UNIVERSITY OF KWAZULU-NATAL ™

INYUVESI YAKWAZULU-NATALI

# The antiviral effects of the crude extract from endophytic fungi, *Alternaria alternata,* on different HIV-1 subtypes and Integrase drug-resistance strains


**By**

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# PREFACE

The research and experimental work described in this dissertation was conducted in the HIV Pathogenesis Programme, Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa from July 2022 to August 2024 while under the supervision of Dr Nompumelelo Mkhwanazi and co-supervisor Dr Sizwe Ndlovu. This study is an original work by the author and has not been submitted in any form for any degree or diploma to any University. Where use has been made of the work of others, it is duly acknowledged within the text.

# PLAGIARISM DECLARATION FORM

I, Darian Naidu, declare that.

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# DEDICATION

I dedicate this Thesis to my mother, father and brother for their unconditional love and support for me throughout my masters degree.

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# RESEARCH OUTPUTS

1. Oral presentation: University of KwaZulu-Natal, Annual research symposium. KRITH building. 15 August 2023.

2. Manuscript (Chapter 3): **J**ournal name: International Journal of Molecular Sciences
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# LIST OF ABREVIATIONS

| | |
|---|---|
| AZT | Azidothymidine |
| AME | Alternariol 5-*O*-methyl ether |
| bNabs | Broadly neutralizing antibodies |
| CA | Capsid |
| CAR | Chimeric antigen receptor |
| cART | Combination antiretroviral therapy |
| CCR5 | C-C chemokine receptor 5 |
| CD4 | Clusters of differentiation type 4 |
| CD8 | Cluster of differentiation type 8 |
| cDNA | Copy DNA |
| CE | Crude extract |
| CRISPR | Clustered regularly interspaced palindromic repeats |
| CXCR4 | C-X-C chemokine receptor 4 |
| DRM | Drug-resistance mutation |
| DNA | Deoxyribonucleic acid |
| dNTP | Deoxynucleotide triphosphate |
| ELISA | Enzyme-linked immunosorbent assay |
| ENV | Envelope |
| FDA | Food and drug administration |
| Gag | Group-specific antigen |
| GC-MS | Gas chromatography-mass spectrometry |
| GFP | Green fluorescent protein |
| HIV | Human Immunodeficiency Virus |
| HIV-1 | Human Immunodeficiency Virus type 1 |
| HIV-2 | Human Immunodeficiency Virus type 2 |
| HLB | Hydrophilic–Lipophilic Balance |
| IN | Integrase |
| INSTI | Integrase strand transfer inhibitor |
| LPA | Latency-promoting agent |
| LRA | Latency-reversing agent |

| | |
|---|---|
| MA | Matrix |
| MAX | Mixed-mode, strong Anion-eXchange |
| MCX | Mixed-mode, strong Cation-eXchange |
| NC | Nucleocapsid |
| Nef | Negative factor |
| NNRTI | Non-nucleoside reverse transcriptase inhibitor |
| NP | Natural product |
| NRTI | Nucleoside reverse transcriptase inhibitor |
| PI | Protease inhibitor |
| PLWH | People living with HIV |
| Pol | Polymerase |
| PR | Protease |
| PrEP | Pre-exposure prophylaxis |
| Rev | Regulator of the expression of virion proteins |
| RNA | Ribonucleic acid |
| RT | Reverse transcriptase |
| SM | Secondary metabolite |
| SPE | Solid phase extraction |
| Tat | Transactivator of transcription |
| TLD | Tenofovir, lamivudine and dolutegravir |
| TOA | Time of addition |
| Vif | Virion infectivity factor |
| Vpu | Viral protein U |
| WHO | World Health Organisation |

# ABSTRACT

**Background**: Antiretroviral drugs have successfully turned HIV/AIDS into a manageable chronic condition and increased life expectancy. The development of drug-resistance, side effects, toxicity and HIV subtype have impeded drug development and cure efforts. Thus, there is a demand of new strategies for developing novel antiretrovirals with decreased toxicity, potent anti-HIV activity, inhibitory activity across HIV subtypes, and activity against drug-resistant strains. This study investigated the anti-HIV effect of the fungal endophyte *Alternaria alternata* PO4PR2 crude extract (CE) on different HIV-1 subtypes and integrase drug-resistant strains.

**Materials and methods**: The secondary metabolites of *Alternaria alternata* PO4PR2 were isolated using methanolic extraction to prepare a CE. The MTT (3-[4,5-dimethylthiazol-2-yl]2,5 diphenyl tetrazolium bromide) assay was then used to determine the cell viability and cytotoxicity of the *A. alternata* crude extract on treated TZM-bl cells. Then, using site-directed mutagenesis, the integrase drug-resistance mutations T66K and S230R were introduced into HIV-1 molecular clone pNL4.3. The anti-HIV activity of the *A. alternata* crude extract against HIV-1 subtype A, B, C, D and integrase drug-resistant strains was then assessed using a luciferase-based antiviral assay. The viral replication stages inhibited by the *A. alternata* crude extract were determined using luciferase-based and p24 ELISA-based time of addition assays.

**Results**: The cell viability of TZM-bl cells treated with *A. alternata* crude extract was greater than 80%, and the CC50 of 300 µg/mL indicated that crude extract was not cytotoxic. The *A. alternata* crude extract inhibited the viral replication of HIV-1 subtypes A, B, C and D viruses with IC50's ranging between 0.10 and 0.60 µg/mL. The calculated SI values for the different subtype viruses were between 493 and 2921 showing therapeutic potential of the crude extract. The *A. alternata* crude extract inhibited the integrase drug-resistant strains T66K and S230R with IC50 of 0.1628 and 0. 0.1961 µg/mL with a 0.7265 and 0. 8751-fold increase in susceptibility to the CE. The *A. alternata* crude extract had activity during attachment (88% viral inhibition; HIV-1 p24 titre =10.38 pg/mL), reverse transcription (81% viral inhibition; HIV-1 p24 titre =13.74 pg/mL), integration (65% viral inhibition; HIV-1 p24 titre =17.74 pg/mL), and proteolysis (9% viral inhibition; HIV-1 p24 titre =49.32 pg/mL) stages of the HIV life cycle.

**Conclusion**: The *A. alternata* crude extract was not cytotoxic and had strong antiviral activity against subtype A, B, C, D and integrase drug-resistant strains. The *A. alternata* crude extract inhibited stages key to the life cycle of HIV. The compounds in *A. alternata* crude extract have potential to develop drug candidates that can inhibit different HIV subtype and drug-resistance.

# CHAPTER 1: INTRODUCTION

## 1.1 Background

Since the Human Immunodeficiency Virus (HIV) was discovered in 1981, there have been 39.9 million people living with HIV (PLWH), and 42.3 million people have succumbed to AIDS-related illnesses (UNAIDS., 2024). Nonetheless, the HIV/AIDS burden has decreased since antiretrovirals were introduced, with 30.7 million PLWH now having access to them in comparison to its scarcity at the beginning of the pandemic. HIV-1 antiretroviral drugs and pre-exposure prophylaxis (PrEP) have effectively reduced the morbidity rate in PLWH and prevented virus transmission (Barnabas *et al*., 2014; Fonner *et al*., 2016). Antiretroviral drugs, however, do not cure HIV, and their efficacy is constrained by the development of HIV subtypes, drug-resistance, viral reservoirs, and side effects (Bhatti *et al*., 2016; Kioko & Pertet, 2017; Hamers *et al*., 2018). Therefore, there is a need to develop new antiviral drugs with fewer drawbacks and susceptibility to drug-resistant mutants and different HIV-1 subtypes. Alternative therapeutic approaches, such as natural products (NPs), are proposed not to share the limitations of antiretroviral drugs (Mandal *et al*., 2020). This study investigates the anti-HIV properties and mode of action of crude extracts (CE) from *Alternaria alternata* against different HIV-1 subtypes and integrase strand transfer-resistant mutant strains.

Human immunodeficiency viruses are divided into type 1 and type 2. Type 1 is further divided into four groups: M, N, O, and P (Bbosa *et al*., 2019). Group M is dominant and comprises nine subtypes: A, B, C, D, F, G, H, J and K (Taylor *et al*., 2008; Bbosa *et al*., 2019). The subtype of HIV-1 must be considered while administering antiretroviral therapy to PLWH since genetic variation influences the development of drug-resistance and susceptibility to antiretrovirals (Lessells *et al*., 2012). Antiretroviral drug resistance reduces the susceptibility of currently available drugs against HIV-1, which limits treatment options for PLWH (Phillips *et al*., 2017).

Drug-resistant mutants have been reported in HIV-1 subtypes A, B, C and D (Soares *et al*., 2007; Ayitewala *et al*., 2020). Furthermore, integrase strand transfer (INSTI) drug-resistance mutations that are resistant to approved integrase inhibitors have been documented in HIV-1 subtype A, C, D, F, G, H, and I (Monleau *et al*., 2012; Han *et al*., 2016; Inzaule *et al*., 2018; Ahmed *et al*., 2019). Therefore, antiviral strategies that can escape the current limitations, such as the emergence of resistant HIV strains, remain a high priority in drug development strategies for the future.

Natural products (NPs) are being investigated as potential alternatives to antiretrovirals to develop new drug choices for PLWHs that can target even drug-resistant strains (Mandal *et al*., 2020). Natural products are chemical compounds found in bacterial, fungal, plant and marine organisms with a broader range of biological activities (Katz & Baltz, 2016). They can be broadly defined as chemicals produced by living organisms (Sorokina & Steinbeck, 2020). Furthermore, bioactive compounds from natural products exhibit high structural hydrophobicity and rigidity, making them more likely to target protein-protein interactions (Fanele & Ndlovu, 2023). Many Current HIV therapies are based on the inhibition of enzymes that are critical for the HIV life cycle. This strategy is prone to mutational changes, which render these antiviral agents ineffective over time. Protein-protein inhibitors are an emerging group of antiviral compounds that can escape these mutation limitations (Markovic *et al*., 2024).

Traditional healer communities have used them as a source of primary health care to manage symptoms from viral, fungal and bacterial infections (Kitazato *et al*., 2007; Li *et al*., 2018; Salam & Quave, 2018). Penicillin and Taxol are two of the most widely used NP-derived medications now being developed by pharmaceutical companies (Fleming, 1929; Wani *et al*., 1971). This evidence of plants and endophytes producing the same NP with medicinal use shifted interest to endophytes associated with medicinal plants (Ancheeva *et al*., 2020). The evolutionary relationship between plants and endophytes has resulted in a shared biosynthetic capacity, enabling both organisms to benefit from an increased acquisition of nutrients, protection against pathogens and increased fitness (Chowdhary *et al*., 2012; Mishra *et al*., 2021). Penicillin is a widely used antibiotic that treats bacterial infections. Alexander Flemings isolated it from a fungus, *Penicillium chrysogenum* which was later reidentified as *Penicillium notatum*, a known endophytic fungus of the medicinal plant *Albizia adianthifolia* (Fleming, 1929). Taxol was isolated from the bark of *Taxus brevifollia* and has been used to treat leukaemia, breast cancer and lung cancer (Wani *et al*., 1971; Wani & Horwitz, 2014).

Many researchers have shifted their efforts in developing tools to exploit the plant microbiome, including fungal endophytes in research strategies. This is also a more sustainable approach to reducing the destruction of plants by opting for a more renewable alternative source of bioactive secondary metabolites (SMs), such as fungal endophytes (Banyal *et al*., 2021). Endophytic fungi often colonise the tissues of plants without causing any apparent or obvious infection (Jia *et al*., 2016). The endophytic fungi can promote plant growth, increase resistance to biotic and abiotic stress and aid the accumulation of secondary metabolites (Waqas *et al*.,

2015; Ye *et al*., 2021; Verma *et al*., 2022). Secondary metabolites are organic compounds not vital to an organism's growth, development and reproduction (Monfil & Casas-Flores, 2014). Natural products are being included in the research to the drug development pipeline.

Pestalotheol C a secondary metabolite isolated from *Pestalotiopsis theae*, is able to block HIV replication (Li *et al*., 2008). Li *et al.* (2008) did not describe a possible mechanism of action of pestalotheol C and its inhibition of HIV-1. Further research into the anti-HIV properties of secondary metabolites in drug-resistant strains and various subtypes is encouraged, as secondary metabolites have been demonstrated to reduce HIV-1 viral replication and inhibit viral enzymes. The sodium butyrate-activated extract of *Penicillium chrysogenum* was found to be a potent inhibitor of HIV-1 replication compared to the control non-activated extract of *P. chrysogenum* (Makhwitine *et al*., 2023). Makhwitine *et al.* (2023), however, did not describe a possible mechanism of action for this potent inhibition of HIV-1 replication. Although the mechanism of action of many secondary metabolites and NPs is unknown, it is important to determine whether these can escape viral mutations that confer drug-resistance. Bevirimat is an unapproved first-in-class maturation inhibitor derived from a betulinic acid compound isolated from *Syzygium claviflorum* a Chinese herb (Sever *et al*., 2024). Naturally occurring chemical structures were incorporated, such as caffeic acid and piperazine, into bevirimat (Zhao *et al*., 2016). The study demonstrated that these bevirimat derivatives could improve the susceptibility against the known bevirimat HIV-1 strain with the V370A mutation. This demonstrates that secondary metabolites and NPs have the potential to escape HIV-1 drug-resistance.

The antiviral effects of secondary metabolites from the fungal endophyte *Alternaria alternata* (PO4PR2), which were isolated from *Hypoxis hemerocallidea,* have been recently demonstrated by our research group against HIV-1 subtype B. (pNL4.3) (Nzimande *et al*., 2022). The results further showed that *Alternaria alternata* (PO4-PR2) crude extract had antiviral activity toward HIV-1 with an $IC_{50}$ of 0.017 ug/ml (Nzimande *et al*., 2022). The *A. alternata* extract selective indexes (SI) were 38.82, 2558.82 and 59.71, indicating high antiviral potency (Nzimande *et al*., 2022). Gas chromatography-mass spectrometry (GC-MS) was used by Nzimande *et al*. (2022) to identify the compounds found in the PO4PR2 extract. Coumarin compounds, which are known to have integrase inhibitory activity, were putatively observed in GCMS data. However, this study did not investigate the mode of action of the *A. alternata* extracts or test them on drug-resistant resistant HIV-1 strains or other HIV-1 strain subtypes that are resistant to current drugs.

The *A. alternata* PO4PR2 crude extract was fractionated using solid phase extraction (SPE) cartridges that were HLB (Hydrophilic–Lipophilic Balance), MCX (Mixed-mode, strong Cation-eXchange), and MAX (Mixed-mode, strong Anion-eXchange) (Kubheka *et al*., 2024). The MCX fraction was found to have potent antiviral activity against HIV NL4.3 of 0.3619 µg/mL. The crude extract and MCX fraction were found to significantly affect reverse transcriptase, integrase and protease (Kubheka *et al*., 2024). The 5% MCX fraction of *A. alternata* crude extract showed that it has both anti-HIV-1 activities and immunological effects (Kubheka *et al*., 2024). Therefore, the secondary metabolites of *A. alternata* have demonstrated promising anti-HIV properties and should be further evaluated for their activity against HIV-1 subtypes and drug-resistant HIV-1 strains.In the current study, we investigate the anti-HIV1 activity of *A. alternata* extracts in different HIV-1 subtypes (A, B, C and D) and in integrase drug-resistance mutants (T66K and S230R). The study will further determine at which stage of the HIV-1 life cycle does the *A. alternata* extracts suppress the virus.

## 1.2 Rationale

The introduction of antiretroviral drugs has lowered the burden of HIV/ AIDS on the global health care system. However, the emergence of drug-resistant strains of HIV combined with non-adherence, virologic failure and side effects compound the difficulty of treating PLWHs. The diversity of HIV-1 subtypes factors into the emerging drug-resistance and influences the development of drug-resistance in PLWH (Wainberg, 2004; Nastri *et al*., 2023). There is a demand for antiretroviral drugs that are that are not impaired by the shortcomings of existing antiretrovirals. To meet this demand, the scientific community is adopting an alternative strategy of bioprospecting secondary metabolites from endophytic fungi associated with medicinal plants as a sustainable source bioactive secondary metabolites to reduce destruction of plants.

## 1.3 Aim

To investigate the anti-HIV activities and mechanism of action of secondary metabolites from *A. alternata* on different HIV-1 subtypes and integrase-resistant mutant strains.

## 1.4 Specific objectives

1. To investigate the antiviral activity of endophytic secondary metabolites on different HIV-1 subtypes using the TZM-bl luciferase-based antiviral assay.
2. To investigate the antiviral effect of secondary metabolites from endophytic fungi *A. alternata* on integrase drug-resistant mutant viruses.
3. To determine the mechanism of action of secondary metabolites on HIV-1 using a time of addition assay.

## 1.5 Overview of this dissertation

This dissertation is divided into 4 chapters, including this one:

**Chapter 1:** This chapter addresses this dissertation's background, rationale, aim and objectives.

**Chapter 2:** This chapter is a literature review describing the history and statistics of the HIV/AIDS pandemic of PLWHs. The classification system describing the diversity of HIV is outlined as well as the distribution of the varieties of HIV. The organisation of the genes in the HIV genome, the function of the viral proteins and the viral life cycle of HIV is detailed. The therapies and strategies used to treat PLWHs are discussed as well as the challenges that impede these therapies and strategies. The medicinal uses and applications of NPs from medicinal plants and associated endophytes such as *Alternaria alternata* used against illness, disease and HIV subtypes and drug-resistant strains is reviewed. This chapter also reviews the methods used in this dissertation as well as in the scientific literature.

**Chapter 3:** This chapter contains the main manuscript of this study entitled ‘’*Alternaria alternata* crude extracts inhibit the HIV-1 replication of different HIV-1 subtypes and Integrase strand transfer drug-resistant strains.’’ This chapter aims to answer research questions and address the research objectives stated in chapter 1.

**Chapter 4:** This chapter is the synthesis of the chapter 3 manuscript. This chapter includes a general discussion, conclusions as well as limitations and recommendations for this study.

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# CHAPTER 2: LITERATURE REVIEW

## 2.1 Human immunodeficiency virus and acquired immunodeficiency syndrome

### 2.1.1 History of HIV

Human Immunodeficiency Virus (HIV) was discovered by two research groups led by Robert Gallo, Barré-Sinoussi, and Luc Montagnier in 1981 (Barré-Sinoussi *et al*., 1983; Gallo *et al*., 1984). HIV is the causal agent of acquired immunodeficiency syndrome (AIDS). A global health crisis was caused by HIV infecting more people decades after its discovery (Frank *et al*., 2019). It was then established that HIV infects CD4+ T cells, which form a critical part of the immune system (Klatzmann *et al*., 1984). The World Health Organization (WHO) noted similarities between HIV and simian AIDS caused by Simian Immunodeficiency Virus (SIV), which is a lentivirus, as early as 1987 (Biberfeld *et al*., 1987). Later studies would confirm HIV as a lentivirus and its relatedness to SIV because of zoonotic transfer from primates (Hahn *et al*., 2000). As early as 1986, it was acknowledged that HIV can be typed as HIV-1 and HIV-2 (Clavel *et al*., 1986). The consumption of flesh from primates such as the sooty mangabey monkeys and chimpanzees is thought to be likely a zoonotic transmission event in which humans first became infected with HIV-1 and HIV-2, respectively (Chen *et al*., 1995; Sharp & Hahn, 2011).

The field of HIV vaccine research has made significant progress in vaccine technology however, there is no vaccine that prevents infection. Vaccine candidates have suffered from poor efficacy in at-risk populations of individuals such as men who have sex with men, sex workers and transgender individuals (Kim *et al*., 2021). The increasing diversity of HIV clades reduces the efficacy of HIV vaccines (Barouch., 2008). Several people have also been reported to be cured or achieved HIV remission. The Berlin patient was able to achieve long-term viral control of HIV (Hütter *et al*., 2009). An allogenic stem transplant from a homozygous donor for the CCR5 Δ32 deletion achieved this. The challenge of the cure strategy previously discussed is donor availability and cost (Hütter and Thiel., 2011).

### 2.1.2 Classification and Distribution of HIV

Human immunodeficiency virus belongs to the lentivirus genus and is classified as either type 1 or 2 (Sharp & Hahn, 2011). The nomenclature of HIV-1 is organised into groups, subtypes, sub-subtypes, and circulating recombinant forms (Robertson *et al*., 2000). In terms of global distribution, HIV-1 is the predominant type, whereas HIV-2 is largely restricted to Western

Africa (UNAIDS & Organization, 2010; Hemelaar *et al*., 2011). Type 2 HIV is distinct from type 1 since type 2 has a lower transmissibility and slower disease progression (Adjorlolo-Johnson *et al*., 1994; Nyamweya *et al*., 2013). HIV-1 is classified into four groups: M, N, O and P based on phylogenetic analysis, of which group M is the most prevalent (Simon *et al*., 1998; HIV, 2000; Robertson *et al*., 2000; Taylor *et al*., 2008). HIV-1, Group M is further divided into nine subtypes: A, B, C, D, E, F, G, H, J and K (Patiño-Galindo & Gonzalez-Candelas, 2017). Figure 2.1 shows the distribution of HIV-1 groups and subtypes across the globe (Bbosa *et al*., 2019). Sub-Saharan Africa, in particular the Southern Africa region is dominated by HIV-1, subtype M, sub-subtype C representing approximately 46% of HIV-1 infections (Hemelaar *et al*., 2011; Bbosa *et al*., 2019; Gartner *et al*., 2020). In Eastern Africa and Russia, subtype A is dominant (Figure 2.1) (Gounder *et al*., 2017; Aibekova *et al*., 2018). Subtype B is most prevalent in North America, Europe, Australia and Middle East Africa (Castley *et al*., 2017; Oster *et al*., 2017; Salam & Quave, 2018; Bbosa *et al*., 2019). In Eastern Africa, subtype D is the dominant circulating strain (Billings *et al*., 2017). Several circulating recombinant forms (CRFs) and unique recombinant forms (URFs) have been reported in South America, Middle East Africa, Asia and Russia (Bbosa *et al*., 2019). It is critical that HIV-1 subtype diversity be considered in the HIV-1 drug research and development pipeline.

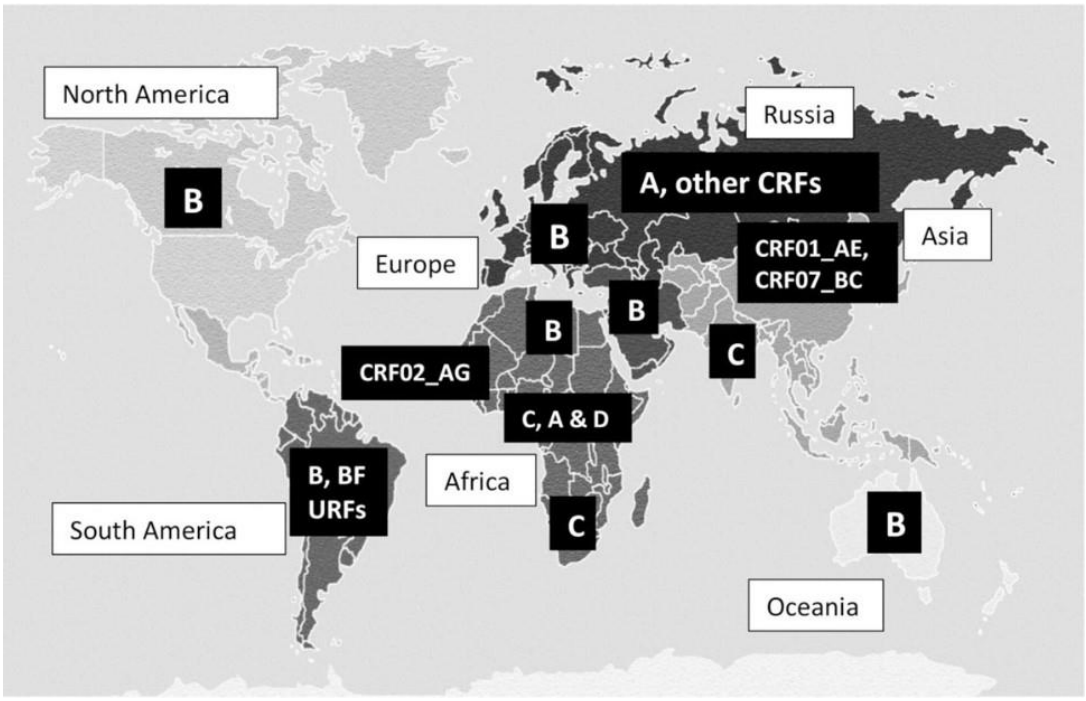


**Figure 2.1**: The global distribution of HIV-1 subtypes, unique recombinant form (URF) and circulating recombinant forms (CRFs) (Bbosa *et al*., 2019).

### 2.1.3 The HIV-1 genome

The HIV viral particle contains two copies of its genome in the form of single-stranded ribonucleic acid (RNA) (Lu *et al*., 2011; Nkeze *et al*., 2015). The genome is approximately nine kilobases long and comprises nine distinct genes that encode fifteen proteins (Li *et al*., 2015). The gene group-specific antigen (*gag*) is cleaved by viral protease to form a mature matrix (MA), capsid (CA), nucleocapsid (NC) and p6 proteins, which are structural proteins of the HIV virion (Figure 2.2)(Freed, 2015; Nkeze *et al*., 2015). The polymerase gene (*pol*) encodes for the viral enzymes protease (PR), integrase (IN) and reverse transcriptase (RT) (Wayengera, 2011). Protease is responsible for the processing of Gag and Pol (Davis *et al*., 2012). The integrase enzyme integrates a copy of HIV deoxyribonucleic acid (DNA) into the host genome and reverse transcriptase converts viral RNA into DNA (Hare *et al*., 2010; Larsen *et al*., 2018). The envelope gene (*env*) encodes for the glycoprotein gp160, which is proteolytically cleaved to form the glycoproteins gp120 and gp41, which form the HIV spike protein (Williamson, 2003; Chen, 2019).

The Vif, Vpu, Tat, Rev and Nef accessory proteins promote viral replication and immune escape (Schubert & Strebel, 1994; Mlcochova *et al*., 2015). The virion infectivity factor gene (*vif*) encodes the Vif protein that blocks host defence factor APOBEC3G which allows for the release of HIV-1 from infected human cells (Bogerd *et al*., 2004). The gene viral protein U (*vpu*) encodes the Vpu protein which is an antagonist of human tetherin (McNatt *et al*., 2009). The transactivator of transcription gene (*tat*) encodes the Tat protein, which enhances the transcription of HIV RNA (Das *et al*., 2011). The Tat protein is also thought to have a fundamental role in reverse transcription. However, conflicting reports have been observed in literature on the nature of this role (Apolloni *et al*., 2007). Harrich *et al*., (1997) suggested that in the absence of functional Tat protein, reverse transcription is inhibited (Harrich *et al*., 1997). In contrast, a recombinant Tat protein inhibited the reverse transcriptase function *in vitro* (Kameoka *et al*., 2001). Due to these conflicting reports, regarding the role of Tat in reverse transcription remains a controversial topic. The *rev* gene encodes for the Rev protein which is a regulator of the expression of virion proteins and exports intron-carrying mRNA (messenger ribonucleic acid) (Jayaraman *et al*., 2019). The negative factor (*nef*) gene encoding for the Nef protein allows for escape from the host immune system and enhances viral replication (Buffalo *et al*., 2019; Omondi *et al*., 2019).

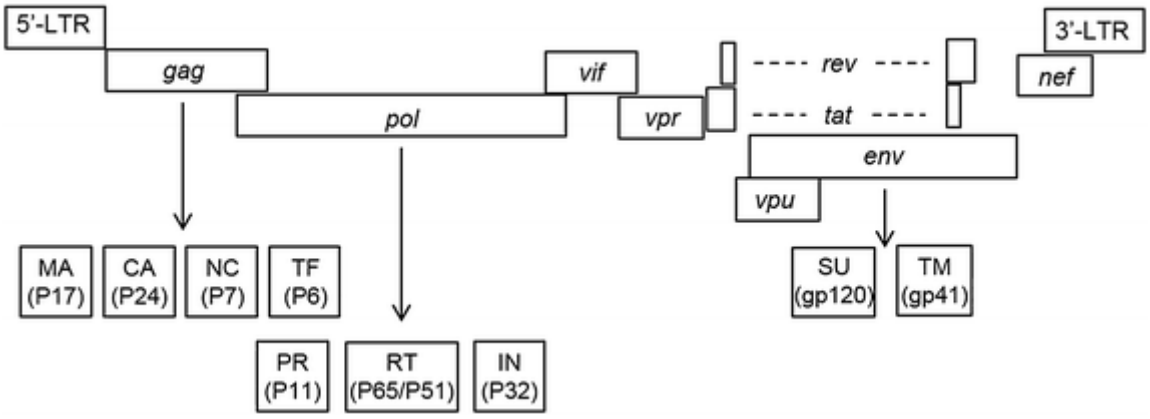


**Figure 2.2:** Organisation and structure of the HIV-1 genome (Nkeze *et al*., 2015).

### 2.1.4 Replication cycle of HIV-1

The HIV-1 life cycle begins with the binding of the virion to the clusters of differentiation type 4+ (CD4+) T cell receptors of T-lymphocytes, monocytes, dendritic and microglial cells (Fanales-Belasio *et al*., 2010; Ramdas *et al*., 2020). Figure 2.3 illustrates the full life cycle of HIV-1 and the steps involved required to complete the infection life cycle thus, during infection the envelope protein (gp120) binds to the C-C chemokine receptor 5 (CCR5) or C-X-C chemokine receptor 4 (CXCR4) of the host CD4+ T cell (Wu *et al*., 1996; Raja *et al*., 2003; Ramdas *et al*., 2020). After binding to the CD4+ T receptor, a conformational change is induced in gp120, which allows binding to the CCR5 and CXCR5 receptors, which permits viral entry into the host cell (Wu *et al*., 1996). Once binding is complete, gp41 is inserted into the host cell membrane, resulting in the formation of a six-helix bundle followed by complete membrane fusion of the viral envelope and host cell membrane in a stage called fusion (2) (Wilen *et al*., 2012). This results in the delivery of the viral core or capsid into the cell cytoplasm in a stage called core delivery or entry (3) (Wilen *et al*., 2012).

Once core delivery or entry is complete and the viral core is delivered, a single-stranded RNA from HIV is enzymatically converted to double-stranded DNA by the (4) enzyme reverse transcriptase (Kati *et al*., 1992). The virus is then disassembled and uncoated upon entering the nucleoplasm (5,6) (Goff, 2001). The viral copy DNA (cDNA) transported to the nucleus will be integrated into the host genome as proviral DNA by the enzyme integrase; this stage is referred to as integration (7) (Figure 2.3) (Vink & Plasterk, 1993). Integration involves introducing viral DNA into the genome of a host cell since proviral DNA is a pre-requisite for completing the viral life cycle of HIV-1 (Delelis *et al*., 2008; Engelman *et al*., 2018).

During the transcription step (8) in Figure 2.3, the proviral DNA serves as a template for the transcription of the viral RNA genome and mRNA (Wu, 2004). The transcription of viral RNA is facilitated by the host cell RNA polymerase II (Ott *et al*., 2011). The transcription of viral RNA is enhanced by Tat, which stimulates transcription by binding to the LTR promoter (Das *et al*., 2011). This allows for the synthesis of full-length RNA transcripts and mRNA (Kirchhoff, 2013). The Rev protein facilitates export to the cytoplasm for translation (9) (Fernandes *et al*., 2016). Then, during assembly, MA, CA, NC, p6 proteins and polymerase enzymes (reverse transcriptase, protease and integrase) assemble (10) into viral core particles containing two copies of the HIV viral RNA (Sundquist & Kräusslich, 2012). During assembly, the exterior of the viral core is encapsulated in envelope proteins gp41 and gp120 (Ganser-Pornillos *et al*., 2011). In the next stage, called budding (11), the immature virus exits the plasma membrane of the infected host cell in a process called budding (Weiss & Göttlinger, 2011). The protease enzyme cleaves the Gag proteins into its mature form, which completes the maturation (12) of the virus and is capable of infecting host cells (Freed, 2015).

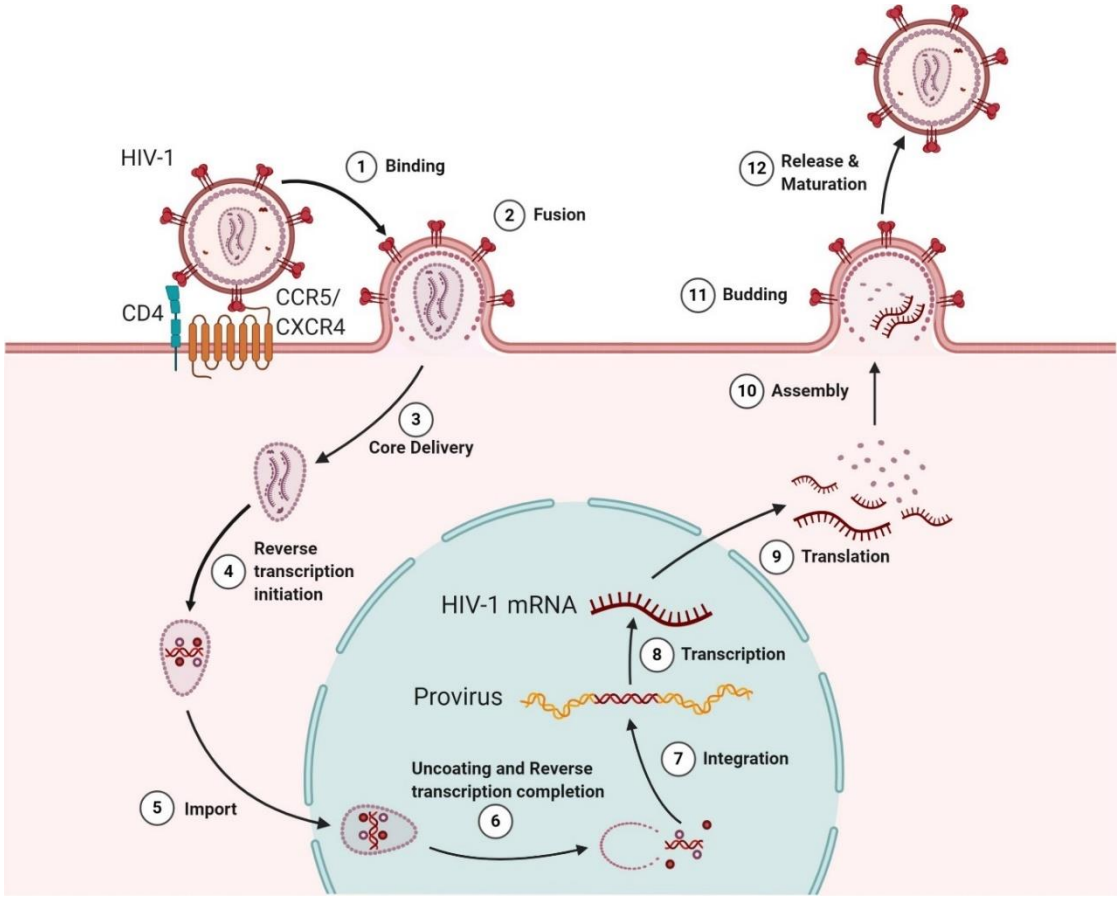


**Figure 2.3**: The life cycle of HIV-1 begins with the attachment and ends with the release of mature viral particles (Ramdas *et al*., 2020). **Steps of HIV-1 replication 1:Binding, 2: Fusion, 3: Core delivery, 4: Reverse transcription initiations, 5: Import, 6: Uncoating and reverse transcription completion, 7: Integration, 8: Transcription, 9: Translation, 10: Assembly, 11: Budding and 12: Release and maturation**.

Viral binding, reverse transcription, integration, translation and proteolysis are key events that are potential targets for developing antiretroviral drugs (Engelman & Cherepanov, 2012). Following this logic, various efforts have produced drugs that strategically interrupt the stages in the life cycle of HIV-1 (Chaudhuri *et al*., 2018). These antiretroviral drugs have thus been categorized according to their mechanistic class, which indicates the key stage in the HIV-1 life cycle that is being inhibited (Kemnic & Gulick, 2018). These antiretroviral classes include NNRTIs (non-nucleoside reverse transcriptase inhibitors), NRTIs (nucleoside reverse transcriptase inhibitors), PIs (protease inhibitors), INSTIs (integrase strand transfer inhibitors),

fusion inhibitors, CCR5 antagonists and CD4 post-attachment inhibitors (Maeda *et al*., 2019; Phanuphak & Gulick, 2020).

### 2.2 HIV therapies and treatment

Antiretroviral therapy and PrEP (pre-exposure prophylaxis) use is estimated to have saved over 9.5 million lives, and by 2030, it is predicted to save an additional 34.9 million lives (Forsythe *et al*., 2019). Pre-exposure prophylaxis is an antiretroviral drug used by at-risk individuals to prevent HIV infection at a rate of more than 90% (Hevey *et al*., 2018). Antiretroviral drugs have been successful in delaying the HIV-1 disease progression in people living with HIV (Zolopa *et al*., 2009). The first antiretroviral approved and nucleoside reverse transcriptase inhibitor (NRTI) by the FDA to treat HIV was zidovudine or azidothymidine (AZT) in 1987 (Kolata, 1987; Khandazhinskaya *et al*., 2010). Despite being approved as an antiretroviral drug, AZT has a poor long-term safety profile since it is associated with hematopoietic toxicity, neutropenia and myelosuppression (Gallicchio *et al*., 1993). Those who were treated long-term with AZT monotherapy developed drug-resistance (Rooke *et al*., 1989). Since 1987, more antiretrovirals have been developed because of AZT's drawbacks and the need for better HIV therapy. As a result, the HIV research pipeline has produced numerous classes of antiretrovirals including combination antiretroviral therapy (Maeda *et al*., 2019).

There are currently 32 antiretroviral drugs that have been licensed by the Food and Drug Administration (FDA) as listed in Table 1.1 (Maeda *et al*., 2019). These antiretroviral drugs can be categorized according to their mechanism of action. Fusion inhibitors such as enfuvirtide bind to gp41, preventing HIV from entering the host cell and infection from occurring (Qadir & Malik, 2010). C-C chemokine receptor five inhibitors/antagonists bind to the CCR5 receptor on susceptible host cells blocking viral entry of CCR5 tropic viruses (Rao, 2009; Tan *et al*., 2013). Attachment inhibitors such as ibalizumab is a monoclonal antibody that prevents co-receptor binding and fusion by blocking conformational changes in gp120 (Emu *et al*., 2018; Blair, 2020).

Nucleoside reverse transcriptase inhibitors (NRTIs) inhibit reverse transcriptase by preventing the synthesis of viral DNA from deoxynucleotide triphosphates (dNTPs) ceasing the synthesis of DNA (Sluis-Cremer *et al*., 2000). Non-nucleoside reverse transcriptase inhibitors function differently from NRTIs by binding to non-substrate binding sites or pockets, which inhibits reverse transcriptase (De Clercq, 1998). Protease inhibitors (PIs) bind to the site that cleaves

the gag-pol polyprotein, blocking the maturation of HIV virions and impeding the infectivity of virions (Flexner, 1998). Integrase strand transfer inhibitors (INSTI) inhibit integrase by binding to the active site of integrase and viral DNA, which prevents the integration of viral DNA into the host cell DNA, thus arresting viral replication (McColl & Chen, 2010; Passos *et al*., 2020). Many antiretroviral drug regimens consist of NRTIs, NNRTI's, PIs and INSTI thus, drug research tends to favour the development of these classes of antiretrovirals since, historically, more drugs from these classes have been approved (Cruciani & Parisi, 2019).

**Table 2.1**: Antiretroviral drugs and classes approved by the FDA (Maeda *et al*., 2019; Phanuphak & Gulick, 2020).

| Name of antiretroviral drug | Class | Year of FDA approval |
|---|---|---|
| Zidovudine (AZT) | Nucleoside Reverse Transcriptase Inhibitors (NRTI) | 1987 |
| Didanosine (ddI) | | 1991 |
| Zalcitabine (ddC) | | 1992 |
| Stavudine (d4T) | | 1994 |
| Lamivudine (3TC) | | 1995 |
| Abacavir (ABC) | | 1998 |
| Tenofovir disoproxil fumarate (TDF) | | 2001 |
| Emtricitabine (FTC) | | 2003 |
| Tenofovir alafenamide (TAF) | | 2015 |
| Nevirapine (NVP) | Non-Nucleoside Reverse Transcriptase Inhibitors (NNRTI) | 1994 |
| Delavirdine (DLV) | | 1997 |
| Efavirenz (EFV) | | 1998 |
| Etravirine (ETR) | | 2008 |
| Rilpivirine (RPV) | | 2011 |
| Doravirine (DOR) | | 2018 |
| Saquinavir (SQR) | | 1995 |
| Ritonavir (RTV) | | 1996 |
| Indinavir (IDV) | | 1996 |
| Nelfinavir (NFV) | | 1997 |
| Amprenavir (APV) | | 1999 |

| | | |
|---|---|---|
| Lopinavir/Ritonavir (LPV/r) | Protease inhibitor (PI) | 2000 |
| Atazanavir (ATV) | | 2003 |
| Fosamprenavir (FPV) | | 2003 |
| Tipranavir (TPV) | | 2005 |
| Darunavir (DRV) | | 2006 |
| Raltegravir (RAL) | Integrase strand transfer inhibitor (INSTI) | 2007 |
| Elvitegravir (EVG) | | 2012 |
| Dolutegravir (DTG) | | 2013 |
| Bictegravir (BIC) | | 2018 |
| Enfuvirtide (T20) | Fusion inhibitor | 2003 |
| Maraviroc (MVC) | CCR5 antagonist | 2007 |
| Fostemzavir (FTR) | Attachment inhibitor | 2020 |
| Ibalizumab (IBA) | CD4 post-attachment inhibitor | 2018 |

Combination antiretroviral therapy (cART) utilizes three or more different antiretroviral drugs that can be taken as a pill or in fixed doses that effectively reduce viral load and mortality (Palella Jr *et al*., 1998; Velasco-Hernandez *et al*., 2002). It is recommended that cART regimens include two NRTIs and another antiretroviral that is an NNRTI, PI or INSTI (Sanchez & Kaul, 2017)(Sanchez and Kaul., 2017). First-line combination therapy recommended by the South African Department of Health, referred to as TLD, consists of tenofovir, lamivudine and dolutegravir as advised by the World Health Organisation (WHO) (Chimukangara *et al*., 2021). While cART has been and continues to be successful at treating PLWH, it is not an adequate long-term solution since it is hindered by many challenges (Agwu & Fairlie, 2013; Rausch & Le Grice, 2020). These include drug-resistance to major antiretroviral classes, HIV subtype, inability to eradicate viral reservoirs, adverse effects and cytotoxicity (Seyler *et al*., 2018; Obeagu & Nwosu, 2019).

## 2.3 The challenges of HIV combination antiretroviral therapy

### 2.3.1 Eradicating viral reservoirs

Viral reservoirs are cells latently infected with HIV that still have replication competency and resist clearing by antiretroviral drugs (Finzi *et al*., 1997; Shen & Siliciano, 2008).

Antiretroviral therapy cannot eradicate the virus from the reservoirs resulting in viral persistence (Dornadula *et al*., 1999; Shen & Siliciano, 2008). In response, several approaches, including “shock and kill”, gene therapy, “Block and lock” and immune-based therapies are being investigated in ongoing research (Figure 2.4) (Abana *et al*., 2022). To implement the “shock and kill” approach, cells latently infected with the HIV-1 provirus must be triggered with a latency-reversing agent (LRA) (Figure 2.4 (A)). Once activated, the cells either die or are destroyed by the host immune system after which antiretroviral therapy is administered (Deeks, 2012). Gene therapy (Figure 2.4 (B) aims to inhibit or alter HIV-1 expression by utilizing gene editing techniques such as clustered regularly interspaced palindromic repeats (CRISPR) Cas9 technology (Ebina *et al*., 2013).

An alternative strategy is called "block and lock" (Figure 2.4 (C)), which uses antiretroviral medications and latency-promoting agents (LPA) to induce profound latency and prevent or reduce the spread of infection (Moranguinho & Valente, 2020). Immune-based therapies (D) involve approaches such as chimeric antigen receptor (CAR) T-cell therapy, broadly neutralising antibodies (bNabs) and therapeutic vaccines. Chimeric antigen receptor T cell therapy requires genetically engineering cluster of differentiation type 8 (CD8)+ T cells to express CD4, which, upon recognition of infected cells, causes a cytotoxic response against the activated cell (Campos-Gonzalez *et al*., 2023). Broadly neutralising antibodies can recognise and bind to a wide range of HIV envelope glycoproteins to prevent entry (Griffith & McCoy, 2021). Apart from the ongoing study, investigating NPs as antiviral, LRAs and LPAs could be an additional approach to eliminating reservoirs.

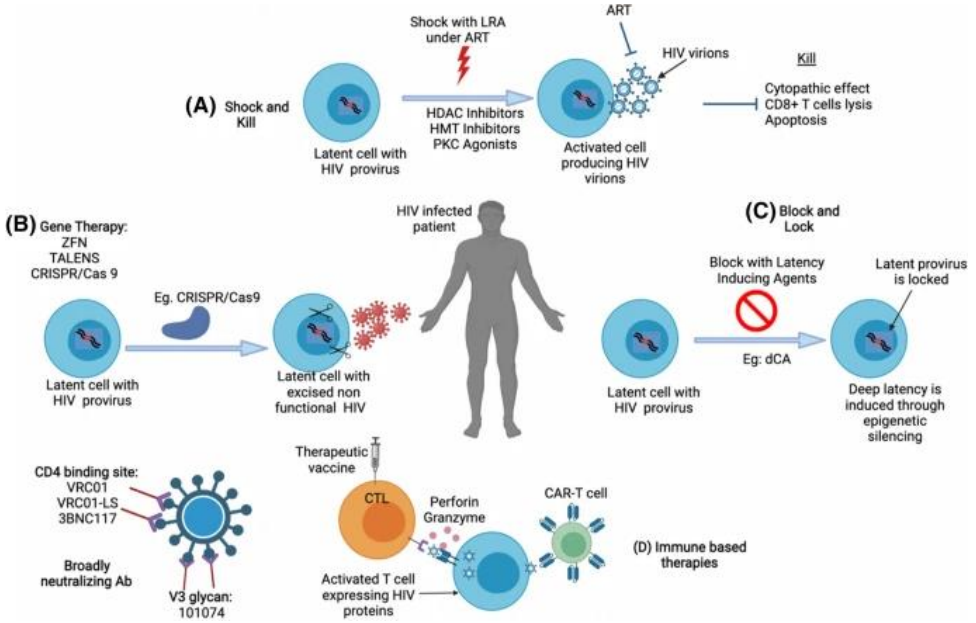

**Figure 2.4**: Strategies proposed to cure people living with HIV (Abana *et al*, 2022). Shock and kill (A). Gene therapy (B). Block and lock (C). Immune-based therapies (D).

### 2.3.2 Adverse effects and cell cytotoxicity

The toxicity of antiretroviral drugs is the next obstacle that needs to be addressed to enhance treatment for PLWHs. Antiretroviral tenofovir is linked to renal damage, and 29.2% of persons experience moderate renal dysfunction per 1000 PLWHs (Quesada *et al*., 2015). The rate of renal dysfunction is concerning, even if moderate, as side effects of this nature contribute to non-adherence in PLWHs (Chesney, 2000). Nucleoside reverse transcriptase inhibitors can diminish mitochondrial function, resulting in mitochondrial toxicity (Foster & Lyall, 2008). Complications such as neuropathy, cardiomyopathy, pancreatitis, and bone marrow suppression have been associated with mitochondrial toxicity (Foster & Lyall, 2008).

Significant weight gain was observed in people receiving INSTI regimens compared to those receiving NNRTI regimens (Bourgi *et al*., 2020). Ritonavir is commonly linked to nausea and vomiting in PLWHs, a symptom that affects approximately 20% of patients using the antiretroviral medication (Barlett, 2004; Mehendale *et al*., 2007). This gastrointestinal stress is hypothesised to be caused by oxidative stress, which is increased by ritonavir (Mehendale *et al*., 2007). The drug raltegravir in high concentrations may intensify or cause severe insomnia in patients with no history of this symptom (Eiden, 2011). Insomnia in patients using ritonavir resolved in those who stopped taking the medication (Gray, 2009; Gray & Young, 2009; Eiden, 2011). Thus, the hunt for an HIV treatment that is both less harmful and capable of eliminating the virus from virus reservoirs, inhibiting viral replication of HIV subtypes and drug-resistant strains must continue. Given that natural anti-HIV products can be non-cytotoxic and cheaper, NPs may reduce the side effects of medication toxicity (Palshetkar *et al*., 2020; Nzimande *et al*., 2022).

### 2.3.3 HIV Drug-Resistance

Another obstacle that needs to be addressed is the spread of drug-resistant HIV-1, as neglecting this issue might have catastrophic consequences for patients and clinical settings. Mutations in the viral genome cause HIV-1 drug-resistance, and viral gene mutations confer resistance to a

drug or drugs (Erickson & Burt, 1996). The acquisition of drug-resistance by HIV-1 strains reduces effectiveness of existing drugs and can result in virological failure (Ross *et al*., 2007; Jordan *et al*., 2008). Drug-resistant mutations conferring resistance to PI, NRTI, NNRTI and INST, major antiretroviral classes, have been reported (Table 1.2). Scholars curate Los Alamos HIV database and the Stanford University HIV drug-resistance database to classify and organise these drug-resistance mutations and are freely accessible for research purposes (Kuiken *et al*., 2003; Shafer, 2006).

The susceptibility of fusion inhibitor enfuvirtide in the presence of drug-resistance mutations was investigated by X-gal staining of HeLa-CD4-LTR-β-gal cells (Izumi *et al*., 2013). I37N, I33S, and N43D point mutations reduced susceptibility to enfuvirtide by 20, 9.6 and 20-fold, respectively (Izumi *et al*., 2013). These findings indicate that the selected mutations result in a major decrease in susceptibility to enfuvirtide. The mutations V169M, L317W and I408T were introduced into the envelope gene by site-directed mutagenesis (Asin-Milan *et al*., 2013).

A luciferase-based assay was used to determine the susceptibility of the mutations V169M, L317W and I408T in the presence of the CCR5 antagonist maraviroc (Asin-Milan *et al*., 2013). The V169M, L317W and I408T have reduced susceptibility of maraviroc by 1.23, 1.52 and 4.00, respectively, suggesting the selected mutations confer resistance to maraviroc (Asin-Milan *et al*., 2013). The V169M, L317W and I408T mutations are situated in the V2, V3 and V4 loop of the envelope glycoprotein and may cause resistance by altering the envelope structure (Asin-Milan *et al*., 2013). Fostemsavir is an attachment inhibitor that was approved for the treatment of multidrug-resistance HIV-1 infection in patients who are failing treatment (Hiryak & Koren, 2021). Surveillance of treatment naïve and antiretroviral experienced cohort in Botswana found that the mutations M434I, M475I and M426L had the highest prevalence and were associated with drug-resistance to fostemsavir (Zuze *et al*., 2023). Zuze *et al.* (2023) further recommended phenotypic studies to improve drug-resistance monitoring like those mentioned above.

The mutation Q151M confers a high resistance to NRTIs zidovudine (Shirasaka *et al*., 1995). The Q151M mutation decreased susceptibility to zidovudine by approximately 100-fold (Shirasaka *et al*., 1995). The susceptibility of the NRTI stavudine decreased in the presence of the M41L mutation, with patients responding poorly to treatment (de Ronde *et al*., 2000). Stavudine was later phased out from many regimens because of the severe side effects and toxicities associated with the drug (Makinson *et al*., 2008). In HIV-1 strains with the K101P

mutation, high-level resistance is conferred to NNRTIs nevirapine, rilpivirine and delavirdine with 400, 28 and 29 median fold changes in susceptibility respectively (Parkin *et al*., 2006). The rare mutation K103H confers up to a 50-fold decrease in susceptibility to NNRTIs nevirapine, delavirdine and efavirenz (Harrigan *et al*., 2005).

Two different commercial phenotypic assays from Antivirogram and Phenosense were used to determine the susceptibility of nelfinavir to the D30N mutation (Santos & Soares, 2011). The D30N mutation caused a 19 and 21-fold reduction in susceptibility using the respective Antivirogram and Phenosense assays (Santos & Soares, 2011). The PI mutation I54V was associated with resistance to atazanavir and increased susceptibility to other protease inhibitors (Rhee *et al*., 2022). Protease mutation L76V was associated with a 2-6 fold decrease in susceptibility to lopinavir, darunavir and amprenavir (Young *et al*., 2010).

Resistance to INSTIs is concerning since many drugs from this class have been approved in the past decade and form part of the South African TLD regimen (Chimukangara *et al*., 2021). Viruses with E92Q mutation showed a 26-fold decrease in susceptibility to raltegravir and cross-resistance to elvitegravir (Abram *et al*., 2013). The T66K mutation confers a 9.6- and 2-fold decrease in susceptibility to INSTIs raltegravir and dolutegravir, respectively (Kobayashi *et al*., 2008; Healthcare, 2013). The R263K mutation confers a 2.5- and 10.4-fold decrease in susceptibility to dolutegravir and raltegravir, respectively (Tsiang *et al*., 2016). The mutation S230R is an accessory drug-resistance mutation that alone can confer a 3.85-, 3.75- and 1.52-fold decrease in susceptibility against dolutegravir, cabotegravir and raltegravir respectively (Pham *et al*., 2018).

**Table 1.3**: Drug-resistance mutations associated with each major antiretroviral class.

| **Antiretroviral class** | **Mutation** | **Phenotypic presentation** | **References** |
|---|---|---|---|
| 1) Protease inhibitor | D30N | Causes 19-to-21-fold change in susceptibility to nelfinavir | (Santos & Soares, 2011) |
| | I54V | Associated with resistance to atazanavir and increased susceptibility to other PIs | (Rhee *et al*., 2022) |
| | L76V | Associated with a 2-to-6-fold decrease in susceptibility to lopinavir, darunavir and amprenavir | (Young *et al*., 2010) |
| 2) Nucleoside reverse transcriptase inhibitor | Q151M | Causes and approximated 100-fold decrease in susceptibility to zidovudine | (Shirasaka *et al*., 1995) |
| | M41L | Poor response to stavudine treatment | (de Ronde *et al*., 2000) |
| 3) Non-nucleoside reverse transcriptase inhibitor | K101P | Causes 400, 28 and 29 median fold decrease in susceptibility to nevirapine, rilpivirine and delavirdine respectively | (Parkin *et al*., 2006) |
| | K103H | Causes 50-fold decrease in susceptibility to NNRTIs nevirapine, delavirdine and efavirenz | Harrigan *et al*., 2005 (Harrigan *et al*., 2005) |
| 4) Integrase strand transfer inhibitor | E92Q | Causes 26-fold decrease in susceptibility to raltegravir and cross-resistance to elvitegravir INSTIs | (Abram *et al*., 2013) |

| | | | |
|---|---|---|---|
| | T66K | Causes 9.6- and a 2-fold decrease in susceptibility to raltegravir and dolutegravir respectively | (Kobayashi *et al*., 2008) |
| | R263K | Causes 2.5- and 10.4-fold decrease in susceptibility to dolutegravir and raltegravir, respectively | (Tsiang *et al*., 2016) |
| | S230R | Causes 3.85-, 3.75- and 1.52-fold decrease in susceptibility against dolutegravir, cabotegravir and raltegravir | (Pham *et al*., 2018) |

## 2.4 The intervention of therapeutic Natural Products

### 2.4.1 Natural products from medicinal plants

Natural products have recently received attention from the scientific community as a source of compounds with medicinal applications (Yuan *et al*., 2016; Atanasov *et al*., 2021). Indigenous communities have used medicinal plants to treat ailments and diseases without modern medicine (Van Wyk, 2011). The rich history of medicinal plant use by indigenous communities has sparked interest in investigating the medicinal benefits of these medicinal plants (Süntar, 2020). As a result, medicinal plants used by indigenous communities to treat diseases such as HIV/AIDS have received attention to investigative these health claims further (Prinsloo *et al*., 2018).

The medicinal plant *Hypoxis hemerocallidea* previously known as *Hypoxis ropeeri* is an African potato used by South Africa indigenous communities (Mofokeng *et al*., 2020). *Hypoxis hemerocallidea* is a medicinal plant used by traditional healers and ethnic communities to treat infertility, mental illness, urinary infection, cancer, tuberculosis and HIV infection (Matyanga *et al*., 2020). South African communication media has previously promoted the used of *H. hemerocallidea* as an immune booster for PLWHs however, these claims require further investigation (Mills *et al*., 2005). The potential anti-HIV properties of *H. hemerocallidea* were evaluated in a clinical study (Albrecht, 1996). Phase 1 clinical trials in PLWHs received 1200 – 3200 mg of a *H. hemerocallidea* dried methanolic extract (Albrecht, 1996). Albrecht *et al.* (1996) reported that the patients receiving the dried extract lived longer by approximately 2 years. The PLWHs had stable CD4+ T lymphocyte counts and decreased serum p24 antigen (Albrecht, 1996). The aqueous and ethanolic extracts of *Hypoxis sobolifera* have also been demonstrated to have anti-HIV reverse transcriptase activity and protease activity (Klos *et al*., 2009).

Healthy male volunteers received doses of efavirenz and the African potato to study drug and medicinal plant interactions (Mogatle *et al*., 2008). Mogatle *et al.* (2008) concluded that the African potato did not affect the clinical usage of efavirenz indicating medicinal plants and antiretroviral drugs can potentially be co-administered in the absence of adverse drug interactions however, this hypothesis requires further investigation. The challenge of using medicinal plants like *Hypoxis hemerocallidea* is its limited supply, overharvesting and the potential environmental damage caused by increased demand (Gail *et al*., 2015; Mofokeng *et al*., 2020). Due to these challenges associated with medicinal plant usage to treat disease the

research interest has shifted to the endosymbiotic microbiome of medicinal plants (endophytic fungi) as a source of bioactive compounds with anti-HIV-1 activity.

### 2.4.2 Antiviral activity of endophytic fungi

A diverse collection of endosymbiotic fungi known as endophytes typically live in the tissues of plants without causing any apparent harm to the host plant (Arnold, 2007). Specifically, the relationship between medicinal plants and endophytes is of interest as endophytes may share medicinal properties. *Hypoxis hemerocallidea/ropeeri* is a medicinal plant used by traditional healers and ethnic communities to treat infertility, mental illness, urinary infection, cancer, tuberculosis and has had positive effects on PLWHs (Albrecht, 1996; Matyanga et al., 2020). The endophytic fungus *A. alternata* was isolated from the medicinal plant of the *Hypoxis* species (Nzimande et al., 2022). The crude extract obtained from *A. alternata* isolates were found to have anti-HIV activity with $IC_{50}$'s ranging between 0.017 – 1.170 µg/mL indicating that these crude extracts have potent anti-HIV activity. The research focus has drifted away from medicinal plants and to the endophytes that inhabit them since these plants are at risk, as overharvesting for medicinal use has become an unsustainable practice threatening the species (Gail *et al*., 2015).

The unique source of active compounds found in endophytic fungi have secondary metabolites with anti-HIV activity hold potential to have fewer adverse effects associated with antiretrovirals (Popović-Djordjević *et al*., 2022). In 2003, Singh and colleagues extracted two new compounds, xanthoviridicatin E and F, from *Penicillium chrysogenum* and an unidentified plant endophyte in Peru. Xanthoviridicatin E and F demonstrated inhibitory action using an HIV-1 integrase assay, with an $IC_{50}$ of 6 µM and 5 µM, respectively (Singh *et al*., 2003). Another study by Singh and colleagues isolated a hinnuliquinone from an unspecified endophyte of *Quercus coccifera* (Singh *et al*., 2004). The inhibitory effect of the compounds against both wild-type protease and the drug-resistant mutant a-44 protease was assessed using an HIV-1 protease test. The hinnuliquinone was found to have inhibitory activity against the protease strains above, with wild-type protease and a-44 protease having potent $IC_{50}$'s of 2.5 µM and 1.8 µM, respectively.

Nzimande *et al*. (2023) provide an in-depth review of secondary metabolites with anti-HIV activity (Nzimande *et al*., 2023). Makhwitine *et al.* (2023) activated the biosynthetic gene clusters of *Penicillium chrysogenum* with sodium butyrate to induce the production of secondary metabolites. The extract of the sodium butyrate activated *Penicillium chrysogenum*

had an $IC_{50}$ of 0.6024 µg/mL compared to the untreated *Penicillium chrysogenum*, which had an $IC_{50}$ of 5.053 µg/mL (Makhwitine *et al*., 2023). This indicates that the induction of secondary metabolites increases the anti-HIV activity of the *Penicillium chrysogenum* secondary metabolites.

The novel metabolite Sch 213766 was isolated from *Chaetomium globosum,* a known endophyte of medicinal plants such as *Ginkgo biloba* (Yang *et al*., 2007). A CCR-5 membrane binding assay determined that the isolated compound was able to inhibit binding with an $IC_{50}$ value of 8.6 µM, indicating potential anti-HIV activity. Furthermore, Li *et al.* (2008) isolated four novel pestalotheols A, B, C and D from the endophyte *Pestalotiopsis theae* obtained from an unidentified plant in China. Using an MTT assay pestalotheol C was found to be non-toxic to C8166 cells with a $CC_{50}$ 163 µM (Li *et al*., 2008). A p24 antigen capture assay found that pestalotheol C had an anti-HIV-1 activity with an $EC_{50}$ of 16.1 µM. Seven isoprenylated chromonone derivatives were isolated from *Pestalotiopsis fici*, an endophyte of an unknown plant in China (Liu *et al*., 2009). The fifth compound was found to have anti-HIV activity using a p24 antigen capture assay and having an $EC_{50}$ of 8 µM. Consequently, endophytic fungi, such as those isolated from medicinal plants, exhibit anti-HIV activity. The anti-HIV-1 properties of the crude extract of *Alternaria alternata* that was isolated from the medicinal plant *Hypoxis hemerocallidea* was investigated in this study.

Endophytic fungi, *Alternaria tenuissima* was isolated from the stems of *Quercus emoryi*, a tree with a history of medicinal applications that showed anti-HIV-1 activity; however, the name of the compound was not defined (Wellensiek *et al*., 2013; Taib *et al*., 2020). Wellensiek *et al*., 2013 fractionated the secondary of an *Alternaria tenuissima* crude extract using solvent-solvent partitioning and gel permeation chromatography. The anti-HIV activity of these fractions was determined using viral replication assay and reverse transcriptase inhibition assay. The DK, DL, DM and DP fractions achieved complete inhibition of reverse transcriptase and HIV-1 viral replication and had the respective $IC_{50}$'s of 0.1 µg/mL, 0.075 µg/mL, 0.03 µg/mL and 0.5 µg/mL. The DK, DL, DM and DP naming convention is based on the purification and fractionation of previous extracts obtained by Wellensiek *et al.* (2013). The study by Wellensiek *et al.* (2013) was informative; however, compounds in the DK, DL, DM and DP were not identified, which was a significant shortcoming of this study.

The antiviral properties of altertoxins 1 to 5 were then evaluated by Bashyal et al. (2014) after separating five altertoxins from *Alternaria tenuissima* secondary metabolites. The antiviral

activity was assessed using reverse transcriptase inhibition and HIV-1 viral replication assays (Bashyal *et al*., 2014). The results showed that altertoxins 1, 2, 3 and 5 achieved complete inhibition of HIV-1 viral replication and had $IC_{50}$'s of 1.42 µM, 0.21 µM, 0.29 µM and 0.09 µM. These compounds displayed potent anti-HIV activity; however, are unsuitable for humans as it is reported that this class of compounds is known to be mutagenic. The mutagenic activity of Altertoxins was demonstrated using the *Ames Salmonella typhimurium* assay in which altertoxins tested positive as mutagens and restored histidine production in *S. typhimurium* caused by genetic mutations induced by altertoxins 1, 2 and 3 (Stack & Prival, 1986; Bashyal *et al*., 2014). The altertoxins 1, 2, 3 and 5 were also found to have low therapeutic indexes of 3, 6.5, 15 and 50, which provides a narrow window for further development or be included in pre-clinical studies (Bashyal *et al*., 2014). Bashyal *et al.* (2014) recommended that epoxy perylene structure may serve as a scaffold for manipulation to develop a potent and non-toxic anti-HIV-1 drug.

An endophyte of the *Alternaria* species was from the plant *Calophylum inophylum* which has been used medicinally (Melappa *et al*., 2015). The crude extract of the isolated Alternaria species was found to almost inhibit integrase by 98% using the multiplate integration assay approach. The coumarin class of antiviral compounds were identified in the crude extract of *Alternaria species* and extracted using a microwave assisted method. The extract of coumarin compounds was assessed for reverse transcriptase and Gp120 binding inhibition by reverse transcriptase inhibition assay and Gp120 binding inhibition assay, respectively. The extracted coumarins from Alternaria species achieved 82.81% inhibition of reverse transcriptase activity and 86.38% inhibition of gp120 binding. Therefore, the *Alternaria* species can potentially be a potent source of antiviral compounds against HIV based on the findings of Mellappa *et al.* (2015).

Vora *et al.* (2021) isolated two fungal endophytes of the *Chaetomium* and *Phoma* species from the medicinal plant *Morus alba* (Vora *et al*., 2021). The anti-HIV activity of *Chaetomium* and *Phoma* species extracts and as well as phytochemical Mulberroside C was evaluated. The β-galactosidase assay and p24 antigen assay were used to evaluate anti-HIV activity. The β-galactosidase assay revealed that *Chaetomium* species extract, *Phoma* species extract and Mulberroside C inhibit HIV replication by more than 50% with $IC_{50}$'s of 10.64 µg/mL, 0.008 µg/mL and < 31.25 µg/mL respectively. The p24 antigen assay using the *Chaetomium* species extract, *Phoma* species extract, and Mulberroside C demonstrated more than 50% HIV inhibition and $IC_{50}$'s 0.1– 10 µg/mL, < 0.001 µg/mL and < 7.8 µg/mL respectively. A cell-free

HIV-1 protease inhibitor screening assay was used to determine a potential mechanism of inhibition of *Chaetomium* species extract, *Phoma* species extract and Mulberroside C. The *Chaetomium* species extract, *Phoma* species extract, and Mulberroside C phytochemical inhibited protease activity by more than 50%. Based on the findings by Vora *et al.* (2021), the extract from the *Phoma* species was the most potent inhibitor of HIV replication and had protease-inhibitory activity. This may suggest that the *Phoma* species has inhibitory activity on HIV-1 viral enzymes.

Endophyte *Penicillium* species BCt was isolated from *Calophyllum tomentosum,* a medicinal plant used to treat ulcers and diseases of the eye (Gupta & Gupta, 2020). Using gas chromatography-mass spectroscopy (GC-MS) identified, a 2H-1-Benzopyran-2-one, a coumarin compound from the extract of *Penicillium* species BCt (Melappa *et al*., 2021). The coumarin compounds in the extract were extracted by microwave-assisted extraction and evaluated for inhibitory activity against HIV-1 viral enzymes. The inhibitory activity of the extracted coumarin compound was evaluated using protease, reverse transcriptase and integrase inhibition assays. The extracted coumarins inhibited protease, integrase, and reverse transcriptase activity by 158%, 118% and 98%, respectively (Melappa *et al*., 2021). These findings may provide a strong rationale for further investigating coumarin compounds as drug candidates however, the findings of their study recommended that *in vivo* research be conducted to confirm the activity of these compounds.

Nzimande *et al.* (2022) isolated the endophyte *A. alternata* from the two medicinal plants *Sclerocarya birrea* (Marula) and *Hypoxis* species (African potato). The secondary metabolites of the endophyte were investigated to determine the compounds present in the crude extract of *A. alternata* using gas chromatography-mass spectrometry (GC-MS) (Nzimande *et al*., 2022). The results of GC-MS putatively revealed the presence of several compounds with bioactive properties, a coumarin, 3.4 dihydro 4,5,7-trimethyl-2- chromanone, which may contribute to anti-HIV activity was identified. However, there were no efforts to isolate, purify and characterize this compound in this report. The anti-HIV activity of the crude extract was evaluated using the peripheral blood mononuclear and CD4+ T cells by HIV p24 ELISA and luciferase-based antiviral assay. The PO2PL1 and PO4PR2 crude extracts suppressed p24 expression and HIV-1 replication in PBMC and CD4+ T cells.

The Kubheka *et al*. (2023) study expanded further on the findings of Nzimande *et al*. (2022) by determining the mechanism of action of the *A. alternata* crude extract and an MCX fraction

of the crude extract by performing time of addition assays to determine the mechanism of action of the anti-HIV activity. The time of addition assay revealed that crude extract inhibited attachment, reverse transcription, integration and proteolysis by 68%, 75%, 98%, and 77% (Kubheka et al., 2024). The MCX fraction of the crude extract had had improved reverse transcription and integration activity of 83% and 100% respectively. These results provide sufficient evidence that *A. alternata* may possess potent and non-toxic anti-HIV-1 secondary metabolites with therapeutic potential. Therefore, elucidating its mechanism of action in different HIV-1 subtypes is necessary. Natural products are indeed capable of inhibiting HIV-1 replication, as established above. The anti-HIV capabilities of NPs should also be investigated further considering HIV-1 subtype and drug-resistance as variables that impact PLWH treatment outcomes (Langs-Barlow & Paintsil, 2014; Nastri *et al*., 2023).

#### 2.4.2.1 Antiviral activity of Natural Products against HIV-1 subtypes

As previously discussed, HIV-1 has sequence variation, resulting in the classification into nine subtypes (Patiño-Galindo & Gonzalez-Candelas, 2017). These subtypes are also not evenly distributed globally, as described by Bbosa *et al*. (2019) as previously discussed in figure 2.1. Antiretroviral and drug-resistance research focuses on Subtype B, meaning there is a research gap in the remaining subtypes (Santoro & Perno, 2013). It is also thought that the HIV-1 subtype may be a predictor of disease progression (Venner *et al*., 2016). Since there is an uneven distribution of subtypes, a subtype B bias in research and subtype is a potential predictor of disease progression, it is important that treatment is effective against HIV-1 at the subtype level. Therefore, it is interesting to demonstrate that NPs have anti-HIV across different subtypes.

Jaduan *et al.* (2023) evaluated the antiviral activity of *Withania somnifera* a medicinal plant commonly used in ayurvedic traditional medicine. The aqueous and hydroalcoholic extracts of *W. somnifera* were then tested for antiviral activity against HIV-$1_{V028}$ subtype C and HIV-$1_{UG070}$ subtype D using a luciferase based antiviral assay and p24 antigen assay (Jadaun *et al*., 2023). The aqueous extract had an $EC_{50}$ of 0.022-0.029 mg/mL and 0.001-0.020 mg/mL for subtype C and D viruses respectively. The hydroalcoholic extract had an $EC_{50}$ of 0.130-0.378 mg/mL and 0.239-0.273 mg/mL for subtype C and D viruses, respectively (Jadaun *et al*., 2023). The aqueous and hydroalcoholic extracts of *W. somnifera* could significantly inhibit the viral replication of the subtype C and D viruses tested.

Ding *et al.* (2017) isolated alternariol 5-*O*-methyl ether (AME) from *Colletotrichum* species (Ding *et al*., 2017). Then using a using a luciferase-based antiviral assay investigated the antiviral activity of the AME compound against subtype B and C viruses pNL4-3 and ZM247Fv1Luc (Ding *et al*., 2017). The AME was able to significantly inhibit the subtype B and C viruses by approximately 40% and 70% however, an $IC_{50}$ for AME was not reported in the text. Therefore, NPs such as those from medicinal plants and fungal endophytes can have antiviral activity across different subtypes. For NPs to be considered further in the drug development pipeline it is necessary to demonstrate that NPs can overcome HIV-1 drug-resistance since this is a variable that effect the treatment outcomes of PLWH.

#### 2.4.2.2 Antiviral activity of NPs against HIV-1 drug-resistance

As previously discussed, HIV-1 drug-resistance reduces the susceptibility of HIV to existing antiretroviral drugs. Since NPs have been shown to have activity against some subtypes as previously discussed the next issue which is drug-resistance must be addressed using NPs. There are few studies that have demonstrated the effectiveness of NPs against HIV-1 drug-resistance which is an identifiable research gap in the NP literature. Natural products are proposed as a strategy to potentially overcome drug-resistance and address a research gap in the literature.

Zhang *et al.* (2017) identified the compound patentiflorin A from the medicinal plant *Justicia gendarussa* and then synthesized the compound. The HIV-$1_{1617\text{-}1}$ zidovudine and $HIV_{N119}$ nevirapine resistant strains were treated with patentiflorin A and antiviral activity was determined using a luciferase-based assay. The HIV-$1_{1617\text{-}1}$ zidovudine resistant strain had an $IC_{50}$ of 3508 nM against zidovudine and 61 nM against patentiflorin A indicating the compound was able to overcome the zidovudine resistance. The $HIV_{N119}$ resistant strain had an $IC_{50}$ of >100000 nM against nevirapine and 5.2 nM against patentiflorin A indicating the compound was able to overcome the nevirapine resistance. Zhao *et al.* (2016) introduced caffeic acid and piperazine structures which are naturally occurring compounds into the bevirimat chemical structure to produce derivatives of the novel drug to increase its activity against HIV-1 and the resistant HIV-1/V370A strain. The bevirimat derivatives of caffeic acid and piperazine had improved activity against the resistant strain by 1.39-51.40-fold indicating the derivatives could overcome resistance to unmodified bevirimat.

The Ding *et al.* (2017) study also evaluated the antiviral activity of AME against the raltegravir resistant HIV-1 strains G140SQ148H, Q148H, V151L and Q148S. The G140SQ148H, Q148H,

V151L and Q148S resistant viruses had a fold decrease in susceptibility of 134, 23, 26 and 5 to raltegraivr (Ding *et al*., 2017). However, when the G140SQ148H, Q148H, V151L and Q148S where treated with AME Ding *et al.* (2017) reported that the viruses were inhibited similarly to wildtype HIV-1 which was susceptible to AME. Natural products as described above have the potential to inhibit drug-resistant HIV-1 and importantly INSTI resistant HIV-1. This supports the rationale of testing the antiviral activity of *A. alternata* crude extract against INSTI resistant strains.

## 2.6 Review of methodology

### 2.6.1 Reporter gene assays

Previous studies conducting antiviral assays have used green fluorescent protein (GFP) and firefly luciferase reporter genes to determine viral replication by dose-response curve (McMahon *et al*., 2007; Nzimande *et al*., 2023). The luciferase-based antiviral assay requires the TZM-bl cell line which was modified to express CD4, CCR5, CXCR4 and firefly luciferase (Sarzotti-Kelsoe *et al*., 2014). Upon infection by HIV, firefly luciferase is expressed, emitting light that can be measured, allowing for quantification of relative infection (Wei *et al*., 2002). This principle has been adapted for the antiviral screening of anti-HIV compounds and substances (Nzimande *et al*., 2023). The antiviral activity of the crude extract from *A. alternata* on HIV-1 subtype B was demonstrated using a TZM-bl luciferase-based assay (Nzimande *et al*., 2022; Nzimande *et al*., 2023). A decrease in luciferase and, therefore, emitted light can be used to infer the antiviral activity of a sample or treatment.

Xing *et al.* (2016) utilised the TZM-bl cell line, which expresses beta-galactosidase that is regulated by HIV-1 Tat in an antiviral assay. Using this approach, HIV-1 inhibitors can be serially diluted, followed by infection with HIV-1 (Xing *et al*., 2016). After 48 hours, the supernatant was removed and then washed, and the β-galactosidase assay reagent was added to each well. The reaction is then stopped with β-galactosidase assay stop solution, and the absorbance is then measured at 420 nm (Xing *et al*., 2016).

Zhang *et al*. (2004) designed a system based on the production of recombinant green fluorescent protein (GFP) NL4-3 envelope-deleted pseudoviruses (Zhang *et al*., 2004). A cell line of Jurkat cells or isolated CD4+ T cells was then treated with the drug or sample of interest, followed by infection with the GFP recombinant pseudovirus (Zhang *et al*., 2004; Shen *et al*., 2008). Infectivity was then measured using flow cytometry by gating for GFP-positive cells (Zhang *et al*., 2004; Shen *et al*., 2008).

### 2.6.2 Biochemical assays

Biochemical assays are used in the drug discovery pipeline to screen for antiviral therapeutics. Potential drug targets of novel HIV-1 therapeutics can be assessed using integrase, reverse transcriptase and protease assays. Hazuda *et al*. (1999) used the strand transfer and 3' processing assays to determine the integrase inhibitory activity of *Fusarium heterosporum* metabolites (Hazuda *et al*., 1999). Subsequently, a fluorescence-based approach to determine the protease inhibitor activity of various tannins and flavonoids (Xu *et al*., 2000). Wellensiek *et al.* (2013) used a modified reverse transcriptase assay based on scintillation from Sears *et al.* (1999) to determine the inhibitory activity of desert plant fungal endophytes on reverse transcriptase. These viral enzyme-based assays are advantageous in determining inhibitory activity and therapeutic mechanisms of action; however, they can be inaccessible to researchers due to a lack of specialised equipment and the financial burden of these experiments. Commercial integrase, reverse transcriptase, and protease activity assay kits are available to researchers; however, despite being convenient, these products are costly.

In this study, the mechanism of action of the *A. alternata* crude extract was determined by time of addition (TOA) assay as described by (Kubheka *et al*., 2024). This assay was based on adding a treatment or sample at different time points post-infection of the cell line and using an indicator to quantify HIV replication at the different stages of viral replication. Lara *et al.* (2014) demonstrated a TOA using the HeLa-CD4-LTR-β-gal luciferase indicator cell line; however, Fleta-Soriano *et al.* (2014) conducted their own TOA assay using the TZM-bl luciferase indicator cell line instead (Fleta-Soriano *et al*., 2014; Lara *et al*., 2014). Fleta-Soriano *et al.* (2014) then added the treatment or samples of interest post-infection with HIV at 0, 2, 4, 6, 8, 10, 15, 18, and 24 hours. Previously, the time of addition method described by Daelemans *et al.* (2011) quantified virus-associated p24 antigen instead of using a luciferase indicator cell line to determine HIV inhibition at different time points (Daelemans *et al*., 2011).

Daelemans *et al.* (2011) added antiretroviral drugs post-infection of the MT-4 cell line in hourly intervals from time point 0 to 24 hours. The virus-associated p24 was then measured using an HIV p24 core profile enzyme-linked immunosorbent assay (ELISA) kit. The ELISA assay is based on the binding of an antigen to a fixed capture antibody and detection antibody tagged with a marker that changes colour when a substrate is added (Aydin, 2015). The ELISA assays can be typed as either homogenous or heterogenous with a washing step absent from homogenous ELISA and present in heterogenous (Aydin, 2015). The HIV-1 viruses used in this study will be generated by transfection of a viral vector into a stable cell line such as the

HEK293T (Merten *et al*., 2016). The HEK293T cell can be transfected with an HIV-1 plasmid using a lipofectamine reagent of commercial reagent such as Fugene and incubated for 48-72 hours (Merten *et al*., 2016). The supernatant should then be collected through a 0.2 μm to 0.45 μm pore size filter to harvest the generated viruses (Merten *et al*., 2016).

**2.7 Development of integrase drug-resistant HIV-1 strains**

This study generated Integrase drug-resistant mutant HIV-1 viruses by site-directed mutagenesis using the pNL4-3 HIV-1 subtype B molecular clone as a template. The site-directed mutagenesis technique was used to introduce mutations such as point mutations into plasmid DNA. The principle of this technique was based on the PCR amplification of a plasmid using complementary mutagenic primers (Yang *et al*., 2022). Parental plasmid DNA was then digested with the DpnI enzyme amplified DNA ligated, and transformed into competent cells (Yang *et al*., 2022). Site-directed mutagenesis using commercially available kits is advantageous since it can be performed with high fidelity, efficiently and quickly. However, commercial kits can be costly, and success can decrease with increased plasmid size (Yang *et al*., 2022).

The integrase drug-resistant mutations E92Q, T66K, S230R and R263K were introduced into the HIV-1 integrase using the New England Biolabs Q5 site-directed mutagenesis kit. Back-to-back forward and reverse primers are designed to introduce the point mutation of interest. Primers were designed using the NEBaseChanger to design primers for the New England Biolabs Q5 site-directed mutagenesis kit. The plasmid containing the gene of interest was then PCR amplified using a high-fidelity polymerase such as Q5 DNA polymerase to produce full-length amplicons of the plasmid containing the desired mutation. The parental plasmid DNA was digested using the DpnI restriction enzyme, and the amplicon was ligated to produce the new mutant plasmid. *Escherichia coli* was then transformed with the desired plasmid for future use (Yang *et al*., 2022). Yang *et al*., 2022 commented on routinely using the New England Biolabs Q5 site-directed mutagenesis kit and QuickChange site-directed mutagenesis kit by Agilent. Successful site-directed mutagenesis was reported for the New England Biolabs Q5 site-directed mutagenesis kit and QuickChange site-directed mutagenesis kit by Agilent (Yang *et al*., 2022).

Alternate approaches for introducing mutations, such as gBlocks available from Integrated DNA Technologies, which are double-stranded DNA fragments, can be used to study mutations similarly to site-directed mutagenesis. The gBlocks from Integrated DNA Technologies can be

modified to contain mutations of interest. However, gene fragments can only synthesised if it is between 125 and 3000 base pairs long. These limitations in gene length must be considered when designing an experiment.

The introduced mutations were then confirmed by Sanger sequencing using the BigDye Terminator v3.1 Cycle sequencing kit (Waltham, Massachusetts, United Stated of America). Sanger sequencing also referred to as chain termination utilizes fluorescently labelled dideoxy nucleotides as a substrate for DNA polymerase to hybridize into a DNA template (Saini *et al*., 2023). The extended DNA separated by capillary electrophoresis and the fluorophores was excited by a laser resulting in a fluorescent colour corresponding to nucleotide (Men *et al*., 2008). The fluorescence was detected by a detector and interprets individual nucleotides in sequence up to 800 base pairs (Chan, 2005). Sanger sequencing can be considered reliable and accurate however, it is labour-intensive and can only sequence short fragments of DNA at a time (Haga *et al*., 2013; Jamwal *et al*., 2017).

## 2.8 Summary

In summary HIV/AIDS is a global health issue that affects healthcare and PLWH. Antiretroviral drugs have saved and improved the lives of PLWH however, is not a perfect solution to the HIV/AID pandemic. Treatment outcomes of PLWH is affected by antiretroviral side effects, HIV-1 subtype and drug-resistance. Despite the success of antiretroviral drugs ongoing research is required to overcome the challenges experienced by PLWH. Natural products have been explored in the literature to overcome the challenges outlined. Current natural product literature research has demonstrated NPs can inhibit the viral replication of HIV-1. The NP research gaps need to address the matter of HIV-1 subtypes and drug-resistance. It is therefore, hypothesized that the secondary metabolites of *A. alternata* will have an antiviral effect against HIV-1 subtypes and Integrase drug-resistant strains.

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# CHAPTER 3: MANUSCRIPT

## *Alternaria alternata* crude extracts inhibit the HIV-1 replication of different HIV-1 subtypes and Integrase strand transfer drug-resistant strains

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### 3.1 Abstract

*Background*: HIV-1 drug-resistance threatens antiretroviral therapies success in combating HIV-1 in different HIV-1 subtypes and in integrase drug-resistant variants. As a result, there is a need for a novel antiretroviral treatment that can inhibit several HIV-1 subtypes and drug-resistance variants. Fungal endophytes, including *Alternaria alternata*, are particularly noteworthy for their potentially antiviral secondary metabolites. Hence, this study intends to investigate the anti-HIV properties and mechanism of action of *A. alternata* crude extract against different HIV-1 subtypes and integrase-resistant mutant strains.

*Material and methods:* The cytotoxicity and viability of *A. alternata* crude extract were determined on TZM-bl cells using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide (MTT) assay, cell cytotoxicity at 50% ($CC_{50}$) was determined. The HIV-1 integrase mutations T66K and S230R were introduced via site-directed mutagenesis. A luciferase-based antiviral assay assessed the antiviral activity of different HIV-1 subtypes (A, B, C, and D) and integrase-resistant drug-resistant strains. The selectivity indices (SI) and inhibitory concentration at fifty percent inhibition ($IC_{50}$) were also calculated. Utilising the luciferase and p24 ELISA-based time of addition test, the mechanism of action of the *A. alternata* CE was assessed.

*Results and Discussion*: The *A. alternata* crude extracts $CC_{50}$ was 300 µg/mL, showing it was not cytotoxic to cell lines. In all HIV-1 subtypes, including A, B, C, and D, the *A alternata* crude extract exhibited 100% inhibition. The results demonstrated that the *A. alternata* crude extract has therapeutic potential. Its $IC_{50}$ values were 0.16, 0.10, 0.23, 0.38, 0.17, 0.10, 0.37, and 0.60 µg/mL. Additionally, its SI values were greater than 10 at 1818, 2915, 1259, 785,

1725, 2921, 790, and 493, respectively. The crude extract demonstrated an $IC_{50}$ between 0.1628 and 0. 0.1961 μg/mL against integrase drug-resistant strains T66K and S230R, with a 0.7265 and 0. 8751-fold increase in susceptibility, showing the *A. alternata* crude extract could potentially prevent drug-resistance. The crude extract exhibited antiviral action, showing that attachment (88%/ 10.38 pg/mL), reverse transcription (81%, 13.74 pg/mL), integration (65%, 17.74 pg/mL), and proteolysis (9%, 49.32 pg/mL) were inhibited.

*Conclusions*: *A. alternata* CE demonstrates strong antiviral activity against many HIV-1 subtypes, including A, B, C, and D, as well as integrase drug-resistance strains. It has been effective in inhibiting various stages of the HIV-1 life cycle. The *A. alternata* CE may contain bioactive compounds that could be utilised to design anti-HIV-1 drugs that inhibit several virus subtypes and overcome drug-resistance. A limitation of this study was that the bioactive compounds of *A. alternata* crude extract were not identified.

Key words: Endophyte, *A alternata*, HIV-1 Subtype, Drug-resistance, Antiviral, Subtype, Drug-resistance

## 3.2 Introduction

According to UNAIDS (2023), there are 39 million PLWHs affected by the HIV pandemic worldwide. AIDS-related mortality and co-morbidities have been successfully decreased with antiretrovirals (UNAIDS., 2023). Antiretroviral drugs successfully reduced the morbidity and mortality rate of PLWH by turning HIV/AIDS into a chronic and manageable illness (Maeda *et al*., 2019; Phanuphak & Gulick, 2020). Notably, HIV-1 is diverse and highly mutational, which results in the fast development of drug-resistance. It comprises nine subtypes, making antiretroviral drugs respond differently (Langs-Barlow & Paintsil, 2014; Patiño-Galindo & Gonzalez-Candelas, 2017). However, antiretrovirals are not an appropriate long-term solution to the HIV/AIDS pandemic due to side effects such as renal dysfunction and weight gain, HIV-1 subtype diversity and emergence of drug-resistance (Chesney, 2000; Lessells *et al*., 2012; Bhatti *et al*., 2016; Hamers *et al*., 2018; Bourgi *et al*., 2020). Thus, there is a research gap which requires HIV therapeutics that do not share the limitations of modern antiretrovirals.

Natural products are being explored as a novel strategy to address antiretroviral challenges (Yuan *et al*., 2016; Atanasov *et al*., 2021). The scientific community has also attempted to address this research gap by bioprospecting of NPs from medicinal plants such as *Hypoxis hemerocallidea* used in traditional medicine to treat HIV (Mofokeng *et al*., 2020; Süntar, 2020). An early phase 1 clinical trial of an *H. hemerocallidea* methanolic extract increased survival of PLWH by years and decreased serum p24 (Albrecht, 1996). Medicinal plants are under threat of over-harvesting, which has the potential to cause irreversible environmental damage (Gail *et al*., 2015; Mofokeng *et al*., 2020). Due to these challenges, the attention has shifted to fungal endophytes of medicinal plants such as *A. alternata*, which have the potential to share medicinal properties of the plant host as well as being a more sustainable strategy to produce and study anti-HIV NPs (Ancheeva *et al*., 2020; Banyal *et al*., 2021; Nzimande *et al*., 2022). Therefore, this study aims to investigate the anti-HIV activities and mechanism of action of secondary metabolites from *A. alternata* on different HIV-1 subtypes and integrase-resistant mutant strains to address the challenges of antiretrovirals and the research gap previously described.

Previous studies have shown that secondary metabolites from endophytic fungi may possess anti-HIV activity (Singh *et al*., 2003; Wellensiek *et al*., 2013; Nzimande *et al*., 2022). A study conducted by Wellensiek *et al.* (2013) showed that fractionated crude extract of *Alternaria tenuissima*, was a strong inhibitor of HIV reverse transcriptase and HIV-1 viral replication;

however, the active compounds were not identified (Wellensiek *et al*., 2013). Subsequently, altertoxins isolated from *Alternaria tenuissima* were found to be potent inhibitors of HIV with $IC_{50}$'s 0.09 - 1.42 μM (Bashyal *et al*., 2014). However, these altertoxins were found to be mutagenic but good candidates as a chemical scaffold for further development (Stack & Prival, 1986; Bashyal *et al*., 2014). Furthermore, coumarin-type compounds isolated from *Alternaria* species inhibited reverse transcription and gp120 binding by 82.81% and 86.38%, respectively (Melappa *et al*., 2015). Recently, analogues of calanolide A, a coumarin-type compound, has been used as a scaffold to synthesise anti-HIV drugs (Khalymbadzha *et al*., 2024).

Shock and kill, gene therapy, block and lock and immune-based therapies have been proposed as strategies to eliminate viral reservoirs (Abana *et al*., 2022). Natural products have also been explored alongside these approaches as a novel strategy (Yuan *et al*., 2016; Atanasov *et al*., 2021). The fractionated crude extract of *Alternaria tenuissima* was found to be a strong inhibitor of HIV-1 reverse transcriptase and viral replication of HIV-1 however, the active compounds involved in HIV inhibition were not identified (Wellensiek *et al*., 2013). Altertoxins isolated from *Alternaria tenuissima* were found to be potent inhibitors of HIV with $IC_{50}$'s 0.09 - 1.42 μM (Bashyal *et al*., 2014). However, these altertoxins were found to be mutagenic but good candidates as a chemical scaffold for further development (Stack & Prival, 1986; Bashyal *et al*., 2014). Coumarin type compounds isolated from *Alternaria* species inhibited reverse transcription and gp120 binding by 82.81% and 86.38% respectively (Melappa *et al*., 2015).

Recently, the crude extract of *A. alternata* isolated from *Hypoxis* species was found to have an $IC_{50}$ of 0.017 – 1.170 μg/mL against HIV-1 (Nzimande *et al*., 2022). Nzimande *et al.* (2022) established that the crude extract of *A. alternata* has strong anti-HIV activity, however, did not determine the mechanism of action, the activity on non-subtype B viruses or drug-resistant strains. Recently it has also been reported that fractioned extracts of *A. alternata* were potent inhibitors of HIV and had attachment, reverse transcription, integration and proteolysis inhibitory activity (Kubheka *et al*., 2024). However, Kubheka *et al*. (2024) did not establish the anti-HIV effects on different HIV-1 subtypes and integrase drug-resistant strains. Therefore, this study investigated the antiviral effect of *A. alternata* crude extract on different HIV-1 subtypes, integrase drug-resistant strains by luciferase based antiviral assay as well as mechanism of action by time of addition assay.

## 3.3 Materials and Methods

### 3.3.1 Preparation of *Alternaria alternata* crude extract

Fungal plugs (6 $mm^2$) of *Alternaria alternata*, PO4PR2, were isolated from *Hypoxis* species (voucher no. 18233) on five-day-old malt agar and identified by Nzimande et al. (2022). The isolated *A. alternata*, PO4PR2, was inoculated into a one-litre Erlenmeyer flask (Rankem, Haryana, India) containing 200 mL malt extract broth and incubated for 21 days at 30 °C under static conditions in the dark. The fungal cultures were then extracted with 200 mL of methanol (Sigma-Aldrich, Johannesburg, South Africa) by shaking at 150 rpm using an Eins-Sci® benchtop Rotary shaker (Reflecta Laboratory supplies, Germiston, South Africa). The fungal cultures were then filtered using sterile gauze to separate the mycelia from the methanol extract was dried at 40 °C under a mild nitrogen gas steam. The dried *A. alternata*, PO4PR2, crude extract was kept at -80 ºC and diluted to a working concentration of 300 µg/mL before using 0.2% DMSO (dimethyl sulphoxide).

### 3.3.2 Maintenance of cell lines

The TZM-bl and HEK 293T cell lines were cultured as a monolayer in sterile 75 $cm^2$ culture flasks using Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, United States of America) supplemented with 10% foetal bovine serum (FBS; heat-inactivated and gamma irradiated) (LTC Biosciences, Gainesville, United States of America), 25 mM HEPES (Thermo Fisher Scientific, Waltham, United States of America), and 50 µL/mL gentamicin (Thermo Fisher Scientific, Waltham, United States of America). The TZM-bl cell line was modified from a HeLa cell line clone to express CD4 and CCR5, allowing for HIV-1 infection and firefly luciferase under the control of the HIV-1 long terminal repeat (LTR) (Wei *et al*., 2002). The HEK 293T cell line is a highly transfectable cell line that produces high titres of HIV-1 virions (Ikeda *et al*., 2003). The TZM-bl and HEK 293T cell lines were incubated at 37 °C with 5% $CO_2$ to confluency. At confluency cells were washed with phosphate buffered saline (PBS) (Thermo Fisher Scientific, Waltham, United States of America). Cell monolayers were then trypsinised with 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, United States of America). Trypsinized cells were then counted using 0.4% trypan blue dye and subcultured till confluency was reached. Trypan blue was utilized since live cells do not absorb the dye while dead cells do, allowing for quantification of cell count and cell viability. The Jurkat cell line, which is an immortalized cell line of human T lymphocyte cells, were

maintained in RPMI-1640 media (Thermo Fisher Scientific, Waltham, United States of America) supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and incubated at 37 °C with 5% $CO_2$.

### 3.3.3 Cytotoxicity and cell viability testing of *A. alternata* crude extract using MTT assay (3-[4,5-Dimethylthiazol-2-yl]-2,5 Diphenyl Tetrazolium Bromide)

*Alternaria alternata* crude extract cell cytotoxicity and viability were assessed by MTT (3- [4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) cell viability assay using the CYQUANT MTT cell proliferation kit (Thermo Fisher Scientific, Waltham, United States of America). A 96-well culture plate was seeded with 15,000 TZM-bl cells/200 µL DMEM per well and incubated at 37 $^oC$ with 5% $CO_2$.). Following this, 10 µL of the drug control azidothymidine and the *A. alternata* crude extract was diluted fivefold, and control wells were neither the control drug AZT nor the crude extract were added, followed by incubation at 37°C with 5% $CO_2$ and 95% humidity for 48 hours. 10 µL of 12 mM of MTT solution was added to each well and incubated for four hours at 37°C with 5% $CO_2$ and 95% humidity. Then, 50 µL of 100% DMSO was added to each well and incubated for 10 minutes. The absorbance was then measured at a wavelength of 540 nm using the Victor Nivo microplate reader (PerkinElmer, Waltham, United States of America). The percentage of cell viability was calculated using the formula below (Nzimande *et al*., 2022).

$$\%Cell\ viability = \frac{Sample\ absorbance - media\ control}{Mean\ media\ control\ absorbance}$$

The drug control azidothymidine and *A. alternata* crude extract $CC_{50}$ (50% cytotoxic concentration) were then determined using GraphPad Prism software (Version 5) by dose-response curve.

### 3.3.4 Plasmid DNA Preparation and Purification

The glycerol stocks of the plasmids pNL4.3 (subtype B), YU2 (subtype B), CM7 (subtype C) and CM9 (subtype C) were cultured in 250 mL of prewarmed LB (Luria-Bertani) broth supplemented with 1% ampicillin at 37°C in a shaking incubator at 230 RPMI. Plasmid DNA was then prepared and purified using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hulsterweg,

Netherlands) following the manufacturer's instructions. The Nanodrop OneTM and 1% gel electrophoresis assessed the plasmid DNA quality and purity.

## 3.3.5 Site-Directed Mutagenesis

### 3.3.5.1 Design of site-directed mutagenesis primers

The Stanford University HIV drug-resistance database was analysed for drug-resistance mutations that confer resistance to raltegravir and dolutegravir (Shafer, 2006). Integrase mutations were selected that conferred resistance to raltegravir and dolutegravir as single mutations and caused a fold change in susceptibility to raltegravir and dolutegravir greater than 1. Integrase inhibitor drug-resistance mutations T66K, E92Q S230R and R263K were then selected based on the parameters described. The primers in Table 2.1 were then designed to introduce the respective mutations T66K, E92Q, S230R and R263K using the NEBaseChanger primer design tool (New England Biolabs, Massachusetts, United States of America). The Q5 site-directed mutagenesis kit was used to introduce the respective T66K, E92Q, S230R and R263K mutations into HIV-1 molecular clone pNL4.3 using the primers designed (Ipswich, Massachusetts, United States of America).

**Table 2.1**: Site-directed mutagenesis primers designed using NEBaseChanger primer design tool to introduce the respective mutations T66K, E92Q, S230R and R263K.

| Primer name | Sequence (5'- 3') |
|---|---|
| T66K_FWD | ATTAGATTGT**AAA**CATTTAGAAGGAAAGA |
| T66K_REV | TGCCATATCCCTGGACTAC |
| E92Q_FWD | TATCCCAGCA**CAA**ACAGGACAAG |
| E92Q_REV | ACCTCTGCCTCTATATATCC |
| S230R_RWD | TTACAGAGAC**CGC**AGAAATCCAC |
| S230R_REV | TAAACCCGAAAATTTTGAATTTTTG |
| R263K_FWD | AGTACCAAGG**AAA**AAAGTAAAAATC |
| R263K_REV | ACCTTTATGTCACTGTTATCTTGTATTAC |

### 3.3.5.2 Site-directed mutagenesis PCR reaction

Following the manufacturer instructions of the Q5 site-directed mutagenesis kit, an exponential amplification polymerase chain reaction (PCR) was conducted for each respective mutation (New England Biolabs, Massachusetts, United States of America). The plasmid pNL4.3 was used as the template for site-directed mutagenesis since it is an HIV-1 recombinant viral clone

of HIV-1. Into a thin-walled PCR tube 12.5 µL of Q5 hot start high-fidelity master mix, 1 µL at 1–25 ng/µL of pNL4.3 DNA template, 9 µL of deionised water and 1 µL of 10 µM the respective forward and reverse primers T66K_FWD-ATTAGATTGT**AAA**CATTTAGAAGGAAAGA, T66K_REV-TGCCATATCCCTGGACTAC, E92Q_FWD- TATCCCAGCA**CAA**ACAGGACAAG, E92Q_REV- ACCTCTGCCTCTATATATCC, S230R_RWD-TTACAGGGAC**CGC**AGAAATCCAC, S230R_REV-TAAACCCGAAAATTTTGAATTTTTG, R263K_FWD-AGTACCAAGG**AAA**AAAGTAAAAATC and R263K_REV-ACCTTTATGTCACTGTTATCTTGTATTAC. The 25 µL PCR reaction was mixed and thermocycled on the Applied Biosystems miniamp thermal cycler (Thermo Fisher Scientific, Waltham, United States of America) using the cycling conditions 98 °C for 30 seconds, 98 °C for 10 seconds then 58-61 °C for 7 minutes 15 seconds then for 25 cycles and 72 °C for 2 minutes.

#### 3.3.5.3 Kinase, ligase and DpnI digestion

The respective thin-walled PCR tubes were then removed from the thermocycler. Then, according to the manufacturer's instructions, a kinase, ligase and DpnI digestion (KLD) reaction was used to digest parental DNA and ligate the PCR product (New England Biolabs, Massachusetts, United States of America). To a thin-walled PCR tube, 1 µL of the PCR products produced in the Site-directed mutagenesis PCR reaction step of 2X KLD reaction buffer, 1 µL 10X KLD enzyme mix, 3 µL nuclease-free water to a final volume of 10 µL. The contents of the PCR tube were mixed and incubated at room temperature for one hour.

#### 3.3.5.4 Transformation of competent Escherichia coli cells

Briefly, competent *Escherichia coli* cells were thawed on ice (New England Biolabs, Massachusetts, United States of America). Then, 5 µL of each respective digested PCR product from the kinase, ligase and DpnI digestion was added to each tube of competent cells and gently flicked 4 times. Each tube was then incubated on ice for 5 minutes, and then subsequently, heat was shocked at 42°C for 30 seconds. Then, to each respective tube of competent cells, 950 µL of super optimal broth with catabolite repression (SOC) medium was added and incubated at 37°C for one hour with shaking at 250 rpm. Then 100 µL of cells from each respective tube

was spread on pre-warmed LB agar plates supplemented with ampicillin and incubated at 37°C overnight.

#### 3.3.5.5 Isolation of Plasmid DNA Using Plasmid Mini-Prep

Colonies from the transformation of competent *Escherichia coli* cells above were picked using a sterile plastic pipette tip and transferred to 11 mL of LB media supplemented with 1% ampicillin. The inoculated LB media was then incubated at 37°C overnight with shaking at 250 rpm. The plasmid DNA was then extracted and purified using the GeneJET miniprep kit following the manufacturer's instructions (Waltham, Massachusetts, United States of America). Then, plasmid DNA quality and purity were assessed using the Nanodrop One$^{TM}$ and 1% gel electrophoresis.

## 3.3.6 Sequencing

#### 3.3.6.1 Integrase PCR amplification

The HotStarTaq Master Mix (QIAGEN, Hulsterweg, Netherlands) was used to PCR amplify the integrase gene of the selected site-directed mutants. The PCR reaction was assembled according to the manufacturer's instructions. In a thin-walled PCR tube 25 µL of HotStarTaq, 1 µL (0.5 µM) forward primer 4155F- GTACCAGCACACAAAGGRATTG, 1 µL (0.5 µM) reverse primer 5264R-CCTGTATGCAGACCCCAATATGTT, 1 µL (< 1 µg) DNA template and 22 µL RNAse-free water was added and mixed (Chrysostomou *et al*., 2020). The reaction mixture was then placed in the Applied Biosystems miniamp thermal cycler (Thermo Fisher Scientific, Waltham, United States of America) using the conditions 95 °C for 5 minutes and 1 cycle, 94 °C for 1 minute, then 55 °C for 1 minute, then 72 °C for 3 minutes and 30 seconds for 30 cycles, 72 °C for 10 minutes and 1 cycle. The integrase PCR amplicons were confirmed and assessed using the Nanodrop One$^{TM}$ and 1% gel electrophoresis.

#### 3.3.6.2 Cycle sequencing reaction

The BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, United States of America) master mix reaction was prepared using 2 µL of v3.1 5X Sequencing Buffer, 3.2 µL 10 µM of the respective forward and reverse primers 4155F-GTACCAGCACACAAAGGRATTG and 5264R-CCTGTATGCAGACCCCAATATGTT, 0.4 µL of BigDye Terminator v3.1 Ready Reaction Mix, 20 ng of DNA template and the reaction

volume was filled up to 10 µL with deionised water. Then 10 µL of the reaction mix was aliquoted into a MicroAmp™ Optical 96-well reaction PCR plate (Thermo Fischer Scientific, Waltham, United States of America) and mixed. The positive control from the BigDye Terminator v3.1 cycle sequencing kit was set up using the forward primer M13-TGTAAAACGACGGCCAGT and PGEM as the template. The MicroAmp™ Optical 96-well reaction PCR plate was then thermocycled using the parameters 96 °C for 1 minute, 96 °C for 10 seconds then 50 °C for 5 seconds then 60 °C for 4 minutes and 25 cycles then kept on hold for 4 °C indefinitely (Applied Biosystems miniamp thermal cycler, Thermo Fischer Scientific, Waltham, United States of America).

#### 3.3.6.3 Cycle-sequencing reaction clean up

To the corresponding wells of the MicroAmp™ Optical 96-well reaction PCR plate (Thermo Fischer Scientific, Waltham, United States of America) 1 µL 125 mM EDTA (pH 8.0) was added and mixed. Then 26 µL of a 3 M NaOAc (pH 5.2) and 100% ethanol solution was added to each corresponding well. The MicroAmp™ Optical 96-well reaction PCR plate was then sealed with an adhesive cover and incubated at room temperature for 10 minutes. Next, the 96-well PCR plate was centrifuged at 3000 g for 20 minutes. After that, sterile paper was placed on top of the 96-well PCR plate, and it was centrifuged at 150 g for 5 minutes. Next, each matching well received 35 µL of 70% cold EtOH, and the wells were centrifuged at 3000 g for 5 minutes. The plate was then inverted again over sterile paper town and centrifuged for 1 minutes at 150 g. The sample were then dried at 50°C for 5 minutes and then sealed with a foil adhesive cover and stored at -20°C. The products were then sequenced by capillary electrophoresis on the 3500 Genetic analyzer (Applied Biosystems, Waltham, United Stated of America). The full-length integrase gene sequences were then assembled and aligned in Unipro UGENE (version 50.0, Unipro) using contig assembly program version 3 (CAP3).

### 3.3.7 Generation of different HIV-1 subtypes by transfection

To prepare the HIV-1 subtypes prepared HEK 293T cells were transfected with the relevant HIV-1 virus using the plasmid stocks generated by plasmid DNA preparation and purification of (pNL4.3, pYU2, pCM7, and pCM9) plasmids donated by Dr Omolara Baiyegunhi (Farinre, 2020). Briefly, $3 \times 10^6$ HEK 293T cells in the T-75 culture flask were seeded and incubated overnight at 37°C with 5% $CO_2$ and 95% humidity. Using 48 µL of Fugene transfection reagent

(Promega, Wisconsin, United States of America), 12 µL of the corresponding plasmid DNA from Table 2.2 transfected HEK 293T cells in the T-75 cell culture flask. After four hours, the transfected HEK 293T cells' medium was changed, and they were then incubated for 48 hours at 37°C, 5% $CO_2$, and 95% humidity. The supernatant of the transfected cells was then harvested and filtered with a 0.45 µm filter aliquoted in 2 mL cryovials, and stored at -80°C.

### 3.3.8 Titration of HIV-1 viruses

The infectivity of the generated HIV-1 viruses was evaluated by infecting TZM-bl cell lines. The viruses' $TCID_{50}$ (50% Tissue culture infectious dose) was determined. Then 100 µl DMEM supplemented with 10% FBS, 25 mM HEPES buffer and 1% penicillin-streptomycin (Pen Strep) was added to a 96-well cell culture plate. In replicates of 4, 25 µL of the virus stock produced by transfection was added to 4 wells in row one and serially diluted 4-fold. Then, 10000 TZM-bl cells containing 37.5 µg/mL DEAE-dextran (Diethylaminoethyl-dextran) to allow for infection were added and incubated for 48 hours at 37°C with 5% $CO_2$ and 95% humidity. From each well, 100 µL of cell culture medium was removed, and in the absence of light, 100 µL of Bright glo$^{TM}$ luciferase reagent was added, except for the control in row 12. The supernatant was aspirated in each well, 150 µL was transferred to the black 96-well plate, and the RLU's (Relative light units) were immediately quantified in a luminometer at 540 nm (PerkinElmer, USA). The $TCID_{50}$ was extrapolated in the plotted graph by selecting the dilution concentration that elicited a minimum of 15000 and maximum of 50000 relative light units (RLU).

### 3.3.9 Luciferase-based antiviral assay

An antiviral assay based on luciferase was used to assess the HIV-1 inhibition of fungal extracts (Modi *et al*., 2013; Nzimande *et al*., 2022; Kubheka *et al*., 2024). The luciferase-based assay was conducted using the viruses generated by transfection. Firstly 150 µL, 100 µL and 140 µL of DMEM was added to a 96- well cell culture plate as a cell control, virus control and sample respectively. Briefly, 11 µL of the drug controls (azidothymidine or raltegravir and dolutegravir) and *A. alternata* crude extract were added to a Corning Costar 96- well cell culture plate (Corning, New York, United States of America), and 3-fold dilution was performed in 140 µL DMEM supplemented with 10% FBS, 25 mM HEPES buffer and 1% Penicillin-Streptomycin. Infected TZM-bl cell line (virus control) and uninfected TZM-bl cell

lines (cell control) were included as the experimental control. Then 50 µL of, PC148, MC2297, pNL4-3, YU2, CM7, CM9, PC60, PC178, T66K, and S230R viruses at $TCID_{50}$ were added to the 96-well cell culture plate and incubated for 1 hour. In each 96-well cell culture plate well, 10,000 cells were added and cultured for 48 hours at 37°C with 5% $CO_2$ and 95% humidity with 37.5 µg/mL DEAE-dextran. After 48 hours of incubation, 150 µL of media was removed and replaced with 100 µL of Bright gloTM luciferase reagent without light exposure. The supernatant was aspirated, and 150 µL of the supernatant and Bright gloTM luciferase reagent mixture was added to a Corning Costar 96-well black plate and was immediately measured in the Victor Nivo microplate reader at 540 nm (PerkinElmer, Waltham, United States of America). Then, the percentage of viral inhibition was calculated using the formula below.

$$\%\ HIV\ inhibition = \frac{Average\ sample - average\ control}{1-(Average\ viral\ control - Average\ control)} x100$$

The $IC_{50}$ (Inhibitory concentration) was then determined by dose-response curve using using GraphPad Prism software (Version 5).

**Table 2.2**: Subtype A and D viruses used in luciferase based antiviral assay

| **Virus** | **Subtype** |
|---|---|
| PC148 | A |
| MC 2297 | A |
| PC60 | D |
| PC178 | D |

### 3.3.10 Time of Addition assay-based inhibitory mechanism in the HIV-1 life cycle

#### 3.10.1 Luciferase-Based Time of Addition Assay

The time of addition assay was used to determine how the anti-HIV drugs interfered with the HIV-1 replication cycle. The luciferase-based time of addition was conducted as described by Lara *et al*. (2014) and Kubheka *et al* 2024. (Lara *et al*., 2014). Briefly, in a 96-well cell culture plate, 150 µL of 10 000 TZM-bl cells were seeded in each well and incubated at 37 °C, 5% $CO_2$. The TZM-bl cells were then infected with 50 µL of each HIV-1 subtype (PC148, MC2297, pNL4-3, YU2, CM7, CM9, PC60, PC178) and T66K and S230R INSTI drug-resistant strains. Then 15 µL of antiretroviral drug controls maraviroc (CCR5 antagonist, 1 hour), azidothymidine (5 hours), raltegravir (8 and 10 hours) and amprenavir (16 hours) were added to a well-contained infected cells. The *A. alternata* crude extract was then added at 1, 3,

5, 6, 8, 10, 16 and 20 hours post-infection. The 96-well cell culture plate was then incubated at 37 °C with 5% $CO_2$ for 48 hours. Then 150 µL of cell culture medium was removed and 100 µL of Bright-Glo luciferase reagent (Promega, Madison, Wisconsin, USA) was added and mixed. Then 150 µL of the Bright-Glo luciferase reagent and cell medium mixture was transferred to a 96-well black plate and measured at 540 nm in luminometer. The percent inhibition was calculated using the formula below.

$$\%\ HIV\ inhibition = \frac{Average\ sample - average\ control}{1 - (Average\ viral\ control - Average\ control)} x100$$

**3.3.10.2 HIV-1 p24 time-based ELISA to measure p24 titre**

The HIV-1 p24-based time of addition assay was performed using a modified protocol (Daelemans *et al*., 2011; Wang *et al*., 2011). The Jurkat cell lines was maintained in RPMI-1640 media supplemented with 10% (v/v) heat-inactivated foetal bovine serum (FBS) and incubated at 37 °C with 5% $CO^2$. To a Corning Costar 24-well cell culture plate (Corning, USA, New York), 500 µL of 500 000 Jurkat cells were maintained under 37°C, and 5% $CO_2$ was seeded in each well. Then, all wells with Jurkat cells were infected with 100 µL of HIV-1 viruses (CM9, MC2297) and integrase drug-resistant strains T66K and S230R. The CM9 and MC2297 viruses were selected since the *A. alternata* crude extract had the most potent anti-HIV activity against these two viruses. The integrase drug-resistant strains T66K and S230R were selected since these were the only functional integrase-resistant strains available. Then 50 µL of Antiretroviral drug controls maraviroc (CCR5 antagonist, 0-1 hour), azidothymidine (nucleoside reverse transcriptase inhibitor 3 hours), raltegravir (INSTI inhibitor 8 - 14 hours) and amprenavir (protease inhibitor 14-24 hours) were added to well contained infected cells. The *A. alternata* crude extract was added to infected cells at 0, 1, 3, 8, 10, 14, 24 and 30 hours post infection. After 48 hours post-infection, the HIV-1 p24 titre was then measured using the Quicktiter Lentiviral Quantification kit (Cell Biolabs Inc., San Diego, United States of America) following the manufacturer's instructions. The HIV-1 p24 was then measured at an absorbance of 450 nm using the Victor Nivo microplate reader (Promega, Madison, Wisconsin, United States of America).

The viral titre of p24 was calculated following manufacturer's instructions below:

The average genome size of lentivirus is 8 kbp; therefore,

1 ng lentiviral RNA = (1x10-9) g / (8,000 bp x 660 g/bp) X 6 x 1023 = 1.1 x 108 VP

$$\text{Virus Titre} = \frac{\text{Amount of lentiviral RNA (ng) X1.1 x 108 VP X (20 µL/5 µL )}}{\text{Viral sample volume (mL)}}$$

$$\text{Virus Titre (VP/mL)} = \frac{\text{Amount of lentiviral RNA (ng) X 4.4 x 108 VP/ ng}}{\text{Viral sample volume (mL)}}$$

## 3.4 Results

### 3.4.1 Cytotoxicity and cell viability of *A. alternata* crude extract using MTT

Figure 3.1A illustrates that the viability of the *A alternata* crude extract is above 80% using MTT *(*3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay on the TZM-bl cell line. Figure 3.1B shows that the CC50 of the crude extract was 300 ug/mL. The cell viability (figure 3.1A) and cell cytotoxicity (figure 3.1 B) of the drug control azidothymidine and 0.2% DMSO were also greater than 80%.

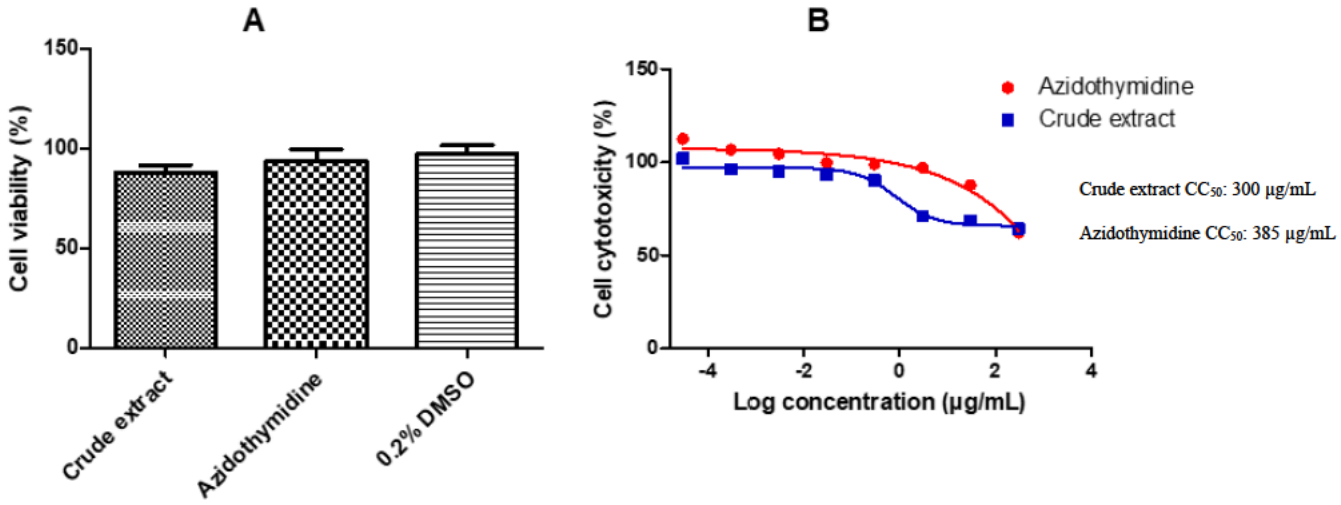



**Figure 3.1**: Cell viability **(A)** and cell cytotoxicity **(B)** of the *A. alternata* crude extract were determined by MTT assay. The Cell viability **(A)** y-axis represents the percentage cell viability of the TZM-bl cells, and the x-axis represents the treatments crude extract, azidothymidine and 0.2% DMSO. The TZM-bl cells treated Dose-response curves **(B)** showing the percentage cell cytotoxicity were used to determine the $CC_{50}$ of the *A. alternata* crude extract blue line ($CC_{50}$= 300 µg/mL), azidothymidine red line ($CC_{50}$= 385 µg/mL). The y-axis of the dose-response curves shows the percent cell cytotoxicity, and the x-axis shows the log concentration of the *A. alternata* crude and azidothymidine. The x-axis concentrations of 300; 30; 3; 0,3; 0.03; 0.003; 0.0003 and 0.00003 µg/mL to a log scale of 2,47; 1,47; 0.47, -0. 52, -1.52; -2,52; -3.52; -4,52.

### 3.4.2 Antiviral activity of *A. alternata* crude extract against different HIV-1 subtypes

The *A. alternata* crude extract antiviral activity was evaluated using a luciferase-based antiviral assay against TZM-bl cells infected with PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 subtype A, B, C and D HIV-1. One dose-dependent curve of 1 virus from each subtype was then presented graphically in (figure 3.2 **A, B, C** and **D**). The reverse transcriptase inhibitor azidothymidine was used as a positive control. At 300 µg/mL, azidothymidine and the *A. alternata* crude extract elicited a 100% inhibition of HIV replication of PC148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 subtype A, B, C and D viruses. An $IC_{50}$ less than 10 µg/mL indicates the treatment was a highly active inhibitor of HIV-1. The $IC_{50}$'s of PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 treated with the *A. alternata* crude extract in (figure 3.2 **A, B, C** and **D**) were less than 10 µg/mL meeting the requirements to be considered highly active against the subtype A, B, C and D viruses.

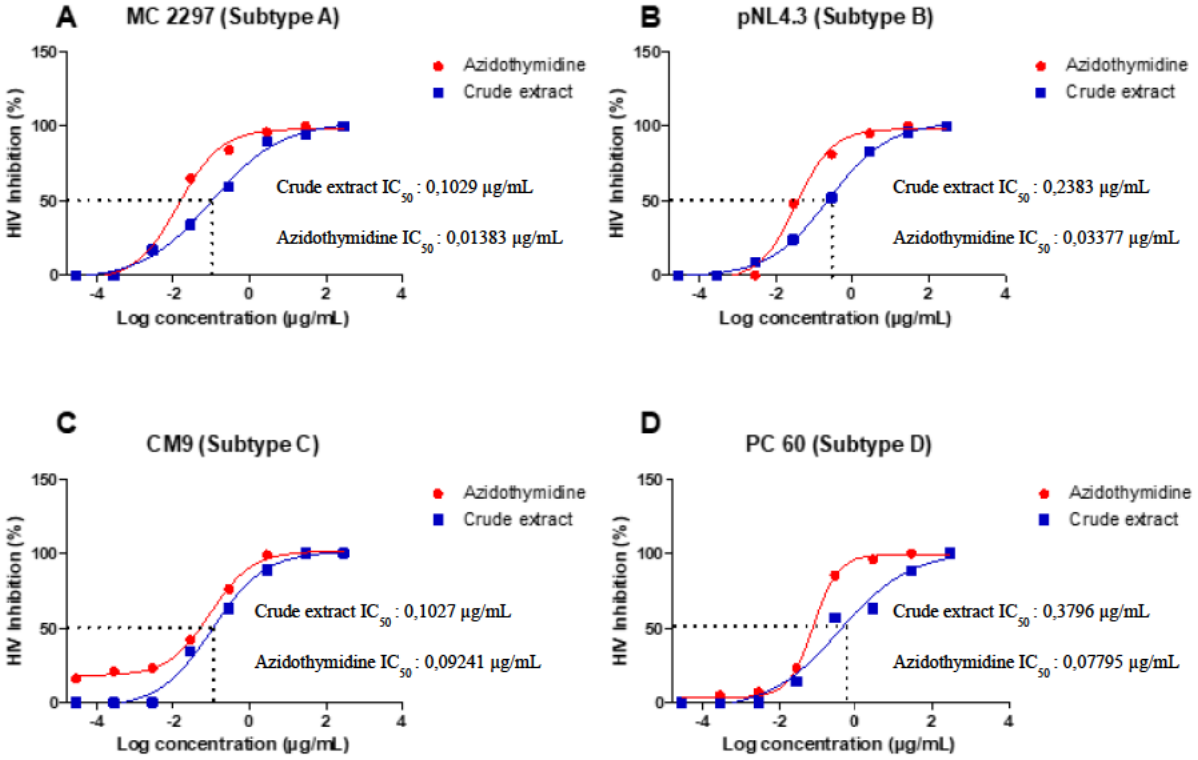



**Figure 3.2**: Dose-dependent curves showing antiviral activity of *A. alternata* crude extract against MC 2297 **(A)**, pNL4.3 **(B)** CM9 **(C)** and PC 60 **(D)** HIV-1 viruses belonging to respective subtypes A, B, C and D. The y-axis of **(A, B, C and D)** shows the inhibition of HIV as a percentage. The x-axis **(A, B, C and D)** shows the log concentration of the crude extract or azidothymidine. The red line shows the positive control azidothymidine and the blue line shows the *A. alternata* CE. The x-axis concentrations of 300; 30; 3; 0,3; 0.03; 0.003; 0.0003 and 0.00003 µg/mL to a log scale of 2,47; 1,47; 0.47, -0. 52, -1.52; -2,52; -3.52; -4,52.

### 3.4.3 Analysis of half-maximal inhibitory concentrations and selective indexes of *A. alternata* crude extract against HIV-1 subtypes A, B, C and D viruses

The $IC_{50}$ data for the *A. alternata* crude extract and Azidothymidine drug control against PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 HIV-1 viruses was summarised in (table 2.3). This data was further used to calculate the selective indexes of positive control Azidothymidine and the *A. alternata* crude extract for PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178. Selective indexes were calculated as the ratio $CC_{50}/IC_{50}$. An SI greater than 10 indicates the treatment or sample has therapeutic potential (Mogatle *et al*., 2008). The SI's of PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 in the table treated with the *A. alternata* crude extract were all greater than 10, meeting the requirement to have therapeutic potential.

**Table 2.3**: Half-maximal inhibitory concentrations ($IC_{50}$) and selectivity indices (SI) of *A. alternata* crude extract and drug control azidothymidine against different HIV-1 subtypes

| Virus | Subtype | $IC_{50}$ µg/mL (Crude extract) | $IC_{50}$ µg/mL (Azidothymidine) | SI (Crude extract) | SI (Azidothymidine) |
|---|---|---|---|---|---|
| PC 148 | A | 0.1650 | 0.04025 | 1818 | 9565 |
| MC 2297 | A | 0.1029 | 0.01383 | 2915 | 27838 |
| pNL4.3 | B | 0.2383 | 0.03377 | 1259 | 11401 |
| YU2 | B | 0.3821 | 0.04226 | 785 | 9110 |
| CM7 | C | 0.1739 | 0.05798 | 1725 | 6640 |
| CM9 | C | 0.1027 | 0.09241 | 2921 | 37487 |
| PC 60 | D | 0.3796 | 0.07795 | 790 | 4939 |
| PC 178 | D | 0.6086 | 0.08129 | 493 | 4736 |

### 3.4.4 Relative fold change in $IC_{50}$'s of *A. alternata* crude extract against different HIV-1 subtypes.

The $IC_{50}$'s of the PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 HIV-1 subtype A, B, C and D viruses was expressed as a fold change in $IC_{50}$ relative to the $IC_{50}$ of pNL4.3 in (figure 3.3) since pNL4.3 is known to be infectious. The fold change in $IC_{50}$ relative to control pNL4.3 was calculated as a ratio of virus subtype $IC_{50}$/ pNL4.3 $IC_{50}$. A fold change in $IC_{50}$ less than 1 indicates an increase in $IC_{50}$ relative to control pNL4.3, indicating greater susceptibility relative to pNL4.3. The viruses CM9, PC 148 and CM7 had fold changes in $IC_{50}$ of less than 1 relative to pNL4.3, indicating greater susceptibility to the *A. alternata* crude extract relative to pNL4.3.

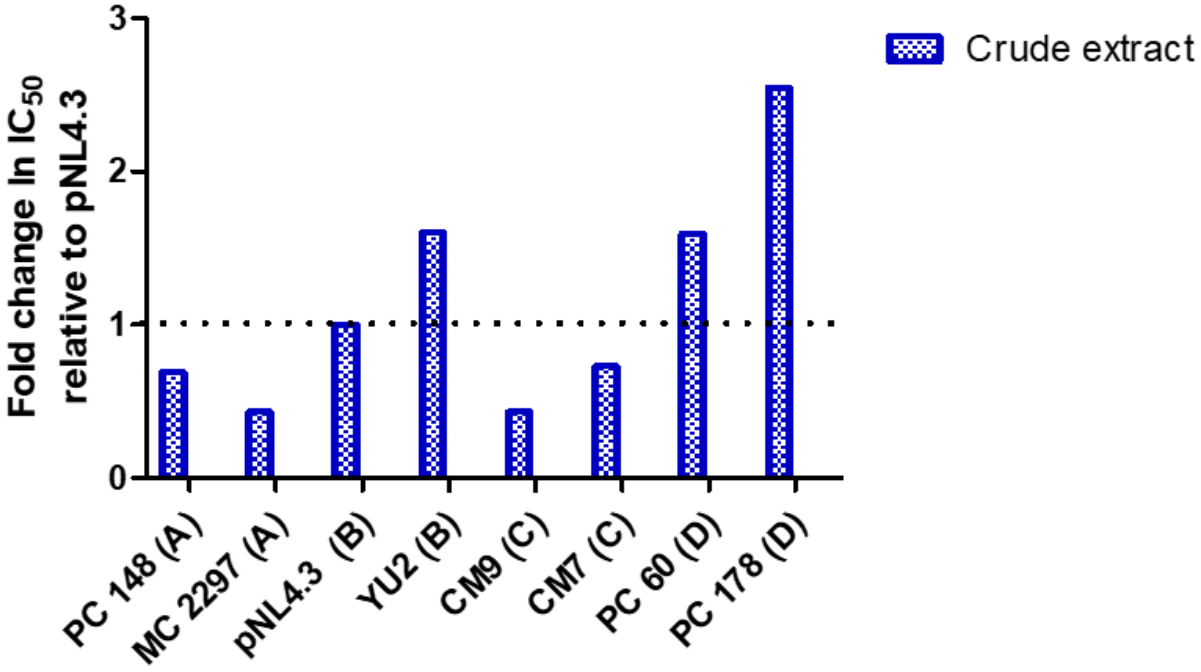



**Figure 3.3**: The fold change in $IC_{50}$ of viruses PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 treated with the *A. alternata* crude extract relative to pNL4.3. The y-axis represents the fold change in $IC_{50}$ relative to pNL4.3. The x-axis represents the virus treated with the *A. alternata* crude extract. The blue bars indicate that *A. alternata* crude extract was the treatment used.

### 3.4.5 Confirmation of mutations introduce by site-directed mutagenesis

The full-length integrase gene sequences of the site-directed mutants were assembled in (figure 3.4). The assembled sequences were aligned with the wildtype integrase gene to confirm the presence of the integrase drug-resistance mutations. In (figure 3.4**A, B, C** and **D**) the mutations T66K, E92Q, S230R and R263K were confirmed.

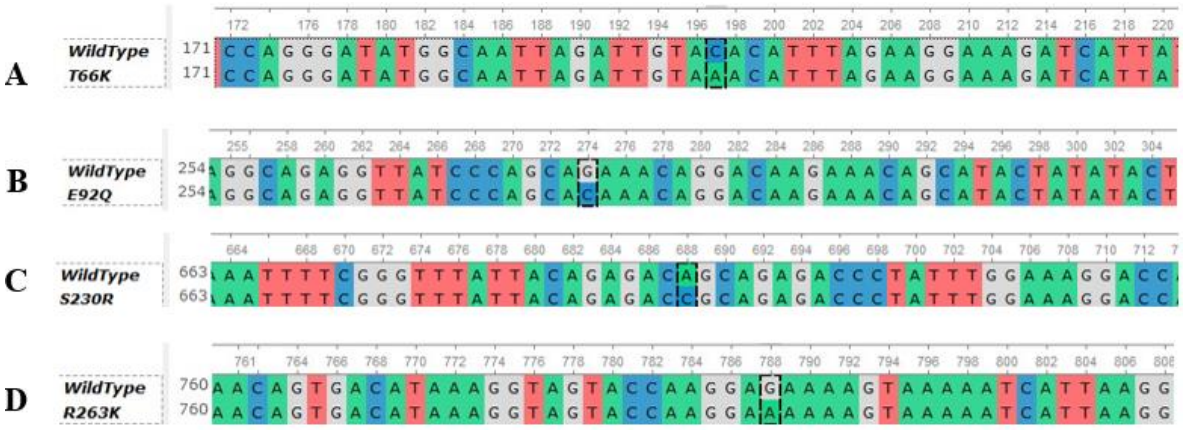



**Figure 3.4**: Sequence alignment of integrase T66K **(A)**, E92Q **(B)**, S230R **(C)** and R263K **(D)** site-directed mutants with wildtype integrase gene.

### 3.4.6 Infectivity of HIV-1 subtypes and Integrase drug-resistant strains T66K, E92Q S230R and R263K

The infectivity of the different subtype HIV-1 viruses PC 148, MC 2297, YU2, CM7, CM9, PC 60 and PC 178 as well as T66K, E92Q, S230R and R263K integrase drug-resistant mutants in TZM-bl cells in figure 3.5**A** and **B** were expressed as relative light units (RLU's) against the wildtype virus pNL4.3. The integrase drug-resistant HIV strains were considered infectious if it was able to produce at least 15000 RLU's. The different subtype HIV viruses PC 148, MC 2297, YU2, CM7, CM9, PC 60 and PC 178 (figure 3.5**A**) as well as integrase drug-resistant HIV strains T66K and S230R (figure 3.5**B**) were considered infectious since both viruses had produced more than 15000 RLU's. However, integrase drug-resistant HIV strains E92Q and R263K in figure 3.5**B** were not considered infectious since they did not produce at least 15000 RLU's.

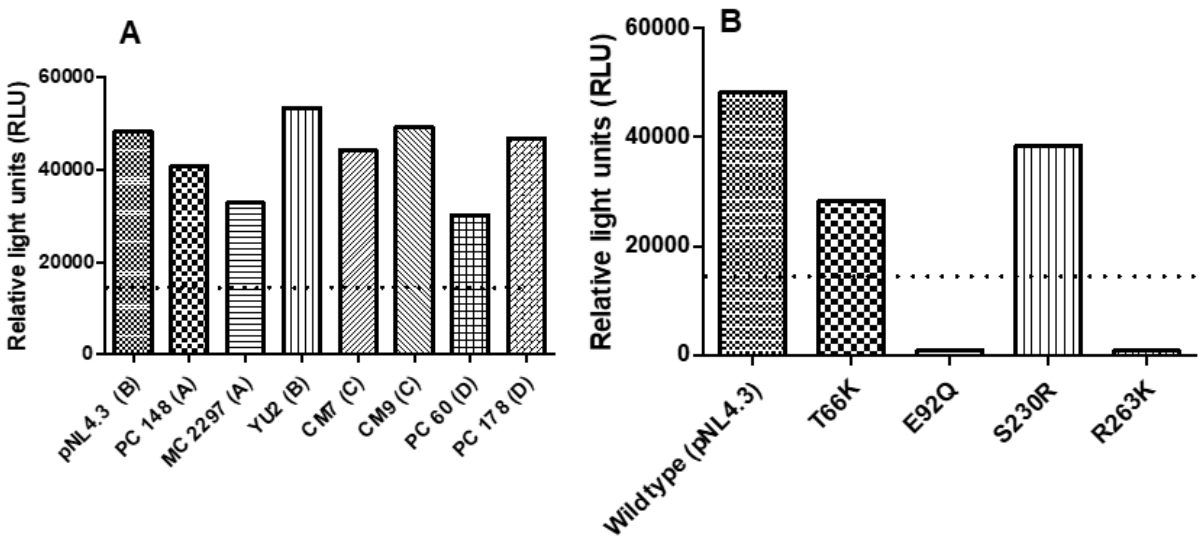
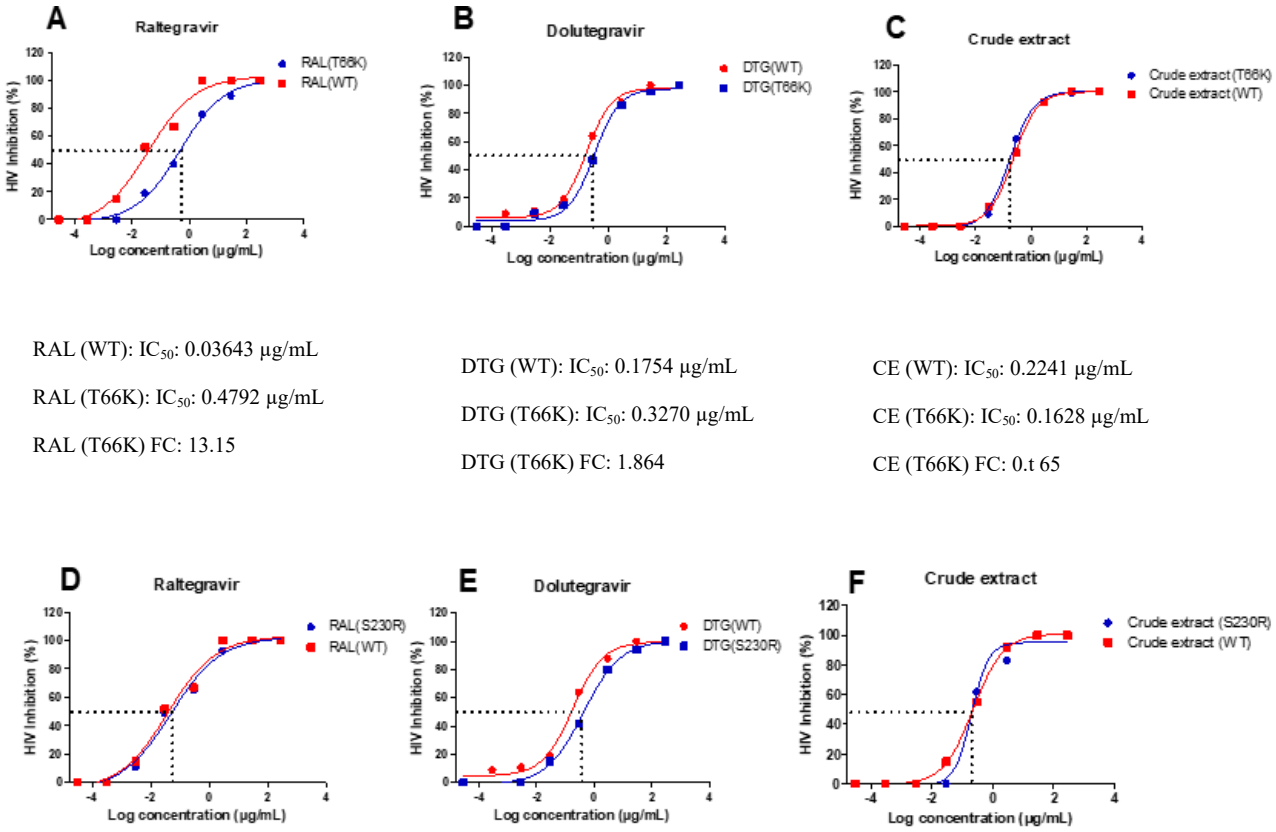



**Figure 3.5**: The infectivity of different subtype HIV-1 viruses PC 148, MC 2297, YU2, CM7, CM9, PC 60 and PC 178 **(A)** T66K, E92Q, S230R and R263K **(B)** integrase drug-resistant mutants relative to control wildtype virus pNL4.3. The y-axis represents the infectivity of **(A and B)** the viruses in relative light units (RLU's). The x-axis of **(A and B)** represents the virus being tested.

### 3.4.7 Antiviral activity of *A. alternata* crude extract against integrase drug-resistant mutants

The antiviral activity of *A. alternata* crude extract and integrase inhibitor drug controls raltegravir (RAL) and dolutegravir (DTG) was evaluated against the integrase inhibitor drug-resistance strains T66K and S230R using a luciferase-based assay (Figure 3.6). At 300 µg/mL raltegravir, dolutegravir and the *A. alternata* crude extract elicited 100% inhibition of HIV replication of T66K and S230R drug-resistant strains. An $IC_{50}$ less than 10 µg/mL indicates the treatment was a highly active inhibitor of HIV. In (figure 3.6) the $IC_{50's}$ of T66K and S230K treated with the *A. alternata* crude extract indicate it was highly active against the integrase drug-resistant strains.




RAL (WT): $IC_{50}$: 0.03643 µg/mL

RAL (T66K): $IC_{50}$: 0.4792 µg/mL

RAL (T66K) FC: 13.15

DTG (WT): $IC_{50}$: 0.1754 µg/mL

DTG (T66K): $IC_{50}$: 0.3270 µg/mL

DTG (T66K) FC: 1.864

CE (WT): $IC_{50}$: 0.2241 µg/mL

CE (T66K): $IC_{50}$: 0.1628 µg/mL

CE (T66K) FC: 0.t 65

RAL (WT): $IC_{50}$: 0.03643 µg/mL

RAL (S230R): $IC_{50}$: 0.04754 µg/mL

RAL (S230R) FC: 1.305

DTG (WT): $IC_{50}$: 0.1754 µg/mL

DTG (S230R): $IC_{50}$: 0.3833 µg/mL

DTG (S230R) FC: 2.166

CE (WT): $IC_{50}$: 0.2241 µg/mL

CE (S230R): $IC_{50}$: 0.1961 µg/mL

CE (S230R) FC: 0.8751

**Figure 3.6**: Dose response curves of *A. alternata* crude extract integrase drug-resistant strains T66K and S230R. The y-axis of **(A, B, C, D, E and F)** shows the inhibition of shows the inhibition of HIV as a percentage. The x-axis **(A, B, C, D, E and F)** shows the log concentration of the *A. alternata* crude extract, raltegravir or dolutegravir. The red lines **(A, D)**, **(B, E)** and **(C, F)** shows raltergravir (WT), dolutergravir (WT) and *A. alternata* crude extract (WT) respectively. The blue lines **(A, B and C)** and **(D, E and F)** shows raltergravir (T66K), dolutergravir (T66K) *A. alternata* crude extract (T66K) and raltergravir (S230R), dolutergravir (S230R) *A. alternata* crude extract (S230R) respectively. The x-axis shows concentrations of 300; 30; 3; 0,3; 0.03; 0.003; 0.0003 and 0.00003 µg/mL to a log scale of 2,47; 1,47; 0.47, -0. 52, -1.52; -2,52; -3.52; -4,52.

### 3.4.9 Time Of Addition

The key inhibitory targets in the HIV life cycle and occurrence post-infection in hours up to 24 hours. The principle of time of addition is that key stages in the life cycle of HIV occur during attachment (0-1 hours), reverse transcription (3-8 hours), integration (6-12 hours) and proteolysis (16-24 hours).

#### 3.4.9.1 Luciferase-Based Time Of Addition

A luciferase-based time of addition assay was used to determine the mechanism of action of the *A. alternata* crude extract. The attachment, reverse transcription, integrase, and protease inhibitors maraviroc, azidothymidine, raltegravir, and amprenavir, respectively, were used as drug controls for each key stage of the HIV life cycle. The *A. alternata* crude extract was added at intervals of 1, 3, 5, 6, 8, 10, 16 and 20 hours. The *A. alternata* crude extract was considered to have activity if the percent HIV inhibition was 50% or more at a time interval. The luciferase-based time of addition assay was performed on viruses PC 148, MC 2297, YU2, CM7, CM9, PC 60 and PC 178 (figure 3.4.9) as well integrase drug-resistant strains T66K and S230R (figure 3.4.9) however, one virus from each subtype in (figure 3.4.9) was graphically represented below.

The *A. alternata* crude extract HIV-1 had overall inhibitory activity during attachment (84%), reverse transcription (80%), integration (64%) and proteolysis (13%) against subtype A, B, C and D (figure 3.7.1**A**) viruses. The *A. alternata* crude extract had inhibitory activity during attachment (88%), reverse transcription (80%), integration (63%) and proteolysis (16%) against the HIV-1 subtype A viruses (figure 3.7.1**B**). The *A. alternata* crude extract had inhibitory activity during attachment (79%), reverse transcription (78%), integration (64%) and proteolysis (11%) against the HIV-1 subtype B (figure 3.7.1**C**). The *A. alternata* crude extract had inhibitory activity during attachment (83%), reverse transcription (84%), integration (69%) and proteolysis (19%) the HIV-1 subtype C (figure 3.7.1**D**) viruses. The *A. alternata* crude extract had inhibitory activity during attachment (86%), reverse transcription (79%), integration (65%) and proteolysis (5%) The HIV-1 subtype D (figure 3.7.1**E**) viruses.

The *A. alternata* crude extract had overall inhibitory activity during attachment (92%), reverse transcription (81%), integration (65%) and proteolysis (5%) against the HIV integrase drug-resistant strains (figure 3.7.2**F**) viruses. The *A. alternata* crude extract had inhibitory activity during attachment (91%), reverse transcription (87%), integration (54%) and proteolysis (2%)

against the integrase drug-resistant virus T66K (figure 3.7.2**G**). The *A. alternata* crude extract had inhibitory activity during attachment (93%), reverse transcription (89%), integration (75%) and proteolysis (7%) against the integrase drug-resistant virus S230R (figure 3.7.2**H**).

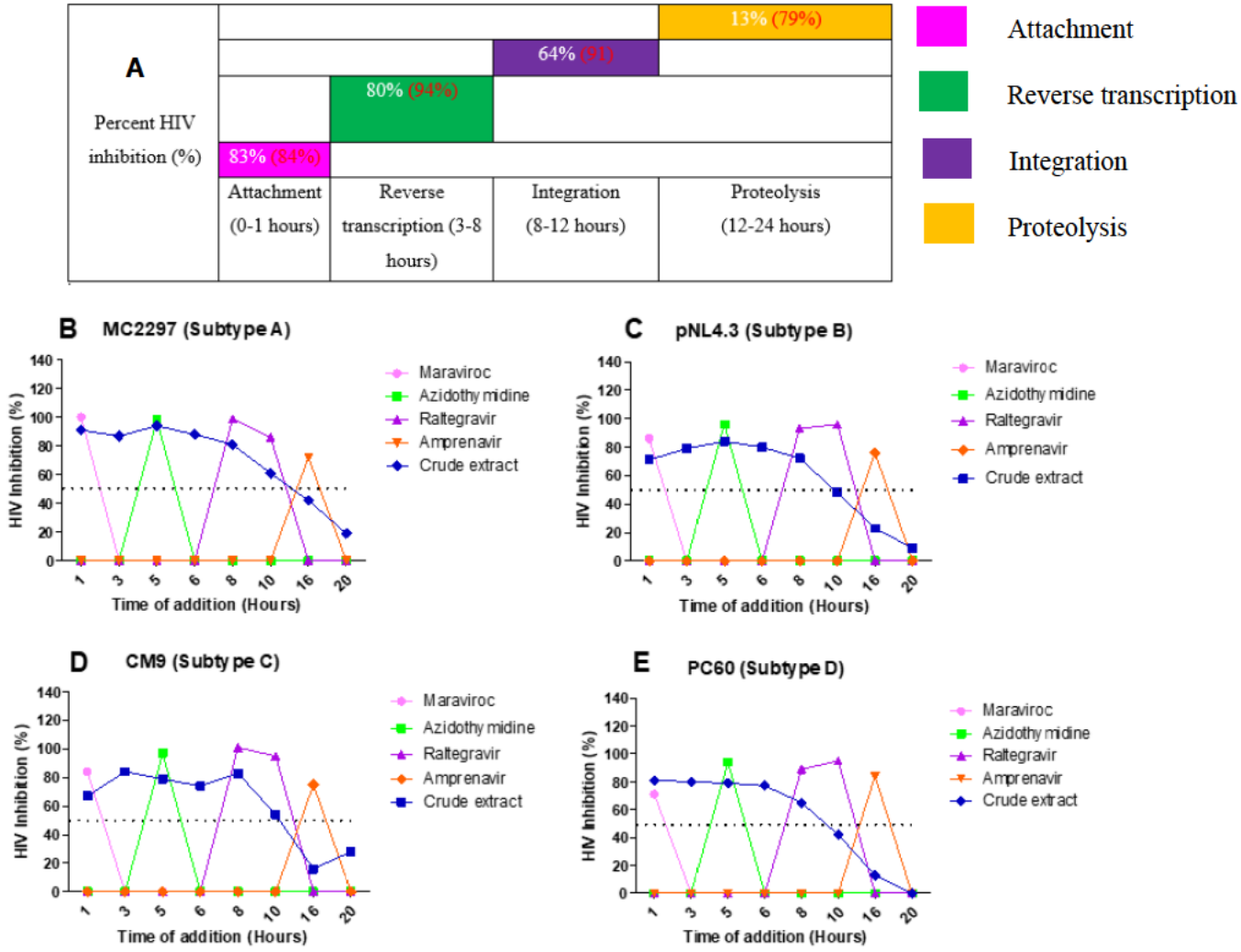



**Figure 3.7.1**: The overall inhibition of the subtype A, B, C and D viruses treated with *A. alternata* crude extract during different stages in the HIV life cycle **(A)**. For **(A)** the y-axis represents the inhibition as a percentage and the x-axis as the stage of in the HIV life cycle.. The red text represents the percent inhibition of the drug controls maraviroc, azidothymidine, raltegravir and amprenavir respectively. The white text represents to percent inhibition of the *A. alternata* crude extract. Luciferase-based time of addition of viruses MC2297 **(B)**, pNL4.3 **(C)**, CM9 **(D)** and PC60 **(E)**. The y-axis of **(B, C, D and E)** the shows the inhibition of HIV as a percentage. The x-axis of **(B, C, D and E)** the shows the time of addition in hours. For **(B, C, D and E)**. The pink, green, purple, orange and blue lines of **(B, C, D and E)** represents maraviroc, azidothymidine, raltegravir, amprenavir and *A. alternata* crude extract respectively. The *A. alternata* crude extract was added at 1, 3, 5, 6, 8, 10, 16 and 20 hours post infection.

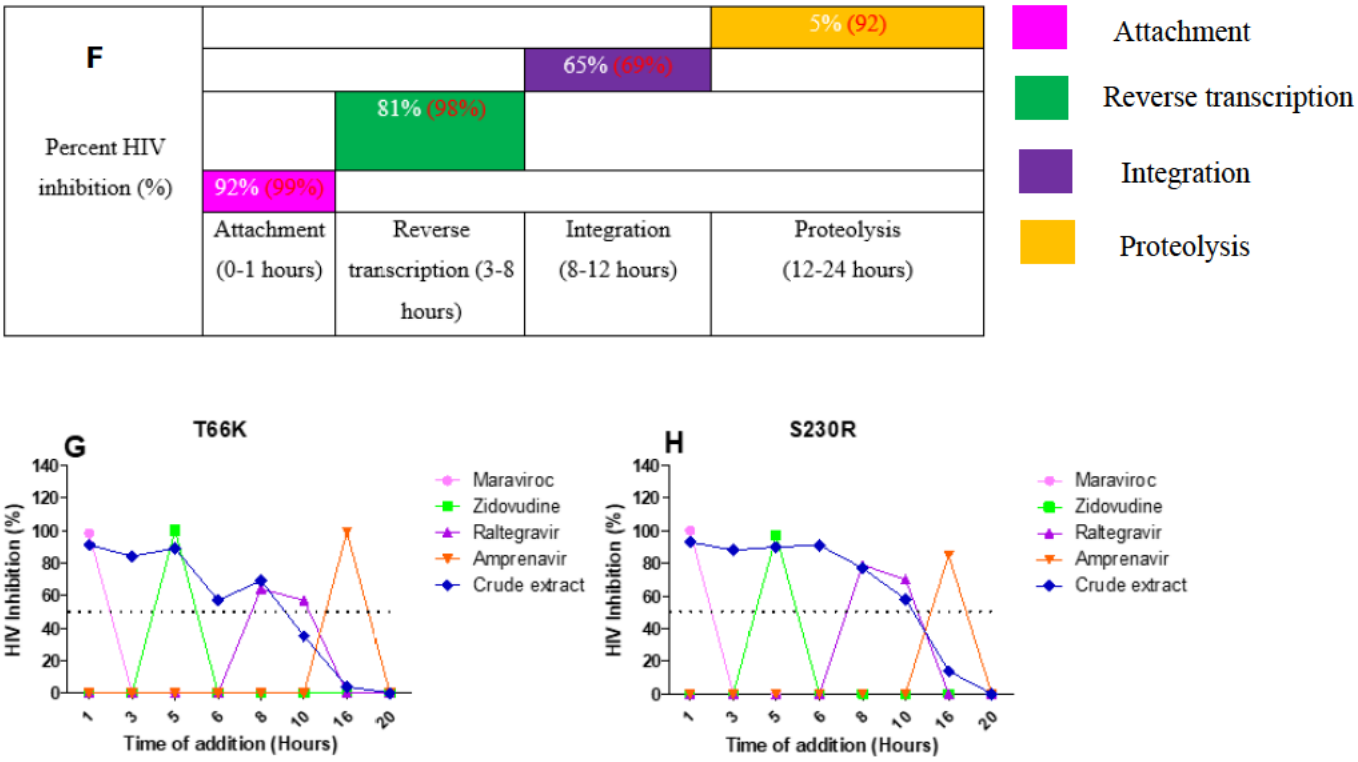



**Figure 3.7.2**: The overall inhibition of the integrase drug-resistant viruses treated with *A. alternata* crude extract during different stages in the HIV life cycle **(F)**. For **(F)** the y-axis represents the inhibition as a percentage and the x-axis as the stage of in the HIV life cycle. The red text represents the percent inhibition of the drug controls maraviroc, azidothymidine, raltegravir and amprenavir respectively. The white text represents to percent inhibition of the *A. alternata* crude extract. Luciferase-based time of addition of integrase drug-resistant viruses T66K **(G)** and S230R **(H)**. The y-axis of **(G and H)** the shows the inhibition of HIV as a percentage. The x-axis of **(G and H)** the shows the time of drug addition in hours. For **(G and H)**. The pink, green, purple, orange and blue lines of **(G and H)** represents maraviroc, azidothymidine, raltegravir, amprenavir and *A. alternata* crude extract respectively. The *A. alternata* crude extract was added at 1, 3, 5, 6, 8, 10, 16 and 20 hours post infection.

### 3.4.9.2 HIV-1 p24 based time of addition assay

A p24-based time of addition assay was used to determine the mechanism of action of the A. alternata crude extract (figure 3.8.1). The attachment, reverse transcription, integrase, and protease inhibitors maraviroc, azidothymidine, raltegravir, and amprenavir, respectively, were used as drug controls for each key stage of the HIV life cycle. The *A. alternata* crude extract was added at intervals of 0, 1, 3, 8, 10, 14, 24 and 30 hours. The viruses CM9 and MC2297 were selected to undergo the p24-based time of addition assay since both displayed $IC_{50}$'s with the highest anti-HIV activity. The integrase drug-resistant viruses T66K and S230R were also selected as integrase drug-resistant viruses to undergo the p24-based time of addition assay. For the viruses, CM9, MC2297 and S230R, an HIV-1 p24 titre equal to 40 pg/mL or less were used to indicate the antiviral activity of *A. alternata* crude extract. For the virus T66K, due to a weaker HIV-1 p24 titre, a titre equal to 20 pg/mL or less was used to indicate the antiviral activity of *A. alternata* crude extract.

The *A. alternata* crude extract had overall inhibitory activity during attachment (10.38 pg/mL), reverse transcription (13.74 pg/mL), integration (17.74 pg/mL) and proteolysis (49.32 pg/mL) against the HIV viruses (figure 3.8.1**A**). The *A. alternata* crude extract had inhibitory activity during attachment (14.53 pg/mL), reverse transcription (14.24 pg/mL), integration (21.19 pg/mL) and proteolysis (49.85 pg/mL) against the HIV-1 subtype A virus MC2297 (figure 3.8.1**B)**. The *A. alternata* crude extract had inhibitory activity during attachment (8.04 pg/mL), reverse transcription (12.20 pg/mL), integration (20.94 pg/mL) and proteolysis (49.67 pg/mL) against the HIV-1 subtype C virus CM9 (figure 3.8.1**C**). The *A. alternata* crude extract had inhibitory activity during attachment (9.28 pg/mL), reverse transcription (16.69 pg/mL), integration (12.34 pg/mL) and proteolysis (31.25 pg/mL) against the INSTI drug-resistant virus T66K (figure 3.8.1**D**). The *A. alternata* crude extract had inhibitory activity during attachment (9.66 pg/mL), reverse transcription (9.68 pg/mL), integration (16.49 pg/mL) and proteolysis (50.28 pg/mL) against the integrase drug-resistant virus S230R (figure 3.8.1**E**).

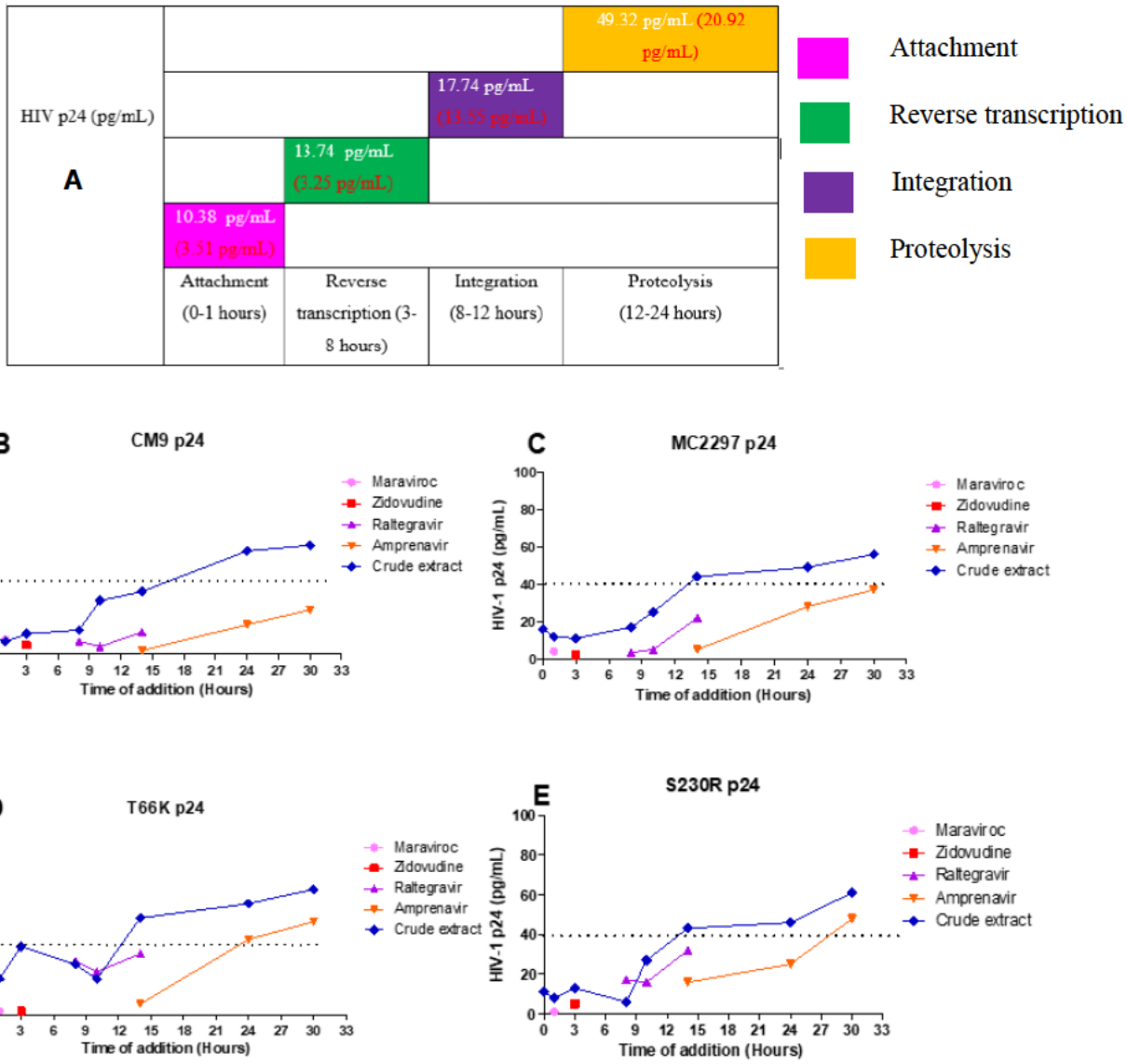



**Figure 3.8.1**: The overall HIV p24 titre (pg/mL) of the CM9, M2297, T66K and S230R viruses treated with *A. alternata* crude extract during different stages in the HIV life cycle **(A)**. For **(A)** the y-axis represents the inhibition as a percentage and the x-axis as the stage of in the HIV life cycle. The red text represents the percent inhibition of the drug controls maraviroc, azidothymidine, raltegravir and amprenavir respectively. The white text represents to percent inhibition of the *A. alternata* crude extract. p24-based time of addition of viruses CM9 **(B)**, M2297 **(C)**, T66K **(D)** and S230R **(E)**. The y-axis of **(B, C, D and E)** shows the concentration of HIV p24 in pg/mL. The x-axis of **(B, C, D and E)** shows the time of addition in hours. The pink, green, purple, orange and blue lines of **(B, C, D and E)** represents maraviroc, azidothymidine, raltegravir, amprenavir and *A. alternata* crude extract respectively. The *A. alternata* crude extract was added at 0, 1, 3, 8, 10, 14, 24 and 30 hours post infection. The pink, green, purple and orange squares represent attachment, reverse transcription, integration and proteolysis respectively.

In (figure 3.8.2**F** and **G**), The inhibition profiles of the integrase-resistant strains T66K and S230R during luciferase and p24-based time of addition assays. In (figure 3.8.2**F**) the T66K virus is inhibited by 7% more during integration by raltegravir than the *A. alternata* crude extract. In (figure 3.8.2**F**) S230R has an identical inhibition profile during integration when treated with raltegravir or the *A. alternata* crude extract. In (figure 3.8.2**G**) the T66K virus has decrease by 1.16 pg/mL when treated with the *A. alternata* crude extract in comparison to raltegravir. For S230R virus the p24 titre differs by 0.26 pg/mL when treated with *A. alternata* crude extract and raltegravir.

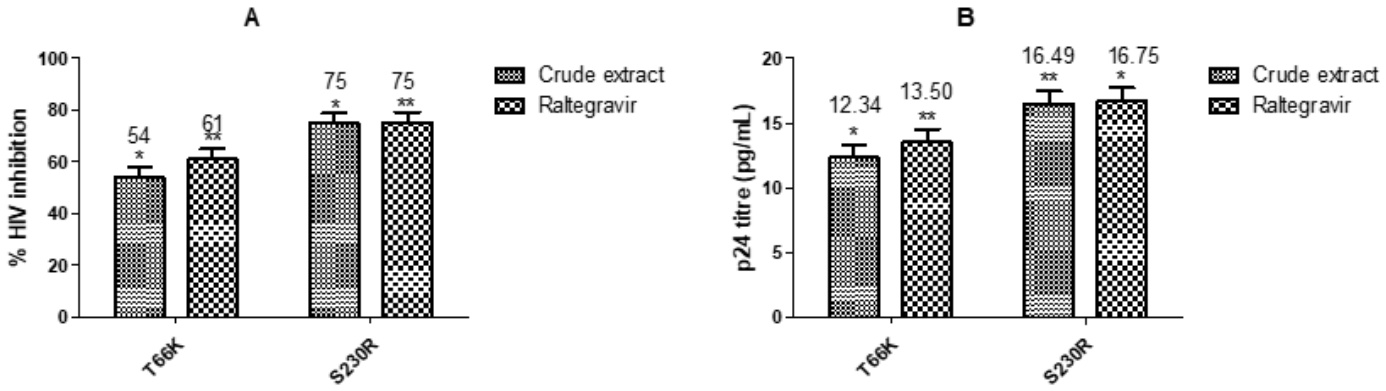



**Figure 3.8.2**: The inhibition profiles of integration of integrase-resistant strains during time of addition. A grouped bar graph illustrating the percent inhibition of *A. alternata* crude extract against T66K and S230R strains during integration **(F)**. The y-axis is the percent of HIV inhibition during integration. The x-axis shows the T66K and S230R strains treated with either the *A. alternata* crude extract or raltegravir. A grouped bar graph illustrating the p24 titre in pg/mL of *A. alternata* crude extract against T66K and S230R strains during integration **(G)**. The y-axis is the p24 titre in pg/mL inhibition during integration. The x-axis shows the T66K and S230R strains treated with either the *A. alternata* crude extract or raltegravir. A significant difference between crude extracts * P< 0.05, a significant difference between raltegravir drug control ** = P< 0.05.

## 3.5 Discussion

Measuring mitochondrial respiration was necessary to determine the cell viability and cytotoxicity of the *A. alternata* crude extract on the TZM-bl cell line. At high concentrations of the *A. alternata* crude extract, the viability of TZM-bl cells decreased due to a decrease in mitochondrial respiration. The decrease in mitochondrial respiration was proportional to the reduction of MTT by mitochondrial succinate dehydrogenase in living cells (Aslantürk, 2018). The cell viability of TZM-bl cells treated with the *A. alternata* crude extract was greater than 80%. A 30% reduction in cell viability suggests that a sample of interest has had a cytotoxic effect; therefore, the *A. alternata* crude extract has not had a cytotoxic effect on TZM-bl cells (Berger *et al*., 2017). A crude extract is said to have *in vitro* cytotoxicity if the $CC_{50}$ of the extract is greater than 30-40 µg/mL (Jimoh *et al*., 2024). Therefore, the *A. alternata* crude extract did not have a cytotoxic effect on the TZM-bl cell line. This finding agrees with the previous study that found *A. alternata* crude extract non-cytotoxic with a $CC_{50}$ of 43.5 µg/mL (Kubheka *et al*., 2024).

The antiviral activity of the *A. alternata* crude extract on the TZM-bl cells infected with the PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 HIV-1 viruses was measured to determine inhibition as a percentage and the $IC_{50}$. At higher and progressively lower concentrations, the *A. alternata* crude extract maintained antiviral activity greater than 50% inhibition. A decrease in antiviral activity was caused by a decrease in the concentration of the *A. alternata* crude extract, resulting in increased infection of TZM-bl cells by the respective viruses and, therefore, an increase in RLU's when measured (Xing *et al*., 2016). An extract can be described as highly active if the $IC_{50}$ is less than 10 µg/ml, according to Moga et al. (2021). The *A. alternata* crude extract had an $IC_{50}$ less than 10 µg/ml against the PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 subtype A, B, C and D viruses.

To date the *A. alternata* crude extract has not been previously reported against PC 148, MC 2297, YU2, CM7, CM9, PC 60 and PC 178 viruses. The studies by Nzimande *et al.* (2022) and Kubheka *et al.* (2024) determined the $IC_{50}$ of *A. alternata* crude extract against pNL4.3 ranges from 2.389 to 0.017 µg/mL. The *A. alternata* crude extract was highly active against the subtype A, B, C and D viruses. Thus, the *A. alternata* crude extract effectively inhibits HIV-1 subtype A, B, C and D viruses. A sample with an SI value of 10 or greater can be said to have therapeutic potential and should be investigated further (Indrayanto *et al*., 2021). The SI values of the *A. alternata* crude extract against PC 148, MC 2297, pNL4.3, YU2, CM7, CM9, PC 60 and PC 178 subtype A, B, C and D viruses were greater than 10 and can be said to have

therapeutic potential. These results agree with those of Kubheka et al. (2024), who found the *A. alternata* crude extract SI value of 2559 against the virus pNL4.3, which was greater than 10, indicating therapeutic potential.

The antiviral activity of the *A. alternata* crude extract, raltegravir and dolutegravir was assessed on TZM-bl cells infected with integrase drug-resistant virus T66K and S230R utilizing the same principle applied as previously discussed. The integrase drug-resistant virus T66K FC in resistance to raltegravir and dolutegravir was 13.15 and 1.864, respectively. It was previously reported that this mutation conferred a FC 9.6 and 2 to raltegravir and dolutegravir respectively (Kobayashi *et al*., 2008; Healthcare, 2013). The FC in susceptibility of integrase drug-resistant virus T66K was therefore, similar to that previously reported by Kobayashi *et al.* (2008) and Healthcare (2013). The integrase drug-resistant virus S230R FC in susceptibility to raltegravir and dolutegravir was 1.305 and 2.166 respectively. It was previously reported that this mutation conferred a FC in susceptibility of 1.52 and 3.85 to raltegravir and dolutegravir respectively (Pham *et al*., 2018). The FC in susceptibility of integrase drug-resistant virus S230R was therefore, similar to that previously reported by Pham *et al.* (2018).

The *A. alternata* crude extract had achieved 100-50% viral inhibition of integrase drug-resistant viruses T66K and S230R in decreasing concentrations from 300 µg/mL. The FC in susceptibility of integrase drug-resistant viruses T66K and S230R treated with *A. alternata* crude extract was 0.7265 and 0.8251 respectively. Since the FC in susceptibility was less than 1 it may suggest the integrase drug-resistance was overcome or another possible mechanism of action. The *A. alternata* crude extract had greater inhibitory activity than raltegravir against the integrase drug-resistant virus T66K. There was not a remarkable difference in inhibition during integration when the integrase drug-resistant virus T66K virus was treated with the *A. alternata* crude extract and raltegravir. Raltegraivr had a greater inhibitory effect than the *A. alternata* crude extract against the integrase drug-resistant virus S230R. The *A. alternata* crude extract and raltegravir had similar inhibitory during integration against integrase drug-resistant virus S230R. Raltegravir is a strand transfer inhibitor and therefore, binds to integrase with greater affinity when the enzyme is in a complex with the viral DNA of HIV (Nguyen et al., 2011). Raltegravir competes with DNA by binding to the DNA binding sites at residues such as N155 and Q148 (Mouscadet & Tchertanov, 2009). Based on the inhibition profiles of the integrase drug-resistant strains an alternate pathway such as attachment and reverse transcription inhibition could explain the activity of the *A. alternata* crude extract against the integrase drug-resistant mutants. However, to validate integrase activity of the *A. alternata* crude extract

during time of addition, integrase activity assays and Alu gag PCR are recommended to determine integrase inhibitory activity of the crude extract.

The mechanism of action of the *A. alternata* crude extract was assessed by luciferase-based and HIV-1 p24-based methods on TZM-bl and Jurkat cells. The luciferase-based method revealed the *A. alternata* crude extract had activity during attachment, reverse transcription and integration when the viruses pNL4.3, YU2, CM9, CM7, PC60, MC2297, PC148, PC178 and T66K were treated. In addition, *A. alternata* crude extract had activity during attachment, reverse transcription and integration activity when CM9, MC 2297 and S230R were treated. The HIV-1 p24-based method revealed that *A. alternata* crude extract had activity during attachment, reverse transcription and integration when the viruses CM9, MC2297 and T66K were treated. In addition, *A. alternata* crude extract had activity during attachment, reverse transcription and integration activity when the virus S230R was treated.

Based on the results above, it is evident that the *A. alternata* crude extract has activity during attachment, reverse transcription and integration; however, activity during proteolysis appears to be weaker. Identifying and isolating the individual compounds that may have anti-HIV-1 activity during these stages is essential. Kubheka *et al. (*2024*)* showed that the *A. alternata* crude extract had a similar activity profile during attachment, reverse transcription and integration; however, it demonstrated greater activity during proteolysis using both luciferase and p24-based time of addition methods against the pNL4.3 virus. In agreement with our finding, The fractionated *A. alternata* crude extract demonstrated an even greater activity during attachment, reverse transcription, integration and proteolysis (Kubheka *et al*., 2024). This may indicate that an active compound is captured in the MCX fraction of the *A. alternata* crude extract, which may explain the increase in activities. The antiviral compounds cyclotrisiloxane octamethyl; propionitrile; pyrrolol[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl) (10.42%); silane, diethyl ethoxy (2-ethoxyethyloxy); coumarin, 3,4-dihydro-4,5,7-trimethyl-4,5,7-trimethyl2-chromanone; and 1,2-cyclobutanedicarbonitrile partially identified by GC-MS could be responsible for these activities described above (Nzimande *et al*., 2022). The coumarin class of compounds has previously been demonstrated to be able to inhibit reverse transcriptase, integrase and protease inhibitory activity of 82.81%, 98% and protease 78%, respectively (Melappa *et al*., 2015). The presence of coumarins potentially may explain the time of addition profile described in this study.

### 3.6 Ethics approval

Ethics approval for this study was obtained from the Biomedical Research Ethics Administration (BREC). The protocol reference number: BREC/00005156/2023 was approved.

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## 3.8 Supplementary figures

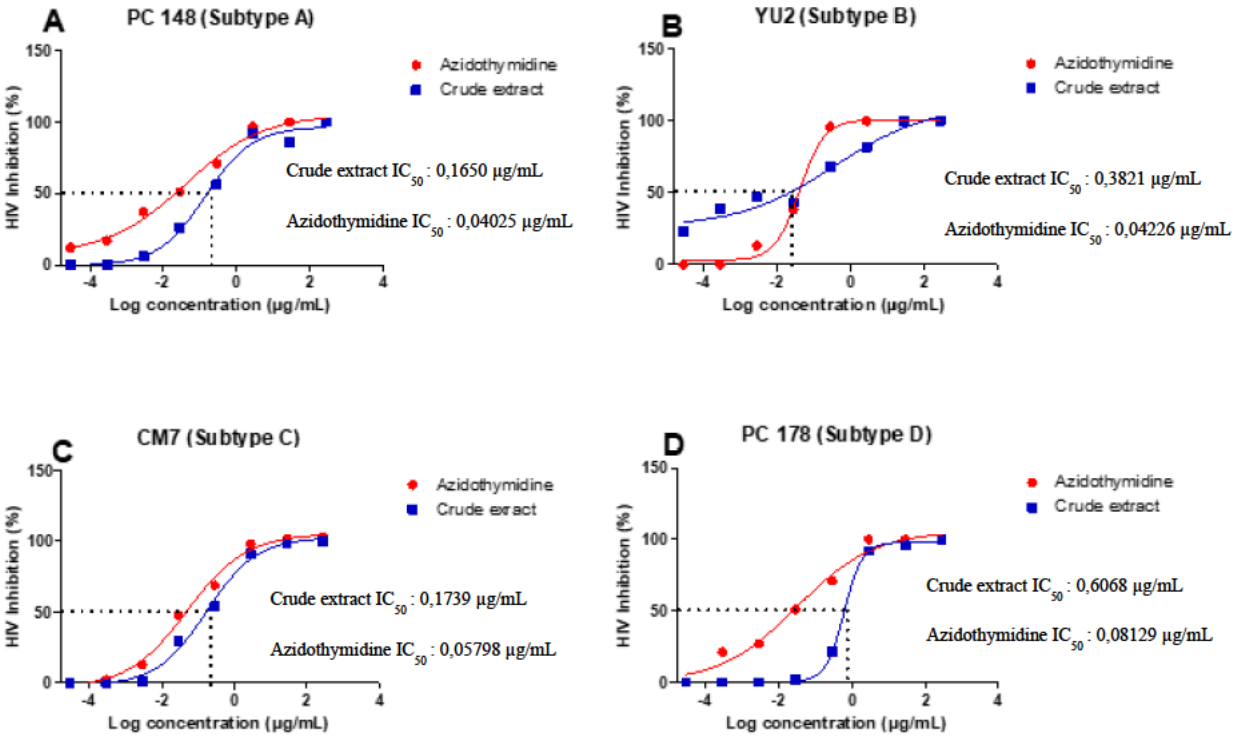


**Figure S3.9.1**: Dose dependent curves showing antiviral activity of *A. alternata* crude extract against PC 148 **(A)**, YU2 **(B)** CM7 **(C)** and PC 178 **(D)** HIV-1 viruses belong to respective subtypes A, B, C and D. The y-axis of **(A, B, C and D)** shows the inhibition of HIV as a percentage. The x-axis **(A, B, C and D)** shows the log concentration of the crude extract or azidothymidine. The red line shows the positive control azidothymidine and the blue line shows the *A. alternata* crude extract. The x-axis concentrations of 300; 30; 3; 0,3; 0.03; 0.003; 0.0003 and 0.00003 µg/mL to a log scale of 2,47; 1,47; 0.47, -0. 52, -1.52; -2,52; -3.52; -4,52

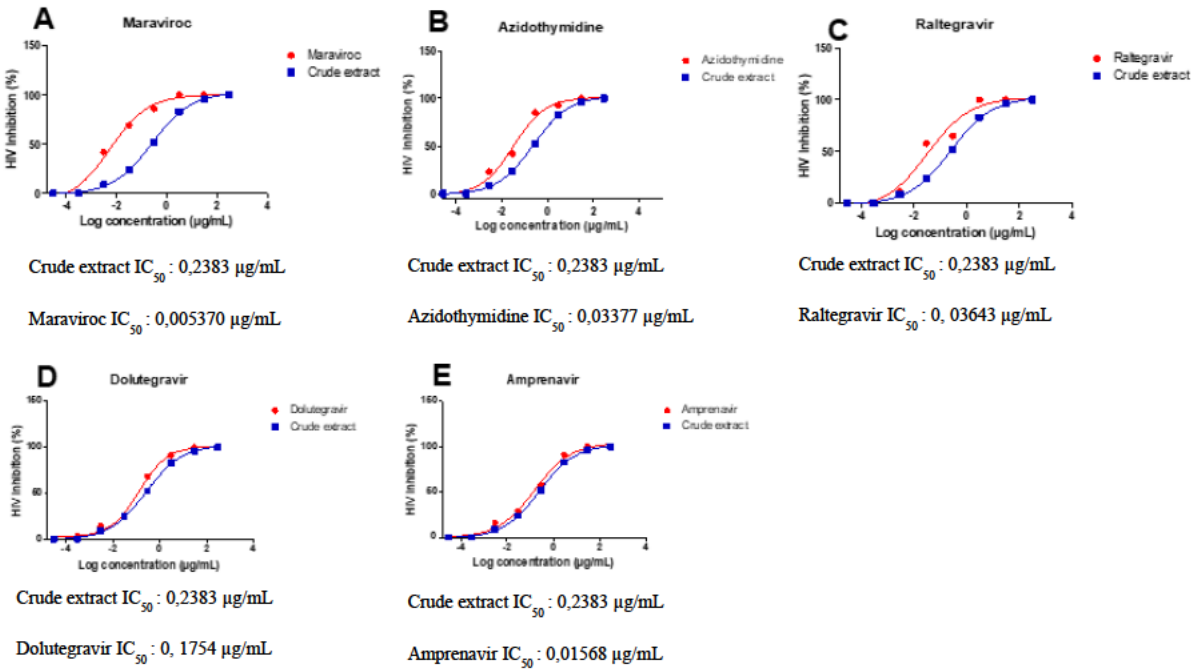


**Figure S3.9.2:** Dose dependent curves showing antiviral activity of the *A. alternata* crude extract and drug controls maraviroc **(A)**, azidothymidine **(B)**, raltegravir **(C)**, dolutegravir **(D)** and amprenavir **(C)** against the subtype B virus pNL4.3. The y-axis shows the inhibition of HIV as a percentage. The x-axis shows the log concentration of the crude extract and positive drug controls **(A, B, C, D and E)**(maraviroc, azidothymidine, raltegravir, dolutegravir and amprenavir). The red line shows the positive drug controls, and the blue line shows the *A. alternata* crude extract. The x-axis concentrations of 300; 30; 3; 0,3; 0.03; 0.003; 0.0003 and 0.00003 µg/mL to a log scale of 2,47; 1,47; 0.47, -0. 52, -1.52; -2,52; -3.52; -4,52.

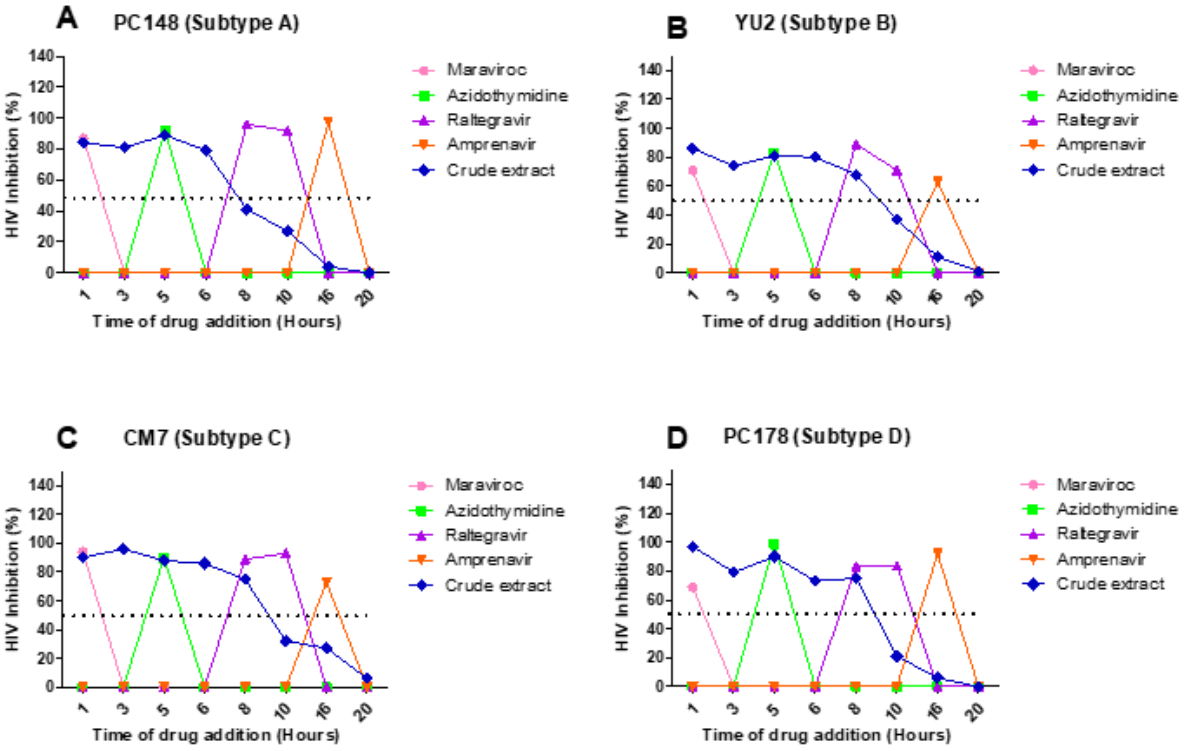


**Figure S3.9.3**: Luciferase based time of addition of viruses PC148 **(A)**, YU2 **(B)**, CM7 **(C)** and PC178 **(D)**. The y-axis of **(A, B, C and D)** the shows the inhibition of HIV as a percentage. The x-axis of **(A, B, C and D)** the shows the time of drug addition in hours. For **(A, B, C and D)**. The pink, green, purple, orange and blue lines of **(A, B, C and D)** represents maraviroc, azidothymidine, raltegravir, amprenavir and *A. alternata* crude extract respectively. The *A. alternata* crude extract was added at 1, 3, 5, 6, 8, 10, 16 and 20 hours post infection.

# CHAPTER 4: SYNTHESIS

## 4.1 General discussion and conclusion

Endophytic fungi are a unique source of anti-HIV secondary metabolites and NPs in the search for compounds that can be included in the drug development pipeline of antiretroviral therapeutics. The anti-HIV activity of secondary metabolites from endophytes of medicinal plants has previously been demonstrated as potent inhibitors of HIV-1 (Wellensiek *et al*., 2013; Bashyal *et al*., 2014; Melappa *et al*., 2021; Nzimande *et al*., 2022). The limitation of these previous studies is that the mechanism of action was not fully elucidated. Furthermore, these studies did not investigate the antiviral effects of the *A. alternata* crude extract in different HIV-1 subtypes and drug-resistance strains. It must be considered that HIV is diverse with different subtypes, and its response to antiretroviral therapy can be different, and drug-resistance development poses a challenge to antiretroviral drug success. Therefore, this study showed that the *A. alternata* crude extract previously isolated from *Hypoxis* species has antiviral activity against different HIV-1 subtypes, integrase drug-resistant strains and the mechanism of action of *A. alternata*.

The TZM-bl cell line was used to assess the cytotoxicity and cell viability of *A. alternata* crude extract using the MTT assay to identify the cell concentration at 50% ($CC_{50}$) and to ensure safety. Based on the MTT assay concept, the *A. alternata* crude extract was safe to use, not cytotoxic to the TZM-bl cell line, and its cell viability was greater than 80%, with the $CC_{50}$ greater than 30–40 µg/mL, respectively (Talib & Mahasneh, 2010; Jimoh *et al*., 2024). These findings were supported by Nzimande *et al.* (2022), who also found that the cell viability and cytotoxicity of the *A. alternata* crude extract was greater than 80% and 30-40 µg/mL using the MTT assay method. The *A. alternata* crude extract were previously used to treat HeLa, MCF-7, H1975 and HepG2 cell lines using the MTT assay method, and it was determined that the extract had inhibitory concentrations ranging from 6.42 µg/mL to 226.59 µg/mL against the cell lines (Ravi *et al*., 2022). It must be acknowledged that toxicity is complex; therefore, *in vitro*, assays must be complemented by animal models or *in vivo* studies to simulate clinical use to predict side effects, safety and biocompatibility in humans (Wang *et al*., 2010; Vajrabhaya & Korsuwannawong, 2018). Cytotoxicity assays can also be used on different cell lines such as astrocytes, HepG2 and human lung fibroblast (HLF) cell lines to study cytotoxicity in brain, liver, and lung anatomical sites (Xue *et al*., 2018; Löfdahl *et al*., 2020; Bhat *et al*., 2024).

A luciferase-based assay was employed to assess the anti-HIV activity of *A. alternata* crude extract on the TZM-bl cell line, and testing subtypes A, B, C, and D viruses. The $IC_{50}$ of the subtype A, B, C and D viruses treated with the *A. alternata* crude extract were less than the standard of 10 µg/mL, showing that the *A. alternata* crude extract was highly active against the different HIV-1 subtypes (Mogatle *et al*., 2008). It was established that a methanol extract of Alternaria species inhibited reverse transcriptase, integrase and protease by 82%, 98% and 78%, respectively (Melappa *et al*., 2015). However, Melappa *et al*. (2015) did not report an $IC_{50}$, and this study was able to determine percent inhibition and $IC_{50}$ values. The anti-HIV activity of altertoxins I, II, III and V isolated *Alternaria tenuissima* was determined by a reverse transcriptase based antiviral assay determined the compounds had $IC_{50}$'s between 0.09 µM and 1.42 µM (Bashyal *et al*., 2014). The limitation of the reverse transcriptase-based antiviral assay is that it requires a recombinant reverse transcriptase enzyme, which is costly to purchase or laborious to produce (Bashyal *et al*., 2014).

The Bashyal *et al.* (2014) and Melappa *et al.* (2015) studies were limited in that $IC_{50}$ or $CC_{50}$ values were not determined. The studies by Nzimande *et al.* (2022), Kubheka *et al.* (2024) and this study determined the $IC_{50}$ and $CC_{50}$ of the *A. alternata* crude extract which is significant progress. However, the Nzimande *et al.* (2022) and Kubheka *et al.* (2024) studies focused only on the subtype B pNL4.3 virus, and this study focused on subtype A, B, C and D viruses. The SI values of the subtype A, B, C and D viruses treated with *A. alternata* crude extract were greater than 10 and had therapeutic potential (Indrayanto *et al*., 2021). Other studies have reported that an SI value can be considered favourable if greater than 1 and largely unfavourable if less than 1 (Lica *et al*., 2021). The high anti-HIV-1 activity and therapeutic potential were supported by Nzimande *et al.* (2022); however, the study only determined the anti-HIV activity and therapeutic potential of the subtype B virus pNL4.3. Our study shows that *A. alternata* crude extract can inhibit different HIV-1 subtypes with high potency.

Although, $IC_{50}$ values can be interpreted to provide useful information for *In vitro* experiments, they do not correspond to therapeutic amounts that patients need to lower viral replication of HIV (Chan & Ray, 2007). In addition, while the SI value of the *A. alternata* crude extract is promising, it is not feasible to study a crude extract in a clinical setting due to the intermingling of bioactive compounds in any crude extract (Newman, 2021). This emphasizes the need to identify the compounds responsible for anti-HIV activity.

Since secondary metabolites are not vital for survival and can be secreted under stressful conditions this may affect the bioactive compounds present in the *A. alternata* crude extract and anti-HIV activity (Monfil & Casas-Flores, 2014). These secondary metabolite pathways could be activated using epigenetic modifiers to induce the expression of biosynthetic gene clusters (Makhwitine *et al*., 2023). The anti-HIV of crude extract of *Penicillium chrysogenum* treated with epigenetic modifier sodium butyrate by 5-fold as demonstrated by Makhwitine *et al.* (2023). Epigenetic modifiers such as sodium butyrate could therefore, be used to improve the anti-HIV of the *A. alternata* crude extract further (Makhwitine *et al*., 2023). Epigenetic activation of *A. alternata* biosynthetic gene clusters would emphasize the importance of SI values as a tool to determine therapeutic potential and antiviral potency. The Nzimande *et al.* (2022) and Kubheka *et al.* (2024) studies also determined the SI values of *A. alterata* crude extract which also indicated therapeutic potential. Thus, SI values are an important preclinical tools in drug development allowing for the assessment of drug candidates without human participants (Muller & Milton, 2012; Tamargo *et al*., 2015).

The development of HIV drug-resistance towards INSTI's is concerning since it is included in the South African TLD regimen (Chimukangara *et al*., 2021). The challenge of drug-resistance is that it renders affected drugs less effective and results in treatment failure in PLWHs (Nastri *et al*., 2023). The current study reports that the *A. alternata* crude extract inhibits the INSTI drug-resistant variant (T66K and S230K) generated through site-directed mutagenesis. In a previous study, patentiflorin A isolated from *Justicia gendarussa* inhibited NNRTI-resistant HIV (Zhang *et al*., 2017). Then, the NP AME was evaluated for its activity against INSTI-resistant HIV and was found to be a potent inhibitor of several raltegravir-resistant viruses (Ding *et al*., 2017). The *A. alternata* crude extract inhibited the T66K virus with higher activity than the S230R virus. The *A. alternata* crude extract did not have a integration activity that was notably greater than raltegravir against the T66K virus. The *A. alternata* crude extract had inhibited the S230R virus during integration at the same level as raltegravir. However, the *A. alternata* crude extract did not appear to inhibit integration significantly better than the drug control raltegravir. This demonstrates that secondary metabolites and NPs can overcome HIV drug-resistance. The integration inhibitory activity of the *A. alternata* crude extract should be validated by *in silico*, integrase inhibition and Alu gag PCR to confirm the degree of activity of the crude extract against integrase.

Site-directed mutagenesis was used since it was a cost-effective strategy to introduce the integrase drug-resistant mutations E92Q, T66K, S230R and R263K with resistance to

dolutegravir and ralftegravir. These integrase drug-resistance mutations have susceptibility scores ranging between 10-30 and 15-60 for dolutegravir and raltegravir respectively according to the Stanford HIV drug-resistance database. The mutations were then confirmed by sequencing. However, only the T66K and S230R integrase resistant strains had viral infectivity and E92Q and R263K integrase resistant strains did not have sufficient viral infectivity. However, other solutions are available, such as the PhenoSense® integrase assay, which is cost-intensive (Labcorp Monogram Biosciences, California, United States of America). The anti-HIV activity of the *A. alternata* crude extract against integrase drug-resistant strains T66K and S230R was evaluated using a luciferase-based assay on the TZM-bl cell. The *A. alternata* crude extract was highly active against T66K and S230R integrase drug-resistance strains according to Mogatle *et al.* (2008). The *A. alternata* crude extract has not been evaluated against any other integrase drug-resistance strains by a previous or drug-resistance study. It can only be concluded that *A. alternata* crude extract is effective against the T66K and S230R strains; however, it can't be concluded that *A. alternata* crude extract can overcome all integrase drug-resistance. The mutations E92Q and R263K were also selected however, these INSTI resistant strains did not have a high enough viral infectivity. Since the crude extract consists of a diverse mixture of compounds that still require characterization and may have diverse mechanisms of action that do not specifically target integrase.

The mechanism of action of the *A. alternata* crude extract was determined using luciferase and HIV-1 p24-based time of addition assays on subtype A, B, C, and D viruses, as well as integrase-resistant viruses (T66K and S230R). The inhibitory activity of *A. alternata* crude extract was observed during attachment, reverse transcription, integration, and proteolysis. This was similar to the recent findings reporting attachment, reverse transcription, integration and proteolysis of the *A. alternata* crude extract (Kubheka *et al*., 2024). However, it is recommended that these results be validated using commercially available reverse transcriptase, integrase and protease activity kits. Integrated DNA can be measured using Alu-gag PCR to validate integrase activity (Donahue *et al*., 2010). Kubheka *et al.* (2024) conducted the luciferase and HIV-1 p24 based time of addition on the subtype B virus pNL4.3, whereas this study used these methods on subtype A, B, C and D viruses. The *A. alternata* crude extract compounds are yet to be identified. However, the presence of coumarin class compounds with reverse transcription and integrationinhibitory activity could explain the activity observed (Melappa *et al*., 2015; Nzimande *et al*., 2022). The active compounds should be identified to confirm the mechanism of action of *A. alternata* crude extract, and attachment, reverse

transcription, integration, and proteolysis assays should be used to confirm the mechanism of action.

The study's goal was ultimately achieved because *A. alternata* crude extract exhibited strong antiviral activity against several HIV-1 subtypes addressing research question 1. The *A. alternata* crude extract exhibited effective anti-HIV activity against strains of the prevalent INSTIs resistant to raltegravir and dolutegravir, that had moderate to high integrase drug-resistance. This finding addressed the research question 2. The T66K and S230R integrase drug-resistance mutations also confer resistance to elvitegravir (40-fold change) and cabotegravir (2 to 3-fold change); however, resistance was not evaluated in the study since these drugs were unavailable (Shafer, 2006). The *A. alternata* crude extract is a potential inhibitor of HIV-1 attachment, reverse transcription and integration, which answers research question 3. The results demonstrate that *A. alternata*, as a source of anti-HIV, contributes to the scientific literature and is a candidate for further prospective research and drug discovery.

### 4.2 Limitations and Recommendations

Although the *A. alternata* had high antiviral activity, the active compounds should be identified using mass spectrometry technologies such as liquid chromatography-mass spectrometry (LC-MS), which can identify a wide range of compounds followed by isolation using a metabolomics approach (Gika *et al*., 2014). It is also recommended that the bioactive compounds of *A. alternata* crude extract be identified and studied *in silico*. Once the bioactive compounds are identified, they are also recommended to be tested further *in vitro* and *in vivo*. Further studies are recommended to identify the *Hypoxis* plant by identifying it at the species level. This study also only evaluated the antiviral activity of *A. alternata* crude extract on two integrase drug-resistant strains, and the integrase inhibitory potential should be evaluated on a broader range of resistant strains. A limitation of this study was that dolutgravir was not included in the time of addition assays due to resource constraints. It is also recommended that the mechanisms of action determined by time of addition be confirmed by attachment, reverse transcriptase, integrase activity, protease activity, colourimetric assays and Alu gag PCR to confirm the mechanism of action. It is also further recommended that latency-inducing and promoting activity be investigated, as HIV latency and viral reservoirs are known to impact treatment in PLWH negatively.

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# APPENDICES

## Appendix 1 ethical clearance for study

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

**14 March 2024**

**Mr Darian Naidu (218052764)**
**School of Laboratory Medicine & Medical Science**
**Medical School**

**Dear Mr Naidu,**

**Protocol reference number: BREC/00005156/2023**
**Project title: Antiviral effects of active compounds from Alternaria alternata on different HIV subtypes and Integrase drug-resistant HIV viruses**
**Degree: MMedSci**

**RECERTIFICATION APPLICATION APPROVAL NOTICE**

| | |
|---|---|
| **Approved:** | **03 February 2024** |
| **Expiration of Ethical Approval:** | **02 February 2025** |

I wish to advise you that your application for Recertification for the above protocol has been **noted and approved** by a sub-committee of the Biomedical Research Ethics Committee (BREC) for another approval period. The start and end dates of this period are indicated above.

If any modifications or adverse events occur in the project before your next scheduled review, you must submit them to BREC for review. Except in emergency situations, no change to the protocol may be implemented until you have received written BREC approval for the change.

The committee will be notified of the above approval at its next meeting to be held on 09 April 2024.

Yours sincerely

[redacted]

Biomedical Research Ethics Committee
Chair: Professor S Singh
UKZN Research Ethics Office Westville Campus, Govan Mbeki Building
Postal Address: Private Bag X54001, Durban 4000
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