 100% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of M2 (methanol 100% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of M2 (methanol 100% - methanolic extract of *Ocimum gratissimum*), indicating M2 (methanol 100% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- M3 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is represented in figure 57.

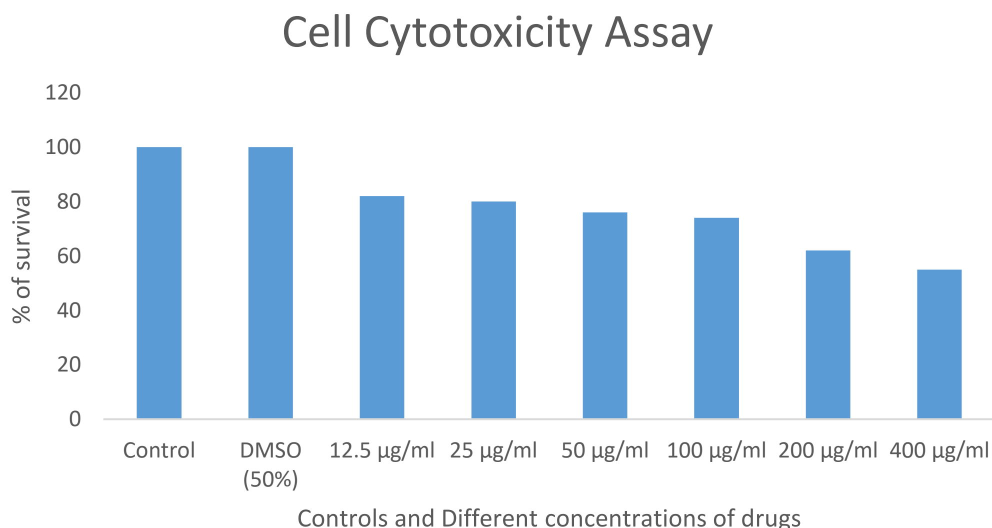


Figure57: Effect of M3 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration M3 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 389.6 µg/ml.

Anti-HIV-1 activity of M3 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 58.

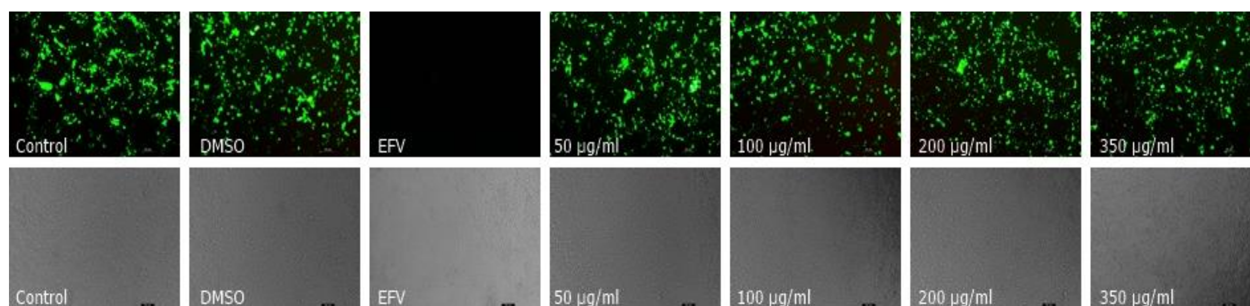


Figure 58- Anti-HIV-1 activity of M3 (methanol 100% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of M3 (methanol 100% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of M3 (methanol 100% - methanolic extract of *Ocimum gratissimum*), indicating M3 (methanol 100% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

Cell cytotoxicity assay- M4 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Cell cytotoxicity assays were performed using the MTT assay as mentioned in the materials and methods. The readings were taken in a plate reader and then plotted in excel and is presented below. Evaluation in cell culture assay was done using drug concentrations below their cytotoxicity level. Results of MTT assay is represented below in figure 59.

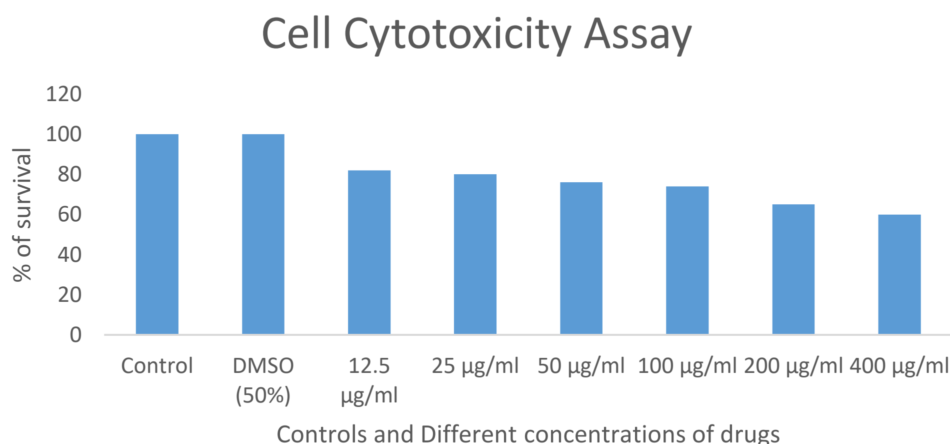


Figure59: Effect of M4 on Cell viability: Huh 7.5 cells were grown in 96 well tissue culture plate (20,000 cells/well) in presence of increasing concentration M4 in triplicate for 72 hours Post incubation MTT assay was performed to evaluate the cytotoxicity. % cell survival was plotted against increasing concentration of extract. The CC_{50} values were calculated after 72 hours and found to be 396.25 μg/ml.

Anti-HIV-1 activity of M4 (methanol 100% - methanolic extract of *Ocimum gratissimum*)

Filter sterilized dissolved dried fractions were evaluated using cell culture based assay as mentioned earlier Decrease in GFP green foci indicates anti-HIV-1 activity of the drug whereas no changes in GFP green foci indicates no detectable activity of the drug. The images below are captured using fluorescence microscope showing green foci. As mentioned earlier the cell culture was performed and observed results are represented below in figure 61.

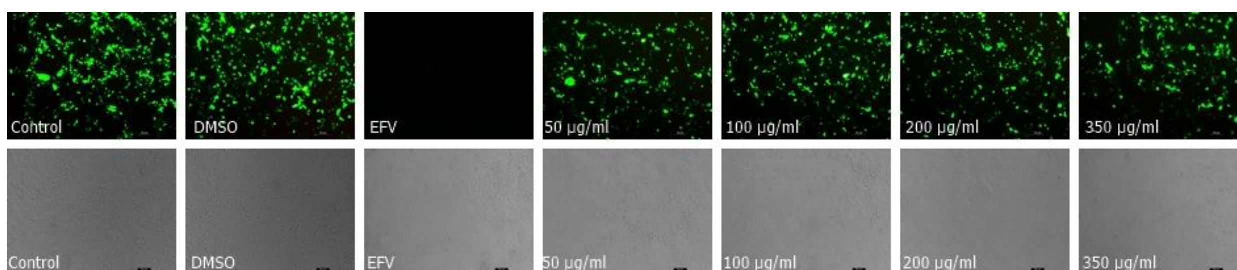


Figure 60- Anti-HIV-1 activity of M4 (methanol 100% - methanolic extract of *Ocimum gratissimum*): In a 96 well plate HIV-1 infected Huh 7.5 cells were grown in presence of different concentrations of M4 (methanol 100% - methanolic extract of *Ocimum gratissimum*). After 48h expression of GFP reporter was evaluated by fluorescent microscopy. As seen in the images above there is no distinct reduction in GFP positive foci in cells treated with increasing concentration of M4 (methanol 100% - methanolic extract of *Ocimum gratissimum*), indicating M4 (methanol 100% - methanolic extract of *Ocimum gratissimum*) have no Anti-HIV-1 activity. Upper panel shows the fluorescence microscopy and the lower plane shows bright filed microscopy.

All twelve fractions were first subjected to MTT assay to check their cytotoxicity level. Then these fractions were serially diluted and the fractions were evaluated for their anti-HIV-1 activity in cell culture-based assays. As mentioned in materials in methods the cells were first plated and maintained. Later the drug was applied in different concentrations. Highest concentration of the drug evaluation was lower than the cytotoxicity level. The GFP expression was then detected using fluorescence microscopy. Along the drug samples, three controls were used. The first control was cells that were not treated using anything or naive cells. The second control was solvent control that is 50% DMSO. The concentration of DMSO that was in the 400µg/ml drug was used in solvent control. And Efavirenz, a well-established anti-HIV-1 drug was used as the drug control.

The number and amount of green foci indicate the activity of the drug as potential anti-HIV-1 agent. The drug control shows no apparent green foci in the field, whereas the bright field of the drug control shows intact cells. It indicates the potent elimination of HIV-1 infection in the cells. All the other fields were evaluated in respect to the Naïve control cells. DMSO or solvent control shows very little to almost no decrease in number of green foci as well as intact cells in the field. It indicates the solvents do not have any potential anti-HIV-1 activity and the concentration of solvent is much below its cytotoxic level.

For these twelve-particular sample, **anti-HIV-1 activity was detected in E3M7 at 200µg/ml and 350µg/ml of its concentrations**. The fraction of methanolic extract of *Ocimum gratissimum* in 30% Ethyl acetate and 70% Methanol in above mentioned concentrations showed significant decrease in the number of green foci whereas the bright filed images of them shown the cells were intact. It indicates the drug at those concentrations does interfere with the HIV-1 and blocks the expression of GFP.

So E3M7 fraction of this crude extract was subjected to further separation by Thin Layer chromatography. The results of the TLC is mentioned next.

Further fractionation using preparative TLC-

Thin Layer chromatography was performed to identify components of **E3M7 (ethyl acetate 30%+ methanol 70% - methanolic extract of *Ocimum gratissimum*).** Three different bands were observed under UV. TLC that was performed using 100% Methanol showed prominent band separation. TLC that were performed using 50% Methanol and 50% water didn't show proper band separation. Other solvents were also tried but prominent band separation was not observed. So, Result of TLC performed using 100% Methanol as solvent is represented in figure 61.

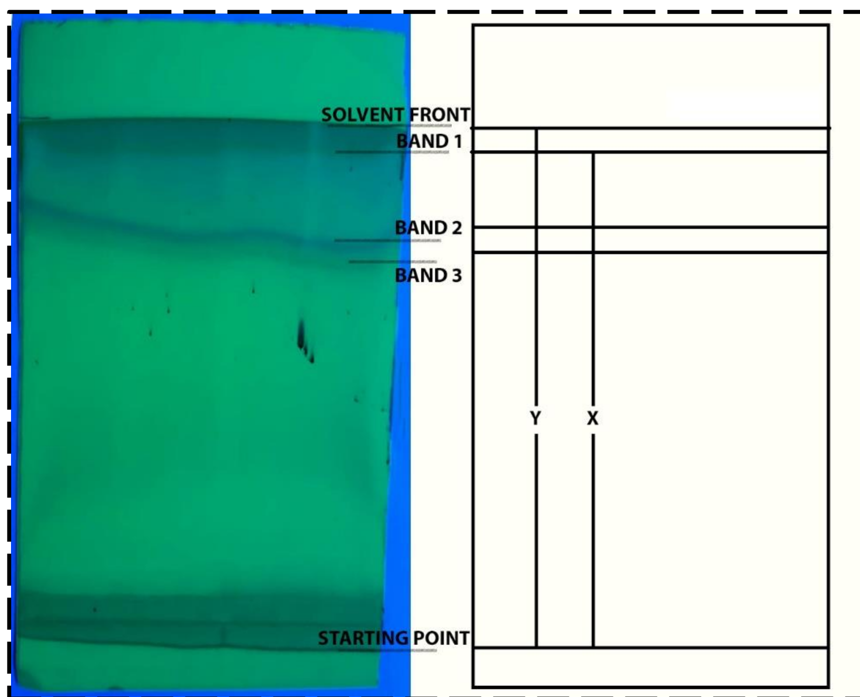


Figure 61: Thin Layer chromatography to identify components of E3M7 (Ethyl acetate 30%+ Methanol 70% - Methanolic Extract of Ocimum gratissimum) showing three bands.

BAND	Y	X	$\frac{X}{Y}$	Rf Value
Band 1	9.8	9.5	$\frac{9.5}{9.8}$	0.96
Band 2	9.8	8.4	$\frac{8.4}{9.8}$	0.857
Band 3	9.8	7.6	$\frac{7.6}{9.8}$	0.775

The three bands were dissolved in Methanol again and the silica is removed from the solution by means of centrifugation. The dissolved bands are then subjected to drying and dissolved in 50% DMSO and made ready for further evaluation in cell culture based assay though due to lack of time these fractions were not evaluated in this project.

Results of Molecular Docking of Plants Compounds with HIV-1 target enzymes-

Docking Results of RT against GC-MS compounds of ***Ocimum gratissimum***and known Drugs-

#Ligand	Total Energy	VDW	HBond	AverConPair	Affinity
3hvt-PI atazanavir-0.pdb	-106.711	-83.1387	-23.5721	16.8627	106.711
3hvt-Structure2D_CID_12409-0.pdb	-101.112	-101.112	0	21.8387	101.112
3hvt-Structure2D_CID_14745-0.pdb	-96.731	-96.731	0	21.7931	96.731
3hvt-PI ritonavir-0.pdb	-90.8774	-89.3666	-1.51074	14.66	90.8774
3hvt-PI saquinavir-0.pdb	-74.3386	-65.2358	-9.10286	14.2653	74.3386
3hvt-Conformer3D_CID_6744-0.pdb	-74.0811	-74.0811	0	20.75	74.0811
3hvt-II raltegravir-0.pdb	-74.0491	-60.4569	-13.5922	18.6333	74.0491
3hvt-RTI abacavir sulfate-0.pdb	-68.9257	-54.2864	-14.6393	29.5238	68.9257
3hvt-PI amprenavir-0.pdb	-68.6608	-42.9135	-25.7473	15.2857	68.6608
3hvt-RTI zidovudine-0.pdb	-67.7416	-52.5057	-15.2359	21.7368	67.7416
3hvt-PI fosamprenavir-0.pdb	-67.2798	-65.8267	-1.45309	12.9487	67.2798
3hvt-RTI Rilpivirine-0.pdb	-67.0039	-63.5039	-3.5	16.8571	67.0039
3hvt-RTI Etravirine-0.pdb	-66.216	-50.6864	-15.5295	14.4286	66.216
3hvt-Conformer3D_CID_2117-0.pdb	-62.2524	-62.2524	0	20	62.2524
3hvt-PI nelfinavir-0.pdb	-62.2152	-55.8588	-6.35642	14.825	62.2152
3hvt-Conformer3D_CID_519857-0.pdb	-60.1869	-54.7879	-5.399	25.1875	60.1869
3hvt-RTI stavudine-0.pdb	-58.872	-35.6924	-23.1796	22.3125	58.872
3hvt-Conformer3D_CID_2237-0.pdb	-58.4775	-52.9346	-5.54296	17.2	58.4775
3hvt-Conformer3D_CID_1742210-0.pdb	-56.11	-56.11	0	23.0625	56.11
3hvt-PI indinavir-0.pdb	-56.0109	-53.0258	-2.98515	13.0889	56.0109
3hvt-PI darunavir-0.pdb	-55.5024	-55.5024	0	14.1579	55.5024
3hvt-Conformer3D_CID_1752-0.pdb	-54.3219	-49.4344	-4.88749	18.7619	54.3219
3hvt-PI tipranavir-0.pdb	-54.257	-43.757	-10.5	13.5526	54.257
3hvt-RTI lamivudine-0.pdb	-54.0768	-42.174	-11.9028	26.8667	54.0768
3hvt-Conformer3D_CID_520384-0.pdb	-53.6675	-53.6675	0	21.85	53.6675
3hvt-Structure2D_CID_18815-0.pdb	-53.575	-51.8515	-1.72349	23.5625	53.575
3hvt-RTI Efavirenz-0.pdb	-53.134	-53.134	0	20.7059	53.134
3hvt-Conformer3D_CID_2758-0.pdb	-52.6474	-52.6474	0	24.3333	52.6474
3hvt-Conformer3D_CID_12302844-0.pdb	-52.6205	-52.6205	0	25.8667	52.6205
3hvt-Conformer3D_CID_441005-0.pdb	-52.6026	-52.6026	0	24.8	52.6026
3hvt-Conformer3D_CID_6918391-0.pdb	-52.1443	-52.1443	0	23.4	52.1443
3hvt-Conformer3D_CID_442393-0.pdb	-51.9784	-51.9784	0	23.4667	51.9784
3hvt-Conformer3D_CID_1930-0.pdb	-51.6863	-51.6863	0	23.4667	51.6863
3hvt-Conformer3D_CID_12302131-0.pdb	-51.5121	-51.5121	0	22	51.5121
3hvt-Conformer3D_CID_91457-0.pdb	-51.1315	-42.6315	-8.5	26.5625	51.1315
3hvt-Conformer3D_CID_3327-0.pdb	-51.1202	-51.1202	0	23.2857	51.1202
3hvt-Conformer3D_CID_7127-0.pdb	-50.757	-50.0568	-0.70026	27.9231	50.757

3hvt-Conformer3D_CID_86609-0.pdb	-50.0965	-50.0965	0	25.2667	50.0965
3hvt-Conformer3D_CID_15560276-0.pdb	-50.0428	-50.0428	0	31.8667	50.0428
3hvt-Conformer3D_CID_3314-0.pdb	-49.5751	-47.7703	-1.80484	28	49.5751
3hvt-Conformer3D_CID_5281516-0.pdb	-49.3781	-49.3781	0	27.4667	49.3781
3hvt-Conformer3D_CID_17900-0.pdb	-49.0874	-45.4528	-3.63462	24.3333	49.0874
3hvt-Conformer3D_CID_23274265-0.pdb	-49.0154	-49.0154	0	23.1875	49.0154
3hvt-Conformer3D_CID_62566-0.pdb	-48.3269	-48.3269	0	23.6	48.3269
3hvt-Conformer3D_CID_12302222-0.pdb	-48.0701	-48.0701	0	21	48.0701
3hvt-Conformer3D_CID_64138-0.pdb	-47.9708	-47.9708	0	30	47.9708
3hvt-Conformer3D_CID_19725-0.pdb	-47.5746	-47.5746	0	24.2667	47.5746
3hvt-Conformer3D_CID_11230-0.pdb	-47.0098	-44.5098	-2.5	26.5455	47.0098
3hvt-Conformer3D_CID_10363-0.pdb	-46.9796	-41.7856	-5.194	27.2727	46.9796
3hvt-Conformer3D_CID_5281515-0.pdb	-46.7487	-46.7487	0	25.4667	46.7487
3hvt-Conformer3D_CID_440967-0.pdb	-46.0269	-43.1581	-2.86881	13.7941	46.0269
3hvt-Conformer3D_CID_62387-0.pdb	-45.9762	-45.9762	0	22.5385	45.9762
3hvt-Conformer3D_CID_6448-0.pdb	-45.4517	-42.9991	-2.45256	24.6429	45.4517
3hvt-Conformer3D_CID_17100-0.pdb	-44.9556	-38.9556	-6	28.0909	44.9556
3hvt-Conformer3D_CID_5317570-0.pdb	-44.766	-44.766	0	19.4	44.766
3hvt-Conformer3D_CID_5281553-0.pdb	-43.4257	-43.4257	0	28.2	43.4257
3hvt-Conformer3D_CID_101629835-0.pdb	-43.3986	-43.3986	0	29.1	43.3986
3hvt-Conformer3D_CID_31253-0.pdb	-43.2088	-43.2088	0	26.7	43.2088
3hvt-Conformer3D_CID_7461-0.pdb	-42.7894	-42.7894	0	34.5	42.7894
3hvt-Conformer3D_CID_6549-0.pdb	-42.2563	-40.439	-1.81733	25.4545	42.2563
3hvt-Conformer3D_CID_18818-0.pdb	-42.2377	-42.2377	0	28.4	42.2377
3hvt-Conformer3D_CID_11463-0.pdb	-41.8923	-41.8923	0	24	41.8923
3hvt-Conformer3D_CID_92138-0.pdb	-41.4412	-36.2605	-5.18065	20	41.4412
3hvt-Conformer3D_CID_64685-0.pdb	-41.3977	-38.8977	-2.5	24.3636	41.3977
3hvt-Conformer3D_CID_12389-0.pdb	-41.3359	-41.3359	0	26.4545	41.3359
3hvt-Conformer3D_CID_2537-0.pdb	-39.8475	-35.2844	-4.56307	26	39.8475
3hvt-Structure2D_CID_440666-0.pdb	-38.6579	-38.6579	0	30.9	38.6579
3hvt-Conformer3D_CID_9018-0.pdb	-38.1477	-38.1477	0	21.6923	38.1477
3hvt-Conformer3D_CID_1105-0.pdb	-36.6295	-36.6295	0	26.1	36.6295
3hvt-Conformer3D_CID_64139-0.pdb	-35.7324	-35.7324	0	25.9	35.7324
3hvt-Conformer3D_CID_6616-0.pdb	-34.3292	-34.3292	0	23.9	34.3292

Docking Results of PR against GC-MS compounds of ***Ocimum gratissimum*** and known Drugs-

#Ligand	**Total Energy**	**VDW**	**HBond**	**AverConPair**	**Affinity**
1dmp-PI ritonavir-0.pdb	-109.458	-103.475	-5.9827	14.82	109.458
1dmp-PI atazanavir-0.pdb	-104.064	-96.8583	-7.20554	13.3137	104.064
1dmp-Conformer3D_CID_6744-0.pdb	-101.495	-101.495	0	19.5	101.495

1dmp-PI amprenavir-0.pdb	-90.4747	-81.8602	-8.61449	18.8286	90.4747
1dmp-PI saquinavir-0.pdb	-85.8884	-81.0293	-4.85911	14.6122	85.8884
1dmp-Structure2D_CID_12409-0.pdb	-82.4555	-77.9005	-4.55497	21.5161	82.4555
1dmp-PI tipranavir-0.pdb	-81.5099	-64.4023	-17.1076	15.5	81.5099
1dmp-Conformer3D_CID_2237-0.pdb	-74.8145	-72.4332	-2.38122	20.9667	74.8145
1dmp-Structure2D_CID_14745-0.pdb	-74.6115	-74.6115	0	17.8276	74.6115
1dmp-Conformer3D_CID_2117-0.pdb	-68.0845	-68.0845	0	22.7895	68.0845
1dmp-RTI abacavir sulfate-0.pdb	-67.3849	-51.9154	-15.4695	22.2857	67.3849
1dmp-PI indinavir-0.pdb	-67.1187	-49.5147	-17.604	13.3111	67.1187
1dmp-II raltegravir-0.pdb	-66.723	-58.0192	-8.70375	14.3333	66.723
1dmp-RTI Etravirine-0.pdb	-63.995	-54.7595	-9.2355	16.7143	63.995
1dmp-RTI zidovudine-0.pdb	-63.3063	-43.5097	-19.6654	18.8947	63.3063
1dmp-Conformer3D_CID_1752-0.pdb	-62.5694	-55.5696	-6.99975	23.619	62.5694
1dmp-PI darunavir-0.pdb	-60.516	-53.1951	-7.32091	13.6579	60.516
1dmp-Conformer3D_CID_12302222-0.pdb	-60.3807	-49.2271	-11.1536	22.875	60.3807
1dmp-RTI lamivudine-0.pdb	-59.9081	-50.3922	-9.51587	22.4	59.9081
1dmp-RTI Efavirenz-0.pdb	-59.8824	-45.6399	-14.2425	24.3529	59.8824
1dmp-RTI Rilpivirine-0.pdb	-59.3746	-44.6096	-14.765	14.1071	59.3746
1dmp-PI fosamprenavir-0.pdb	-57.3958	-42.9879	-11.9081	14.4615	57.3958
1dmp-Conformer3D_CID_92138-0.pdb	-57.0271	-51.0271	-6	21.875	57.0271
1dmp-Structure2D_CID_18815-0.pdb	-56.9323	-49.9323	-7	20.5625	56.9323
1dmp-Conformer3D_CID_7127-0.pdb	-56.7127	-45.2918	-11.4208	25.6154	56.7127
1dmp-Conformer3D_CID_17900-0.pdb	-56.6079	-43.0963	-13.5115	24.9167	56.6079
1dmp-Conformer3D_CID_520384-0.pdb	-56.3073	-50.7176	-5.58968	19.7	56.3073
1dmp-Conformer3D_CID_91457-0.pdb	-56.2818	-49.5956	-6.68619	22.4375	56.2818
1dmp-RTI stavudine-0.pdb	-56.1361	-49.2055	-6.9306	22.8125	56.1361
1dmp-PI nelfinavir-0.pdb	-55.9618	-45.6113	-10.3506	13.125	55.9618
1dmp-Conformer3D_CID_441005-0.pdb	-53.2762	-53.2762	0	24.0667	53.2762
1dmp-Conformer3D_CID_1930-0.pdb	-53.1052	-53.1052	0	24.4	53.1052
1dmp-Conformer3D_CID_62566-0.pdb	-52.4108	-52.4108	0	23	52.4108
1dmp-Conformer3D_CID_6918391-0.pdb	-52.3092	-52.3092	0	23.2667	52.3092

1dmp-Conformer3D_CID_442393-0.pdb	-52.2367	-52.2367	0	22.9333	52.2367
1dmp-Conformer3D_CID_1742210-0.pdb	-52.0866	-47.1984	-4.88826	22.625	52.0866
1dmp-Conformer3D_CID_12302131-0.pdb	-52.0195	-52.0195	0	22.4667	52.0195
1dmp-Conformer3D_CID_86609-0.pdb	-51.7906	-51.7906	0	21.9333	51.7906
1dmp-Conformer3D_CID_5281516-0.pdb	-51.2779	-51.2779	0	22.8	51.2779
1dmp-Conformer3D_CID_23274265-0.pdb	-51.1799	-47.4928	-3.68715	20.5	51.1799
1dmp-Conformer3D_CID_19725-0.pdb	-49.4943	-49.4943	0	22.1333	49.4943
1dmp-Conformer3D_CID_3327-0.pdb	-49.4067	-49.4067	0	23.7143	49.4067
1dmp-Conformer3D_CID_2758-0.pdb	-49.2165	-49.2165	0	22.2	49.2165
1dmp-Conformer3D_CID_519857-0.pdb	-49.1188	-43.3047	-5.81407	21.1875	49.1188
1dmp-Conformer3D_CID_15560276-0.pdb	-48.9389	-48.9389	0	22.1333	48.9389
1dmp-Conformer3D_CID_440967-0.pdb	-48.5879	-48.5879	0	14.4706	48.5879
1dmp-Conformer3D_CID_12302844-0.pdb	-48.2932	-48.2932	0	20.3333	48.2932
1dmp-Conformer3D_CID_5281515-0.pdb	-47.9867	-47.9867	0	21.1333	47.9867
1dmp-Conformer3D_CID_3314-0.pdb	-47.2277	-36.5617	-10.666	24.4167	47.2277
1dmp-Conformer3D_CID_5317570-0.pdb	-46.7533	-46.7533	0	24	46.7533
1dmp-Conformer3D_CID_6448-0.pdb	-45.7107	-40.7107	-5	20.7143	45.7107
1dmp-Conformer3D_CID_6549-0.pdb	-44.1472	-39.7902	-4.35704	23	44.1472
1dmp-Conformer3D_CID_11230-0.pdb	-43.8664	-36.5329	-7.3335	24.3636	43.8664
1dmp-Conformer3D_CID_10363-0.pdb	-42.6259	-37.2057	-5.42024	21.9091	42.6259
1dmp-Conformer3D_CID_2537-0.pdb	-42.512	-36.7965	-5.7155	21.3636	42.512
1dmp-Conformer3D_CID_62387-0.pdb	-41.876	-35.8602	-6.0158	20.9231	41.876
1dmp-Conformer3D_CID_17100-0.pdb	-41.4773	-34.8632	-6.61406	24.8182	41.4773

1dmp-Conformer3D_CID_9018-0.pdb	-40.649	-40.649	0	20.2308	40.649
1dmp-Conformer3D_CID_64685-0.pdb	-40.3693	-32.057	-8.31233	22.8182	40.3693
1dmp-Conformer3D_CID_18818-0.pdb	-39.2232	-39.2232	0	24.1	39.2232
1dmp-Conformer3D_CID_11463-0.pdb	-38.9245	-38.9245	0	24.7	38.9245
1dmp-Conformer3D_CID_7461-0.pdb	-38.8552	-38.8552	0	23.6	38.8552
1dmp-Conformer3D_CID_6616-0.pdb	-38.5745	-38.5745	0	25.1	38.5745
1dmp-Conformer3D_CID_31253-0.pdb	-38.0575	-38.0575	0	24.1	38.0575
1dmp-Conformer3D_CID_1105-0.pdb	-37.4449	-37.4449	0	23.8	37.4449
1dmp-Structure2D_CID_440666-0.pdb	-36.9342	-36.9342	0	24.5	36.9342
1dmp-Conformer3D_CID_12389-0.pdb	-36.9283	-36.9268	-0.00155	21.3636	36.9283
1dmp-Conformer3D_CID_5281553-0.pdb	-36.6185	-36.6185	0	22.6	36.6185
1dmp-Conformer3D_CID_64138-0.pdb	-36.0169	-36.0169	0	24.2	36.0169
1dmp-Conformer3D_CID_64139-0.pdb	-35.8181	-35.8181	0	23.5	35.8181
1dmp-Conformer3D_CID_101629835-0.pdb	-34.2378	-34.2378	0	21	34.2378

Docking Results of INT against GC-MS compounds of ***Ocimum gratissimum***and known Drugs-

#Ligand	TotalEnergy	VDW	HBond	Elec	AverConPair	Affinity
3lpt-Structure2D_CID_12409-0.pdb	-108.639	-108.639	0	0	20.6452	108.639
3lpt-PI saquinavir-0.pdb	-98.3394	-84.5608	-13.7786	0	13.3265	98.3394
3lpt-PI ritonavir-0.pdb	-94.2226	-94.2226	0	0	15.26	94.2226
3lpt-Structure2D_CID_14745-0.pdb	-90.9257	-90.9257	0	0	18.8621	90.9257
3lpt-Conformer3D_CID_6744-0.pdb	-81.1868	-81.1868	0	0	13.85	81.1868
3lpt-PI tipranavir-0.pdb	-76.5653	-76.5653	0	0	14.6316	76.5653
3lpt-PI atazanavir-0.pdb	-75.7894	-66.5636	-9.22577	0	13.1176	75.7894
3lpt-RTI Etravirine-0.pdb	-74.0026	-65.3068	-8.69577	0	15.8929	74.0026
3lpt-Conformer3D_CID_2117-0.pdb	-68.7086	-68.7086	0	0	21.4211	68.7086
3lpt-RTI Rilpivirine-0.pdb	-68.5171	-56.7257	-11.7913	0	17.8929	68.5171

3lpt-PI indinavir-0.pdb	-68.1244	-65.3368	-2.78758	0	12.7778	68.1244
3lpt-II raltegravir-0.pdb	-64.6281	-59.4472	-5.18096	0	13.6	64.6281
3lpt-RTI lamivudine-0.pdb	-63.0723	-43.7818	-19.2905	0	24.6667	63.0723
3lpt-PI amprenavir-0.pdb	-61.4115	-55.8206	-5.59091	0	15.3429	61.4115
3lpt-RTI zidovudine-0.pdb	-60.8377	-57.6496	-3.5	0.311878	23.3158	60.8377
3lpt-RTI stavudine-0.pdb	-60.8361	-46.8637	-13.9724	0	27.6875	60.8361
3lpt-Conformer3D_CID_2237-0.pdb	-57.7553	-57.7553	0	0	14.6667	57.7553
3lpt-RTI abacavir sulfate-0.pdb	-57.5325	-51.7902	-5.74222	0	19.3333	57.5325
3lpt-PI fosamprenavir-0.pdb	-56.9151	-53.4151	-3.5	0	13.2051	56.9151
3lpt-Conformer3D_CID_440967-0.pdb	-54.4088	-52.5422	-1.86665	0	12.7647	54.4088
3lpt-Conformer3D_CID_5281516-0.pdb	-53.2866	-53.2866	0	0	23.8	53.2866
3lpt-PI darunavir-0.pdb	-53.0902	-45.3057	-7.7845	0	14.6316	53.0902
3lpt-Structure2D_CID_18815-0.pdb	-53.0526	-45.5956	-7.45705	0	22.0625	53.0526
3lpt-Conformer3D_CID_19725-0.pdb	-51.8324	-51.8324	0	0	32.6	51.8324
3lpt-Conformer3D_CID_17900-0.pdb	-51.7958	-35.1041	-16.6917	0	25.4167	51.7958
3lpt-Conformer3D_CID_64138-0.pdb	-51.0339	-51.0339	0	0	33.6	51.0339
3lpt-Conformer3D_CID_12389-0.pdb	-50.9938	-50.9938	0	0	32.1818	50.9938
3lpt-PI nelfinavir-0.pdb	-50.5877	-49.8758	-0.7119	0	13.3	50.5877
3lpt-Conformer3D_CID_1105-0.pdb	-50.4848	-50.4848	0	0	31.5	50.4848
3lpt-Conformer3D_CID_92138-0.pdb	-50.4314	-42.3593	-8.07206	0	21.125	50.4314
3lpt-Conformer3D_CID_441005-0.pdb	-50.3386	-50.3386	0	0	24.0667	50.3386
3lpt-Conformer3D_CID_5281515-0.pdb	-49.8815	-49.8815	0	0	26.2667	49.8815
3lpt-Conformer3D_CID_62566-0.pdb	-49.7642	-49.7642	0	0	26.0667	49.7642
3lpt-Conformer3D_CID_3314-0.pdb	-49.5828	-46.4122	-3.17068	0	29.8333	49.5828
3lpt-Conformer3D_CID_2537-0.pdb	-49.1317	-45.9262	-3.20546	0	32.1818	49.1317
3lpt-Conformer3D_CID_64685-0.pdb	-48.9166	-46.3248	-2.59181	0	31.5455	48.9166
3lpt-Conformer3D_CID_519857-0.pdb	-48.6308	-43.75	-4.88086	0	24.0625	48.6308

3lpt-Conformer3D_CID_11230-0.pdb	-48.5382	-46.3782	-2.15997	0	29.0909	48.5382
3lpt-Conformer3D_CID_64139-0.pdb	-48.0815	-48.0815	0	0	32.3	48.0815
3lpt-Conformer3D_CID_1752-0.pdb	-47.7485	-44.2485	-3.5	0	15.3333	47.7485
3lpt-Conformer3D_CID_62387-0.pdb	-47.4103	-44.9735	-2.43678	0	24.7692	47.4103
3lpt-Conformer3D_CID_12302222-0.pdb	-47.4045	-41.3121	-6.09248	0	18.6875	47.4045
3lpt-Conformer3D_CID_6448-0.pdb	-47.3927	-47.3927	0	0	31.1429	47.3927
3lpt-Conformer3D_CID_6918391-0.pdb	-46.1223	-46.1223	0	0	20.4667	46.1223
3lpt-Conformer3D_CID_12302844-0.pdb	-46.0572	-46.0572	0	0	21.2	46.0572
3lpt-Conformer3D_CID_101629835-0.pdb	-45.7977	-45.7977	0	0	33.2	45.7977
3lpt-Conformer3D_CID_18818-0.pdb	-45.7644	-45.7644	0	0	31.6	45.7644
3lpt-Conformer3D_CID_91457-0.pdb	-45.7199	-40.7199	-5	0	21.8125	45.7199
3lpt-Conformer3D_CID_17100-0.pdb	-45.4563	-42.5746	-2.88167	0	25.2727	45.4563
3lpt-Conformer3D_CID_15560276-0.pdb	-45.2932	-45.2932	0	0	22.2	45.2932
3lpt-Conformer3D_CID_10363-0.pdb	-45.2685	-42.1196	-3.1489	0	28.5455	45.2685
3lpt-Conformer3D_CID_1930-0.pdb	-45.1162	-45.1162	0	0	19.8	45.1162
3lpt-RTI Efavirenz-0.pdb	-44.9601	-32.4867	-12.4735	0	15.1176	44.9601
3lpt-Conformer3D_CID_12302131-0.pdb	-44.4537	-44.4537	0	0	20	44.4537
3lpt-Structure2D_CID_440666-0.pdb	-44.3779	-44.3779	0	0	32.3	44.3779
3lpt-Conformer3D_CID_7127-0.pdb	-44.2921	-43.5715	-0.7206	0	26.7692	44.2921
3lpt-Conformer3D_CID_1742210-0.pdb	-44.1652	-44.1652	0	0	26.1875	44.1652
3lpt-Conformer3D_CID_23274265-0.pdb	-44.0651	-44.0651	0	0	18.625	44.0651

3lpt-Conformer3D_CID_520384-0.pdb	-43.9882	-40.8493	-3.13892	0	19.05	43.9882
3lpt-Conformer3D_CID_11463-0.pdb	-43.3943	-43.3943	0	0	30.5	43.3943
3lpt-Conformer3D_CID_6616-0.pdb	-42.5071	-42.5071	0	0	32.9	42.5071
3lpt-Conformer3D_CID_31253-0.pdb	-42.4714	-42.4714	0	0	29.3	42.4714
3lpt-Conformer3D_CID_86609-0.pdb	-41.6208	-41.6208	0	0	19.8	41.6208
3lpt-Conformer3D_CID_2758-0.pdb	-41.4825	-41.4825	0	0	23.2667	41.4825
3lpt-Conformer3D_CID_5281553-0.pdb	-40.9654	-40.9654	0	0	28.6	40.9654
3lpt-Conformer3D_CID_9018-0.pdb	-40.8335	-40.8335	0	0	19.6923	40.8335
3lpt-Conformer3D_CID_6549-0.pdb	-40.4837	-28.7044	-11.7793	0	21.6364	40.4837
3lpt-Conformer3D_CID_3327-0.pdb	-40.0196	-40.0196	0	0	19.3571	40.0196
3lpt-Conformer3D_CID_5317570-0.pdb	-38.4677	-38.4677	0	0	24.9333	38.4677
3lpt-Conformer3D_CID_442393-0.pdb	-38.2663	-38.2663	0	0	20.2667	38.2663
3lpt-Conformer3D_CID_7461-0.pdb	-37.4088	-37.4088	0	0	33.9	37.4088

The docking results show some compounds are interacting with HIV-1 RT as efficiently as some Anti-HIV-1 drugs. The Atazanavir ($C_{38}H_{52}N_6O_7$, CAS ID: 198904-31-3, Molar mass: 704.856 g/mol, Trade name: Reyataz, Evotaz, others) interacts with HIV-1 RT with highest affinity (106.711). Whereas Efavirenz ($C_{14}H_9ClF_3NO_2$, CAS ID: 154598-52-4, Molar mass: 315.675 g/mol) interacts with an affinity of 53.134.

Many Plant compounds lie in between these range, which could be regarded as strong binder for HIV-1 RT. It includes CID-14745 that is 3-isopropoxycholest-5-ene ($C_{30}H_{52}O$, Molecular Weight: 428.745 g/mol), CID-1742210 that is Caryophyllene oxide ($C_{15}H_{24}O$, Molecular Weight: 220.356 g/mol) and CID-520384 that is beta-Thujene ($C_{10}H_{16}$, Molecular Weight: 136.238 g/mol). These compounds are potential Anti-HIV-1 targets.

These three compounds are selected and again docked with some cellular homeobox proteins and cancer suppressing proteins namely 2mwy- Cellular tumor antigen p53, 5vzu- S-phase kinase-associated protein 1, 4bhb- O-6-methylguanine-DNA methyl transferase, 2dat- SWI/SNF, 2e2w- BRCT domain of human DNA ligase IV, 4nrv- Crystal Structure of non-edited human NEIL1 to check whether they can cause any harmful effect in the cell or not. They only interacted very

weakly with the selected proteins. This indicates they are potential Anti-HIV-1 RT drug candidates. Here is the result of that docking-

#Ligand	TotalEnergy	VDW	HBond	AverConPair	Affinity
model1_2dat-Structure2D_CID_14745-0.pdb	-68.666	-68.666	0	17.4828	68.666
model1_2e2w-Structure2D_CID_14745-0.pdb	-75.4676	-75.4676	0	17.8621	75.4676
model1_2mwy-Structure2D_CID_14745-0.pdb	-70.2339	-70.2339	0	16.5172	70.2339
4bhb-Structure2D_CID_14745-0.pdb	-79.6	-79.6	0	16.7241	79.6
4nrv-Structure2D_CID_14745-0.pdb	-66.5335	-66.5335	0	16.931	66.5335
5vzu-Structure2D_CID_14745-0.pdb	-56.8326	-56.8326	0	15.8966	56.8326
model1_2dat-Conformer3D_CID_1742210-0.pdb	-37.4437	-37.4437	0	22	37.4437
model1_2mwy-Conformer3D_CID_1742210-0.pdb	-35.8943	-32.3943	-3.5	19.25	35.8943
4bhb-Conformer3D_CID_1742210-0.pdb	-43.619	-43.619	0	22.375	43.619
4nrv-Conformer3D_CID_1742210-0.pdb	-34.3079	-29.7683	-4.53957	20.4375	34.3079
5vzu-Conformer3D_CID_1742210-0.pdb	-42.5248	-39.0248	-3.5	24.5	42.5248
model1_2dat-Conformer3D_CID_520384-0.pdb	-46.3624	-46.3624	0	19.35	46.3624
model1_2mwy-Conformer3D_CID_520384-0.pdb	-37.4487	-34.7326	-2.71609	20.05	37.4487
4bhb-Conformer3D_CID_520384-0.pdb	-40.7397	-38.9137	-1.82604	18.75	40.7397
4nrv-Conformer3D_CID_520384-0.pdb	-27.1509	-24.4077	-2.74322	18.25	27.1509
5vzu-Conformer3D_CID_520384-0.pdb	-3.42096	2.59487	-6.01583	20.45	3.42096

Many compounds also very strongly interacted with the HIV-1 PR and HIV-1 INT. But HIV-1 RT was selected as our target HIV-1 enzyme because in the next phages of the study plant components can be tested with HIV-1 RT in in-vitro assays. In absence of efficient in-vitro INT and PR assays evaluating plant components against those enzymes are not possible yet in this project.

Protein production purification and in-vitro assay

Both the HIV-1 RT51 and HIV-1 RT66 was cloned in *E. coli* strain *BL21(DE3)* in vector *pET-28-a* under an IPTG inducible promoter and Kanamycin selection gene and fused with six Histidine tags. The next process was done separately for RT51 and RT66 clones. For RT production first and 5ml culture was seeded in liquid LB media and kept in 37°C from a stab of stock culture that was stored in -80°C. From the seed culture primary culture was seeded and kept in shaker at 37°C. After 3 hours secondary culture was seeded in 4 250ml culture in 1 lit conical flask. The culture was kept in shaker at 37°C. OD of the secondary culture was checked in a spectrophotometer at 600nm wavelength until the OD reached 0.6. Both the primary and secondary culture was maintained in presence of Kanamycin to reduce any chance of contamination. After the secondary culture reached an early log phase (OD of 0.6 in 600nm) IPTG was added for the induction and the secondary culture was kept in a shaker for 10-12 hours at 28°C.Before induction little amount of sample collected in an Eppendorf tube to check unindicted protein in SDS-PAGE. Then the cells were harvested from the secondary culture by

centrifugation at 6000rpm for 6mint. Then the cell pellet are washed with Pellet Wash Buffer. Little amount of sample collected in an Eppendorf tube to check if protein production was induced in SDS-PAGE. Then a SDS-PAGE was performed to check the induction of protein. BSA was used as a standard. The induced protein appears as large band in the gel. Then the cells are sonicated using Cell lysis buffer. 50 cycles of 10sec pulse with 10sec gap of midrange sonication was used for cell lysis. After the cells lysis was done the debris are pelleted down using centrifugation at 13000rpm for 15min twice and the soup was collected and kept in ice. The soup contains our protein of interest RT51 and RT66 that are fused with Hexa-HIS tags.

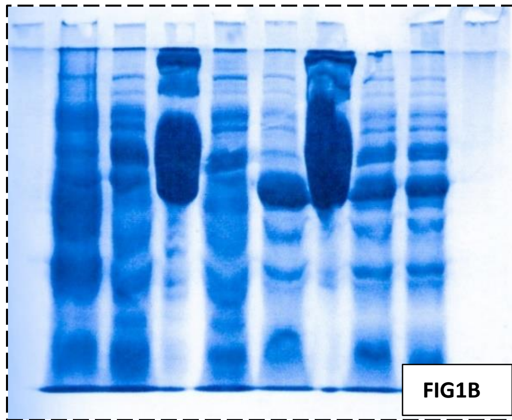
The proteins are then purified using Nickel immobilized metal affinity chromatography. First a small column was taken and sepharose beads were loaded. The beads were first washed using water, EDTA and ethanol. Then the bead was recharged using Nickel Hydroxide and then binding Buffer was added and kept for sometimes to allow the nickel to bind with the beads. Then the proteins isolated was taken in a falcon tube, the recharged beads were mixed with the protein and kept overnight on a rocker so that the hexa-his tagged proteins can bind with the beads. The whole process of column recharge and protein purification was done in a cold room at 4°C. After protein binding the protein bead mix was loaded into the column and let the column run for the beads to get packed. The flow through was collected and checked in a SDS-PAGE. Then binding buffer was added. After it washing off of other proteins were performed by applying washing buffer. The samples are checked in a SDS-PAGE. After proper washing elution buffer was added and the eluted proteins were collected in Eppendorf tube. The eluted samples were checked in a SDS-PAGE. After checking the eluted samples were taken in a dialysis membrane and kept in dialysis buffer-1 for overnight and dialysis buffer-2 for 3hours for concentrating the isolated proteins. The proteins were collected checked in SDS-PAGE and kept in -20°C for future use.

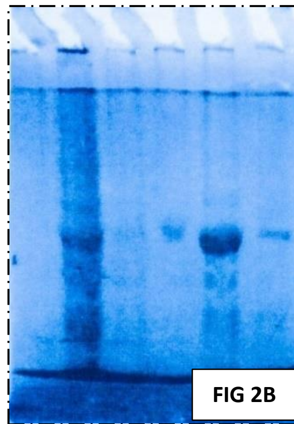
To check the RT activity RNA was produced using an in-vitro transcription procedure. The purified RNA was then used along the isolated RT66 to run a RT PCR. The result of RT PCR was positive indicating the isolated and purified RT66 was functional.

To evaluate the crude extracts from plants and other drugs for their anti-HIV-1 activity setting up an in-vitro assay was necessary. The final optimization process was initiated for the pre-established fluorescence based pico-green assay. But due to some unforeseen circumstances the pico-green assay was not completed. So, a radioactivity-based primer extension assay was proposed. But the initial setting up procedure of this assay took very long and the process needed to be first optimized well. So due to lack of time the in-vitro assays were not performed.

The quantification of Proteins and the results of SDS-PAGEs that were performed during the protein production and protein purification are given in next pages of this project.

Following the induction with IPTG, SDS-PAGE results are obtained after negative staining of the gel as shown below. BSA (0.5 µg/µl) is taken as the standard. RT was quantified using Bradford assay, BSA (0.5 µg/µl) is taken as the standard.





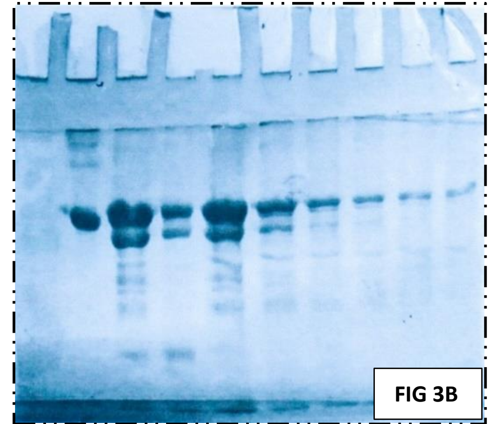


FIG1B. Gel showing induction with IPTG. Lane 1: 1x dye, Lane 2: RT66 (uninduced), Lane 3: RT66 (induced), Lane 4: BSA, Lane 5: RT51 (Uninduced), Lane 6: RT51 (Induced), Lane 7: BSA, Lane 8 & 9: RT51+RT66

FIG2B. Gel showing Flow through in Lane 1, 2 and 3. Lane 4: BSA, Lane 5: Last elution

FIG3B. Gel showing Elution. Lane 1: 1x dye, Lane 2: BSA, Lane 3-10: Every Odd elution.

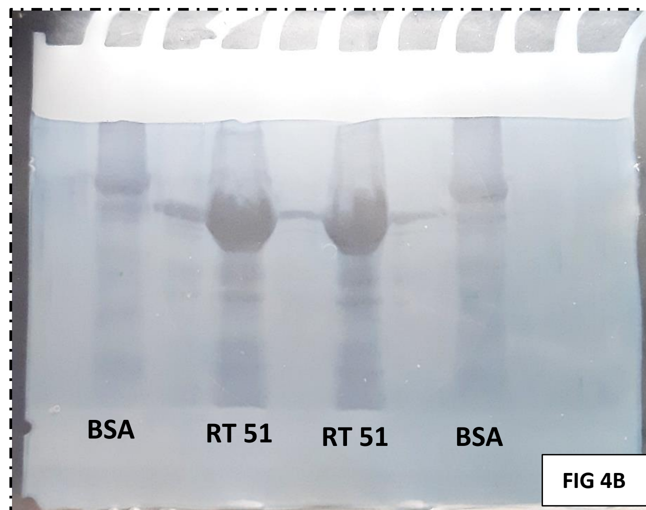
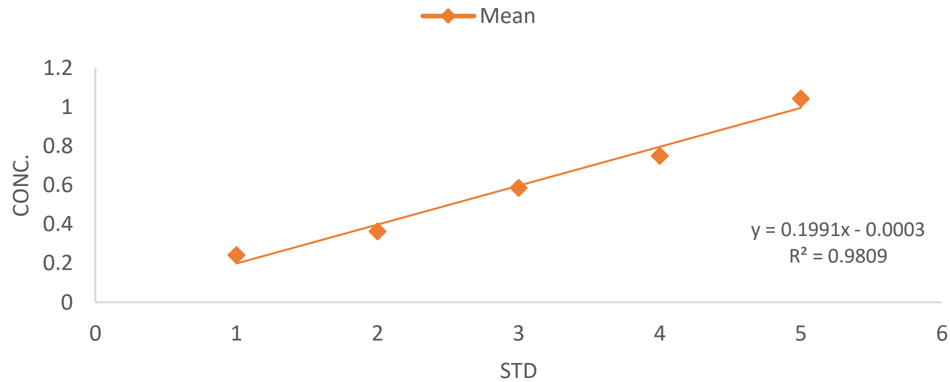


FIG 4B. Gel showing RT51 after second dialysis. Lane 2, 8: BSA, Lane 4, 6: RT51

Protein Quantification-

Standard Curve is prepared using known concentrations of BSA. The Quantification is given below.

Std	Conc	Well	Replicates	Mean	SD	%CV
1	0.5	F12	0.173	0.242	0.111	45.73
		G12	0.183			
		H12	0.37			
2	1	F11	0.367	0.363	0.068	18.82
		G11	0.293			
		H11	0.43			
3	2	F10	0.403	0.586	0.107	21.15
		G10	0.497			
		H10	0.617			
4	4	F9	0.803	0.75	0.174	24.73
		G9	0.807			
		H9	0.503			
5	8	F8	1.043	1.044	0.035	3.352
		G8	1.08			
		H8	1.01			



Standard curve was generated from the values obtained from BSA standard solutions by plotting them in excel graph.

Plotting the values of unknown proteins (RT51. RT66) in the standard curve gives the quantity of the purified proteins.

Std	Conc	Well	Replicates	Mean	SD
SO1	**3.7**	C12	0.683	0.633	0.692
		D12	0.583		
SO2	5.819	C11	0.913	0.85	0.876
		D11	0.787		
SO1B	**4.2**	H5	0.804	0.78	0.179
		G5	0.807		
SO2B	6.127	C5	0.926	0.91	0.792
		D5	0.939		

SO1, SO2: RT51 1µg/ml and 2 µg/ml, SO1B, SO2B: RT66 1µg/ml and 2 µg/ml.

The concentration of RT51 is 3.7 µg/ml and the concentration of RT66 is 4.2 µg/ml.

Quantification of RNA-

For Picogreen based assay RNA was prepared and quantified through nanodrop spectrophotometer by using A260/A280. The concentration of RNA is 815.65 ng/µl.

RT PCR-

RT PCR was performed to check the activity of RT66. RT PCR worked and band of PCR product seen using running agarose gel electrophoresis.

Fluorescence based in-vitro Assay-

Initial optimization for Pico green assay was initiated but further experiment was not carried out due to some unexpected incidents.

Summary-

- Selection of plants was done.
- Collection of plants, drying, grinding and preparation for extraction was done.
- Cold extraction of dried plant samples with solvents of different polarities was done.
- Rotary Evaporation and drying of extracted crude was done.
- Crude drugs were weighed and dissolved in DMSO. Filter sterilization of dissolved crude was done.
- Cell containing pNL4-3 ΔEnv EGFP and pVSV-G was cultured and plasmid isolation was done with alkaline lysis method for co-transfection process.
- Pseudo virus production with pNL4-3 ΔEnv EGFP and pVSV-G was done in HEK293 T-cells using Calcium Phosphate transfection protocol.
- Produced virus used to infect Huh7.5 Cells.
- Cytotoxicity assay of crude extracts were performed.
- Plant crudes evaluated using Cell culture based assay to check reduction in GFP expression.
- *Ocimum gratissimum* methanolic extract, and *Tinospora sp.* petroleum benzene extract showed positive anti-HIV-1 activity, i.e. reduction in green foci under fluorescence microscope.
- Reporting of anti-HIV-1 activity in methanolic extract of *Swertia bimaculata* was confirmed.
- The anti-HIV-1 activity of *Ocimum* was much prominent than *Tinospora*. So further fractionation of methanolic Extract of *Ocimum* using column chromatography was performed.
- Cytotoxicity of each fraction was performed.
- E3M7 fraction (Separated with 30% Ethyl acetate and 70% Methanol) of methanolic extract of *Ocimum* showed positive anti-HIV-1 activity.
- Anti-HIV-1 activity was not detected in no other fractions as well as in no other crude extract tested.
- Thin Layer Chromatography or TLC was used for separation of E3M7. 3 Bands were detected and collected.
- GC-MS compounds of *Ocimum gratissimum* used as ligands and molecular docking was performed using iGemDock against HIV-1 RT (3hvt.pdb).
- Multiple compounds detected that interact with HIV-1 RT.
- HIV-1 RT51 and HIV-1 RT66 was isolated and purified for in-vitro assay.
- For protein production primary culture, secondary culture, cell harvesting, cell lysis, protein isolation, Ni-iMAC for protein purification was performed.
- Protein quantification was done.
- Dialysis of purified proteins were performed. And stored.
- To check RT activity, in-vitro transcription and RT PCR was performed. RT was active.
- Optimization for fluorescence based pico-green assay was performed but was not completed.
- Optimization for radioactivity based primer extension assay was initiated but was not completed due to lack of time.

Discussion-

The present therapy finds its limitations in the emergence of multidrug resistance and accordingly finding new drugs and novel targets are the need of the hour. Molecules from plants have enormous structural diversity which fits criteria of anti-HIV-1 molecules despite India's rich biodiversity very little exploration has been done in this field. Hence more plants should be screened for their anti-HIV-1 potential.

Crude extracts showing anti-HIV-1 activity can be purified and further studied to identify lead molecules and their mechanism of HIV-1 inhibition.

In this dissertation project six plants namely *Catharanthus roseus, Ocimum gratissimum, Mangifera indica, Tinospora sp., Solanum sp., Swertia bimaculata* were selected and screened in cell culture system for their potential anti-HIV-1 activity. The cell culture assay used here was a GFP expression-based assay. Where pseudovirus was first produced with pNL4-3 ΔEnv EGFP and pVSV-G. And it was used to infect Huh7.5 cells. The active proviral particles express GFP and that become visible in fluorescence microscopy. Cells were treated with different concentrations of drugs along with positive negative and shuttle control. The expression of GFP was then measured in the fluorescence microscope. Reduction in GFP expression from negative control indicates anti-HIV-1 activity by the drug. Among the tested crude extracts methanolic extract of *Ocimum gratissimum* has shown adequate anti-HIV-1 activity. Petroleum benzene fraction of *Tinospora* sp. has shown some anti-HIV-1 activity. And the previous report of anti-HIV-1 activity in a methanolic fraction of *Swertia bimaculata* has been confirmed. For the next phase of the work, *Ocimum gratissimum* was selected because of the easy availability of the material and the reduction in GFP in *Ocimum* was much more prominent than *Tinospora.* Subsequent fractionation of its methanolic crude extract was performed and each fraction was again evaluated for their anti-HIV-1 activity below its cytotoxic level. One fraction, E3M7 among twelve has shown adequate anti-HIV-1 activity. Thin layer chromatography was performed to identify components of this E3M7 fraction. Three bands were observed under UV. Bands were eluted and collected for further evaluation. But no further evaluation was performed due to lack of time.

For the next phase of the work in-silico studies was performed with GC-MS records of *Ocimum gratissimum*. The list of GC-MS compounds was made available from published reports [see materials and methods section] and was not performed in this project. A few molecules were detected to be interacting very strongly with HIV-1 RT, PR, and INT.

For the Next Phase of the study, HIV-1 RT was selected as the target due to the presence of established in-vitro RT Assays in the lab. For the next stages, E. coli cloned with HIV-1 RT51 and RT66 were cultured, harvested, RT51 and RT66 were isolated and purified using Nickel immobilized metal affinity chromatography. For in-vitro assay RNA isolation from Huh 7.5 cells were done. RT-PCR was performed. But the fluorescence-based pico-green assay was not completed due to some inevitable circumstances. So, work on radioactivity based primer extension assay was initiated. But primary optimization and troubleshooting of this assays require a sufficient span of time. But due to lack of time, it was not completed. Hence, I have to wrap up my dissertation project to whatever is done to date.

Evaluated plants showed anti-HIV-1 potential. In-silico analysis showed potential RT binders present in the plant. But whether the extracts that showed anti-HIV-1 activity in cell culture assay targets HIV-1 RT or it's any other enzyme was not identify. To evaluate if the target is RT requires in-vitro assay. And the reporting that is made based on the cell culture-based assays are needed to be confirmed in in-vitro assays. In the future, this project can be continued and further progress can be made.

The limitation of this project is the reporting of the planned crude and fractions having anti-HIV-1 activity is not confirmed using any in-vitro assays due to lack of time. And the target for the phytochemicals in HIV-1 life cycles is now known. The results of molecular docking indicate the site of interaction of phytochemicals present in the *Ocimum gratissimum* could be HIV-1 RT or INT or PR. The fractions that showed positive anti-HIV-1 activity was not analyzed through GC-MS. So, if the reported fractions that showed anti-HIV-1 activity in this project are the same that shows positive interaction with HIV-1 enzymes in the molecular docking is the same molecules or completely different molecules is not known.

The cell culture-based assay depends on the GFP expression and the evaluation of drug depends on the reduction of number in green foci under the fluorescence microscope. Although negative and positive controls, as well as shuttle controls with DMSO, were taken, the number of green foci can be variable in number for other reasons. And the reduction in the number of green foci was estimated solely with human observation in relation to its controls. Human error can interfere with the data.

No quantitative evaluation was done. Detestation in total fluorescence of each individual wells and could have been helpful in this aspect. But it was not done due to the absence of such instruments.

Evaluation of plant crudes and each fraction were though evaluated using in triplicates the results were not confirmed by evaluating plant materials from different places and different environmental conditions.

So results in this project are just initial reporting. These results are needed to be evaluated again with different plant materials. And the plant materials that showed positive anti-HIV-1 activity in this project are needed to be evaluated in in-vitro assays. The methanolic fraction of *Ocimum gratissimum* have potential anti-HIV-1 activity is reported in this project. And its further fractionation and evaluation of the individual fraction were done. And it is reported in this project the E3M7 fraction do have anti-HIV-1 activity. The E3M7 fraction was again separated using TLC and three bands were detected. Chemical analysis of the TLC plate may show other bands that were not visible in UV. The compounds from three bands were collected but not evaluated again. Evaluation of those bands and if an anti-HIV-1 activity is found in any of them then again further separation or analysis through GC-MS or HPTLC is required to identify the individual candidate molecule that interacts with the HIV.

Evaluation of *Tinospora sp*. also indicates it has some potential anti-HIV-1 activity. Though apparently, it seemed like the anti-HIV-1 activity of *Ocimum* was much more prominent than *Tinospora*. Thus no further fractionation and evaluation of the fractions were don for *Tinospora sp.* So that part of the work is left undone.

The anti-HIV-1 activity in methanolic fraction of *Swertia bimaculata* was initially reported by Mr. Vivek Gairola in the same lab [unpublished]. In this project, his work is again repeated and a positive result is detected. Hence this project confirms his findings.

The results in this project indicate that the evaluation of various medicinal plant can be helpful in detecting anti-HIV-1 activities of different phytochemicals as it is mentioned in the literature review section. So, more medicinally important plants are needed to be examined thoroughly for their anti-HIV-1 properties. Evaluation of natural compounds from naturally occurring medicinal plants or naturally occurring wild plants could be helpful in new therapeutic studies for the development of new anti-HIV-1 drugs. In this project, only six plants were evaluated and two of them indicates possible anti-HIV-1 activities. It suggests it is important that other medicinal plants and traditionally used plants are needed to be screened for their potential anti-HIV-1 activity.

Though evaluation of the plants for their potential anti-HIV-1 activity is only the beginning of the process. Further in-vitro studies and in-depth studies in an actual animal model is required for a meaningful therapeutic evaluation. If these kinds of studies are continued then potentially new drugs can be developed from the plant sources that can be incorporated in the HAART program as it is mentioned in the literature review. So, this project left open multiple areas in which further studies and further experiments are needed to back these findings up that are stated in this project.

Conclusion-

The problem with anti-HIV-1 drugs are constantly evolving anti-drug resistance variety of HIV-1 renders the drug useless after a certain period of time so constant research for new drug development is required. Traditional plant materials can be proven as a great source for new HIV-1 drug development. Already there are thousands of known traditional plants that possess some sort of medicinal importance. Some of those plants have anti-viral activities. So those medicinally active plants needed to be screened for their anti-HIV-1 activity. Multiple medicinally activated plants have already been screened for their anti-HIV-1 potentials and some of them had shown potential result and can be used in the next level of drug researches. Plants are source for naturally occurring thousands of molecules. Some of them may interact with the viral life cycles and can hinder it without hampering any cellular activities of the host. So many plants can be proven to be good anti-HIV-1 drug sources. Systematic studies designed to discover anti-HIV-1 drugs from plants are few in number, but proper evaluation of more plants are required.

The drugs that can be produced from the plant source will not definitely replace the HAART drugs and will obviously not replace the need for the development of anti-HIV-1 vaccines but will be helpful if incorporated in HAART program due to a constant need for new drugs. Israel and China had already invested millions on a project for developing a new anti-HIV-1 drug named ‘Gammora’ from a plant source. So far published reports on more than 50 plants shows a promising role of phytochemicals in the irradiation of HIV-1 virus and new drug development research can be started using those plants.

Although there are reporting of various naturally occurring phytochemicals as anti-HIV-1 components from different plants are made, not many serious efforts of chemotherapeutic

studies are performed on them. So in vivo testing and, ultimately, human clinical trials need to be carried out on key lead plants and phytochemical isolates. In addition, continuous evaluation of medicinal plants for the anti-HIV-1 activity should be pursued.

Another plant-based anti-HIV-1 research includes the development of GM crops to produce anti-HIV-1 drugs in plants. The pharma-planta program is already working on this project.

Another dimension in the plant-based anti-HIV-1 studies includes evaluation of the secondary metabolites produced by the endophytes of the plant. Already there are some reports of positive anti-HIV-1 activity found in secondary metabolites of endophytic bacteria and fungi of some desert plants. These kinds of studies can be very helpful because the secondary metabolites can be produced from the endophytes in large scale commercially viable fermenter if proper media formulation and process optimization can be done.

Development of a successful new drug after reporting of anti-HIV-1 components includes various steps that include in-vitro studies, trial in animals and at last stage human trials. In this process, multiple limitations can arise. The plants some time produces some components only in special conditions and only when they attain a special stage of development. So a reported phytochemical may or may not be produced by the plants all the time and everywhere so before going to the next level of study understanding the metabolic pathway that produces that particular phytochemical is necessary. Plants may produce those phytochemicals in them only in limited amount so new drug development study may not be commercially viable. Understanding these challenges is required before new drug development from any plant source. A better approach can be if possible a potent phytochemical with therapeutic activity can be brought in a microbial system through intensive molecular biology approach that includes metabolic engineering and then it can be produced in a commercially viable manner.

So developing anti-HIV-1 drugs from naturally occurring phytochemicals is not limited just to the evaluation of different plants for their anti-HIV-1 activity. Rather multiple fields of researches are open in this subject that needed to be explored in a holistic approach.

APPENDIX

- SDS-PAGE Resolving Gel (10%) -5ml

DD Water	1.9 ml
30% acrylamide mix	1.7 ml
1.5M Tris-Cl (PH 8.8)	1.3 ml
10% SDS	0.05 ml
10% APS	0.05 ml
TEMED	0.002 ml

- SDS-PAGE Stacking Gel (5%) -2ml

DD Water	1.4 ml
30% acrylamide mix	0.33 ml
1.0 M Tris-Cl (PH 6.8)	0.25 ml
10% SDS	0.02 ml
10% APS	0.02 ml
TEMED	0.002 ml

- Protein isolation and purification

Pellet Wash Buffer	200mM NaCl, 10mM Tris-Cl, pH 8.0
Binding Buffer	50mM Tris (PH 8.0), 500mM NaCl, 5mM imidazole
Lysis Buffer	Binding Buffer, 0.05% NP-40, 1mM PMSF, Lysozyme
Wash Buffer	20mM Tris (PH 7.5), 500mM NaCl, 75mM imidazole
Elution Buffer	50mM Tris (PH 7.5), 500mM NaCl, 500mM imidazole
Dialysis Buffer 1	50mM Tris-Cl, 100mM NaCl, 1mM EDTA, Glycerol 30%
Dialysis Buffer 2	50mM Tris-Cl, 100mM NaCl, 1mM EDTA, Glycerol 50%

- Primer Extension Assay

Polymerizing Buffer	120mM Tris-Cl, 120mM KCl, 16mM $MgCl_2$, 0.5M DTT, 100mM dTTPs, water to makeup 1ml
Reaction Buffer	50mM Tris-Cl (PH 7.8), 5mM $MgCl_2$, 60mM KCl, 100µg/ml BSA, 1mM DTT, 2µM dTTPs, 0.5µCi ^{3}HdTTPs, Poly rA-dT Template-Primer dimer, HIV-1 RT66, Water to makeup reaction volume

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