The antiviral effects of *Alternaria alternata* PO4PR2 crude extracts on integration stage and integrase drug-resistant variants

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PREFACE

The research and experimental work detailed in this dissertation were carried out in the HIV Pathogenesis Programme at the Doris Duke Medical Research Institute, College of Health Sciences, University of KwaZulu-Natal, Durban, South Africa, between January 2024 and November 2025 under the supervision of Dr. Nompumelelo Mkhwanazi. This study is the author's own work and hasn't been submitted in any way to any university for a degree or diploma. Where someone else's work has been used, it is properly acknowledged in the text.

PLAGIARISM DECLARATION FORM

I, Ndzalo Mashabele, declare that

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DEDICATION

I dedicate this thesis to my mother, who has provided me with unwavering support throughout my educational journey, and to my younger brother, who has kept me grounded and reminded me to keep laughing, even in the toughest of times.

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RESEARCH OUTPUTS

Conferences:

1. College of Health Sciences research symposium, 25 August 2025. Oral presentation: Ndzalo Mashabele; Darian Naidu; Nompumelelo Mkhwanazi: "The antiviral effects of endophytic fungi secondary metabolites on integration site and integrase drug-resistant variants."
2. Mangosuthu University of Technology 2nd Regional Traditional Medicine Symposium: *"Harnessing African Indigenous Knowledge for Good Health and Well-being",* 28 August 2025: Poster presentation: Ndzalo Mashabele; Darian Naidu; Nompumelelo Mkhwanazi: *Alternaria alternata* Crude Extract Suppresses HIV-1 Integrase Mutants and Blocks Viral Integration."
3. 23rd International Conference on AIDS and STIs in Africa Conference 3 - 8 December 2025, Accra - Ghana. Virtual Poster (Poster number THPEA008) presentation: Ndzalo Mashabele; Darian Naidu; Nompumelelo Mkhwanazi: "The antiviral effects of endophytic fungi secondary metabolites on integration site and integrase drug-resistant variants."

Publications:

1. In preparation: Ndzalo Mashabele; Darian Naidu; Nompumelelo Mkhwanazi: *Alternaria alternata* (PO4PR2) Crude Extract Suppresses HIV-1 Integrase Mutants and Blocks Viral Integration."

Achievements:

1. Runner-up in the category for best poster: Mangosuthu University of Technology 2nd Regional Traditional Medicine Symposium: *"Harnessing African Indigenous Knowledge for Good Health and Well-being".* Poster presentation: Ndzalo Mashabele; Darian Naidu; Nompumelelo Mkhwanazi: *Alternaria alternata* Crude Extract Suppresses HIV-1 Integrase Mutants and Blocks Viral Integration."

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LIST OF ABBREVIATIONS

A. alternata	*Alternaria alternata*
AIDS	Acquired Immunodeficiency Syndrome
ART	Antiretroviral Therapy
AZT	Azidothymidine (Zidovudine)
BIC	Bictegravir
BSA	Bovine Serum Albumin
CAB	Cabotegravir
CC_{50}	50% Cytotoxic Concentration
$CD4^+$	Cluster of Differentiation 4 Positive T Cell
CE	Crude Extract
CHS	College of Health Sciences
CO_2	Carbon Dioxide
DMEM	Dulbecco's Modified Eagle Medium
DEAE-dextran	Diethylaminoethyl Dextran
DNA	Deoxyribonucleic Acid
DRM(s)	Drug Resistance Mutation(s)
DTG	Dolutegravir
DMSO	Dimethyl Sulfoxide
Env	Envelope Glycoprotein
EtOH	Ethanol
EVG	Elvitegravir
FBS	Fetal Bovine Serum
GAPDH	Glyceraldehyde-3-Phosphate Dehydrogenase
GC-MS	Gas Chromatography–Mass Spectrometry
GM	Growth Medium

HES / HEPES	4-(2-Hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HIV-1	Human Immunodeficiency Virus Type 1
HEK293T	Human Embryonic Kidney 293T Cells
HPP	HIV Pathogenesis Programme
IC_{50}	50% Inhibitory Concentration
IN	Integrase
INSTI	Integrase Strand Transfer Inhibitor
LB	Luria-Bertani Broth
LTR	Long Terminal Repeat
MEA	Malt Extract Agar
MTT	3-(4,5-Dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide
NaOAc	Sodium Acetate
PBMC(s)	Peripheral Blood Mononuclear Cell(s)
PBS	Phosphate Buffered Saline
PDA / PDB	Potato Dextrose Agar / Potato Dextrose Broth
PCR	Polymerase Chain Reaction
PGEM	Plasmid Template for Sequencing Control
RAL	Raltegravir
RLU(s)	Relative Light Unit(s)
RNA	Ribonucleic Acid
RPM	Revolutions Per Minute
RT	Reverse Transcriptase
$TCID_{50}$	Tissue Culture Infectious Dose 50%
TZM-bl	HeLa-derived HIV Reporter Cell Line

ABSTRACT

Background: HIV-1 antiretroviral drugs have reduced the burden of people living with HIV-1. However, the development of drug resistance has reduced the effectiveness of current therapies, including HIV integrase inhibitors, highlighting the need for novel agents targeting viral integration. Natural products derived from endophytic fungi have emerged as a promising source for the development of new antiviral drugs. Hence, this study investigates the anti-HIV potential of the *Alternaria alternata* PO4PR2 crude extract against integrase drug-resistant viruses and explores its mechanism of action in inhibiting the viral integration stage.

Materials and Methods: Major drug-resistant mutations (DRM) in HIV-1 integrase (G118R, N155H, R263K, and Y143R) were identified using the Stanford HIV Drug Resistance Database and introduced into the HIV-1 pNL4.3 molecular clone via site-directed mutagenesis. Successful introduction of these mutations was confirmed by Sanger sequencing. Integrase-resistant HIV-1 variants were generated by transfection in HEK293T cells, and their infectivity was tested on TZM-bl cell lines. The cytotoxicity of the *Alternaria alternata* PO4PR2 was assessed using the 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay. A luciferase-based antiviral assay was used to measure the antiviral activity of *A. alternata* PO4PR2 in TZM-bl cells infected with the pNL4.3 virus and integrase mutant viruses. HIV-1 integrase activity assay and *Alu-gag* PCR were used to confirm the inhibitory effects of *A. alternata* PO4PR2 on the integration step and its impact on the integration phase. Molecular docking was performed to predict the binding affinity and interaction profiles of the *A. alternata*-derived secondary metabolites against HIV-1 integrase.

Results: The *A. alternata* PO4PR2 crude extract showed potent inhibition of the G118R, N155H, Y143R, and R263K mutants while maintaining low toxicity and high cell viability in TZM-bl cell lines. The extract also demonstrated direct inhibition of HIV-1 integrase enzymatic activity, as confirmed by the HIV-1 integrase activity assay, with effects comparable to those of the established integrase strand-transfer inhibitor Raltegravir. Real-time *Alu-gag* PCR further confirmed a significant reduction in integrated HIV-1 DNA in PBMCs treated with the crude extract, with inhibition levels comparable to Dolutegravir and approaching those of Raltegravir, indicating direct interference with the integration step. Docking analysis revealed that *A. alternata* PO4PR2 metabolites bind strongly to the HIV-1 integrase catalytic pocket across both wild-type and resistant variants, demonstrating moderate binding affinities and maintaining interactions with key catalytic residues, Mg^{2+} cofactors, and DNA bases, even in the presence of resistance-associated mutations.

Discussion and Conclusion: *A. alternata* PO4PR2 is a promising source of antiretroviral compounds that target drug-resistant HIV-1 integrase mutations, thereby supporting its potential as a source of novel antiretroviral agents against resistant HIV-1 integrase. Several metabolites within the *A. alternata* crude extract interact directly with the HIV-1 integrase catalytic pocket, consistently

engaging the key catalytic residues, and positioning themselves near the Mg^{2+} cofactors in both wild-type and resistant variants, mirroring the interaction patterns of clinically approved strand-transfer inhibitors such as Raltegravir and Dolutegravir. The *Alu-gag* PCR showed a marked reduction in proviral DNA integration, and the integrase activity assay demonstrated direct enzymatic inhibition of HIV-1 integrase, strongly suggesting that the *A. alternata* metabolites primarily inhibit the strand-transfer step of integration. These observations indicate that the *A. alternata* extract contains natural compounds with INSTI-like properties capable of retaining activity against key drug-resistant integrase mutations. Building on these findings, future studies should focus on isolating and characterizing the active compounds responsible for the observed activity to further validate the therapeutic potential of *A. alternata.*

CHAPTER 1: INTRODUCTION

Background

More than four decades since its discovery, the Human Immunodeficiency virus continues to pose a global health challenge, with an estimated 40.8 million people living with HIV, 1.3 million new infections, as well as 630,000 AIDS-related deaths reported in 2025 (UNAIDS, 2025). Despite extensive prevention strategies and awareness campaigns, the virus continues to spread, and Eastern and Southern Africa remain the most impacted, contributing to substantial morbidity and mortality (UNAIDS, 2025; Parker et al., 2017). HIV primarily targets and depletes $CD4^+$ T lymphocytes, leading to progressive immune dysfunction and, ultimately, acquired immunodeficiency syndrome (AIDS) if left untreated (Février et al., 2011).

Problem Statement

The advent of combination antiretroviral therapy (cART) has transformed HIV infection from a fatal disease into a manageable chronic condition, markedly reducing viral replication, transmission, and AIDS-related deaths (Eissa, 2025). However, ART does not eliminate the virus completely due to the persistence of latent viral reservoirs within long-lived immune cells (Chou et al., 2024). These reservoirs represent a major obstacle to achieving a functional or sterilizing cure, as they can reignite systemic infection once treatment is interrupted (Kreider & Bar, 2022). Moreover, the emergence of drug-resistant HIV strains and treatment-related toxicities further complicate long-term management (Apetroaei et al., 2024). Consequently, there is a growing need to identify novel therapeutic targets and agents that can suppress viral replication through alternative mechanisms. Natural products serve as an important source for the discovery of new antiviral compounds.

Rationale

One critical stage in the HIV replication cycle is viral integration, a process mediated by the viral enzyme integrase (Elliott & Kutluay, 2020). During this step, the reverse-transcribed viral DNA is inserted into the host cell's genome, establishing a proviral state that enables persistent infection and the formation of a latent reservoir (Anderson & Maldarelli, 2018). Integration is therefore a pivotal event in the establishment of HIV persistence and a major barrier to viral eradication (Maldarelli, 2016; Pasternak & Berkhout, 2023). Although integrase strand transfer inhibitors (INSTI) have been successfully incorporated into current ART regimens, the emergence of resistant variants and cross-resistance among drugs in this class highlight the urgent need for novel integrase-targeting compounds (Anstett et al., 2017; Jóźwik et al., 2020; Mbhele et al., 2021; X. Z. Zhao et al., 2016).

Integration occurs in two distinct stages. First, during 3′-end processing, the integrase enzyme removes two nucleotides from the 3′ ends of the viral DNA. This is followed by strand transfer, where the processed viral DNA is inserted and covalently linked to the host cell's DNA (Craigie & Bushman,

2012). Most integrase inhibitors currently target the strand transfer step, creating a need for new drugs that can block earlier stages of integration, such as nuclear import and 3′-end processing (Bonnenfant et al., 2004; Wainberg et al., 2012). *A. alternata* may produce a range of compounds capable of inhibiting multiple phases of the integration process, and previous studies have shown that metabolites such as alterotoxins and coumarins can interfere with key integration steps (Sharapov et al., 2023; Xu et al., 2021).

In recent years, natural products have gained renewed attention as a valuable source of bioactive compounds with potential antiviral activity (Musarra-Pizzo et al., 2021; Ribeiro et al., 2025). Endophytic fungi, which reside asymptomatically within plant tissues, are known to produce a wide range of secondary metabolites with diverse pharmacological properties (Liao et al., 2025; Nazir & Rahman, 2018). Among these, species such as *Alternaria alternata* have demonstrated promising antimicrobial and antiviral effects (Elghaffar et al., 2022).

Previous studies have demonstrated that endophytic fungi isolated from desert plants represent a promising source for discovering potent compounds that inhibit HIV-1 replication (Bashyal et al., 2014; Wellensiek et al., 2013). *In vitro* assays showed that the methanol extract of *Penicillium* species BCt significantly inhibited three major HIV-1 enzymes: reverse transcriptase (RT), integrase (IN), and protease (PT), suggesting that fungal coumarins effectively block key viral replication processes, indicating their potential as natural anti-HIV agents (Govindappa et al., 2021). The study by Ding et al (2017) found that alternariol 5-O-methyl ether (AME) effectively inhibited multiple HIV-1 strains, including those resistant to the integrase inhibitor Raltegravir, showing equal or greater efficacy than the wild-type virus, and at higher concentrations, completely suppressed viral replication (up to 100%) in primary human immune cells (PBMCs), thereby demonstrating its strong and broad-spectrum antiviral potential against both drug-sensitive and resistant forms of HIV-1.

Previous studies reported that secondary metabolites from *Alternaria alternata* PO4PR2 showed potent anti-HIV activity, with IC_{50} values ranging from 0.017–1.170 μg/ml in TZM-bl cells and a demonstrated ability to reduce viral replication in both PBMCs and $CD4^+$ T cells (Nzimande et al., 2022). Recently, it was further confirmed that secondary metabolites from *Alternaria alternata* PO4PR2 exhibit broad-spectrum and potent anti-HIV-1 activity, effectively targeting multiple HIV subtypes and HIV-1 drug-resistant integrase mutants, notably by blocking the integration step with 98-100% efficiency, thereby positioning these metabolites as promising natural candidates for the development of new anti-HIV therapeutics, particularly against integrase inhibitor-resistant viral strains (Naidu et al., 2025; Kubheka et al 2024). However, despite demonstrating strong antiviral effects, these studies did not elucidate the precise mechanism through which these metabolites inhibit HIV-1, thereby creating a critical knowledge gap that the present study aims to address.

Building on these findings, exploring endophytic fungal metabolites as inhibitors of HIV-1 integrase

offers an innovative approach to suppress viral integration and potentially overcome limitations associated with current ART regimens, including drug resistance and incomplete viral eradication (Singh, Jayasuriya, et al., 2003). This study, therefore, investigates the anti-HIV potential of *Alternaria alternata* PO4PR2, with a particular focus on its inhibitory effects on HIV-1 integrase and integrase drug-resistant variants. To achieve this, we identified clinically relevant INSTI-resistant mutations across all HIV-1 subtypes using the Stanford HIV Drug Resistance Database and generated the corresponding resistant viruses through site-directed mutagenesis. The anti-HIV-1 activity of the *Alternaria alternata* PO4PR2 crude extract was then evaluated against both the wild-type pNL4.3 strain and the engineered drug-resistant variants. Furthermore, integrase inhibition was validated using a colorimetric HIV-1 integrase activity assay, and the suppression of viral integration was confirmed through *Alu-gag* real-time PCR. Molecular docking analyses were performed to characterize the binding interactions between the *Alternaria alternata* fungal metabolites and the HIV-1 integrase catalytic core, providing structural insights into their potential mechanism of inhibition and their capacity to retain activity against drug-resistant variants.

Study aim and objectives:

Aim:

This study aims to elucidate the anti-HIV-1 activity of *Alternaria alternata* PO4PR2 crude extract against integrase drug-resistant variants and its mechanism of action at the HIV-1 integration stage.

Specific objectives:

1. To assess the prevalence of integrase drug-resistant mutation in HIV-1 subtypes by analysing the integrase mutation sequences in the Stanford drug resistance database.
2. To develop integrase drug-resistant viral mutations using Site-Directed Mutagenesis, followed by testing the antiviral activity of *A. alternata* PO4PR2, on these resistant viruses using a luciferase-based antiviral assay
3. To confirm the inhibitory effects of the *A. alternata* PO4PR2 crude extract on the HIV- 1 integration stage by real-time PCR, HIV-1 integrase activity assay and molecular docking.

Hypothesis

Alternaria alternata PO4PR2 crude extract exhibits potent anti-HIV-1 activity by suppressing the integration stage of replication and overcoming resistance conferred by major integrase drug-resistant mutations.

Research question(s):

What is the impact of *Alternaria alternata* PO4PR2 crude extract on HIV-1 integration and on integrase drug-resistant variants?

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CHAPTER 2: LITERATURE REVIEW

2.1 History and Epidemiology of HIV

2.1.1 History of HIV-1

Human immunodeficiency virus was first discovered in 1981 by French scientists Barre-Sinoussi and David Gallo, and was later reported to be the major causative agent of Acquired Immune Deficiency (AIDS) syndrome (Fauci, 2003; Sharp & Hahn, 2011). Four previously healthy men were admitted to the hospital with a history of oral and perianal candidiasis, fever, malaise, and generalized lymphadenopathy, along with bilateral lung infiltrates and severe immunodeficiency (Gallo et al., 1984; Gottlieb et al., 1982). At the time, the disease was prevalent among homosexual men, who presented with cytomegalovirus (CMV) pneumonia and *Pneumocystis carinii* pneumonia, which was highly associated with severe immune suppression (Clavel et al., 1986; Follansbee et al., 1982; Noruzi et al., 2025). Though the aetiology was not yet known, it was suggested that it was transmitted by a sexually transmitted infectious agent or through exposure to blood and blood products (Pollack, 2001; Stoto et al., 1995). It was further discovered that HIV is divided into HIV-1 and HIV-2 (Saxon & Campen, 1988; Seitz, 2016; Swinkels et al., 2024). Research conducted in West Africa in 1986 led to the discovery of HIV-2, a virus that causes AIDS and is different from the previously recognized HIV-1 in terms of morphology (Clavel et al., 1986; Swinkels et al., 2024). Numerous characteristics of HIV-1 and HIV-2 are identical, such as their fundamental gene organization, mechanisms of transmission, intracellular replication pathways, and clinical outcomes, which both lead to AIDS (Saleh et al., 2017). Nonetheless, HIV-2 is distinguished by its decreased transmissibility and decreased risk of developing into AIDS (Nyamweya et al., 2013). HIV mainly infects the CD4 T cells, resulting in the deterioration of immune cells and the development of opportunistic infections (Dalgleish et al., 1984; Habeshaw & Dalgleish, 1989; Redfield & Burke, 1988).

2.1.2 HIV-1 Epidemiology

The Joint United Nations Programme on HIV/AIDS has reported that 88.4 million people have become infected with HIV, as of 2023, and 42.3 million people have died from AIDS-related illnesses since the start of the epidemic (UNAIDS, 2025). It is estimated that 40.8 million people globally were living with HIV as of 2025 (Figure 1). There were 630,000 people who succumbed due to AIDS-related deaths in 2023. South Africa was estimated to have 7.6 million people living with HIV as of 2025, ranking the country among the top five countries with the highest number of HIV cases worldwide (UNAIDS, 2025). Within South Africa, the KwaZulu Natal province was found to have the highest number of HIV infections (Zuma et al., 2014, 2022). Figure 1 shows the global HIV infection epidemiology of individuals. As of 2023, 20.8 million individuals in Eastern and Southern Africa were living with HIV, making this region the most severely affected (UNAIDS, 2025).

2.2 Classification of HIV-1

HIV is classified into two forms, HIV-1 and HIV-2, and is a member of the Orthoretrovirinae subfamily of the Retroviridae family under the genus Lentivirus (Blut, 2016; Cullen, 1991; Luciw, 1996; Miller et al., 2000; Pieniazek et al., 1998). HIV-1 occurs more frequently in Eastern and Western Africa and is the most commonly studied type of HIV (Westby & Dalgleish, 1998). The two types of HIV are almost identical in morphology; however, HIV-1 is more infectious and more prevalent than HIV-2 (Blut, 2016; Nyamweya, Hegedus, Jaye, Rowland-Jones, et al., 2013). HIV-1 is subdivided into groups M (major), O (outlier), N (non-M, non-O), and P (Archer, 2008; Hemelaar, 2012; Hemelaar et al., 2006; Plantier et al., 2009). Group M viruses are further subdivided into subtypes A-D, F, H, J and K (Blut, 2016; Buonaguro et al., 2007; Leitner, 1996). The African continent contains the greatest diversity of HIV-1 subtypes, particularly in West Central Africa (Bbosa et al., 2019). HIV-1 subtype B dominates in Europe, North and South America, as well as Australia, while HIV-1 subtype C is the predominant virus circulating in Southern Africa, responsible for the majority of cases (Matume et al., 2020; Musyoki et al., 2015). HIV-2 also exists in eight different types, namely HIV-A to HIV-H. HIV-2 group A has been reported in and around the Sub-Saharan region, while HIV-2 subtype B commonly occurs in the Ivory Coast (de Silva et al., 2008; Ishikawa et al., 2001). Groups C to H are classified as "dead-end" transmissions as they are infrequent in nature and rarely cause further infections (Sharp & Hahn, 2011).

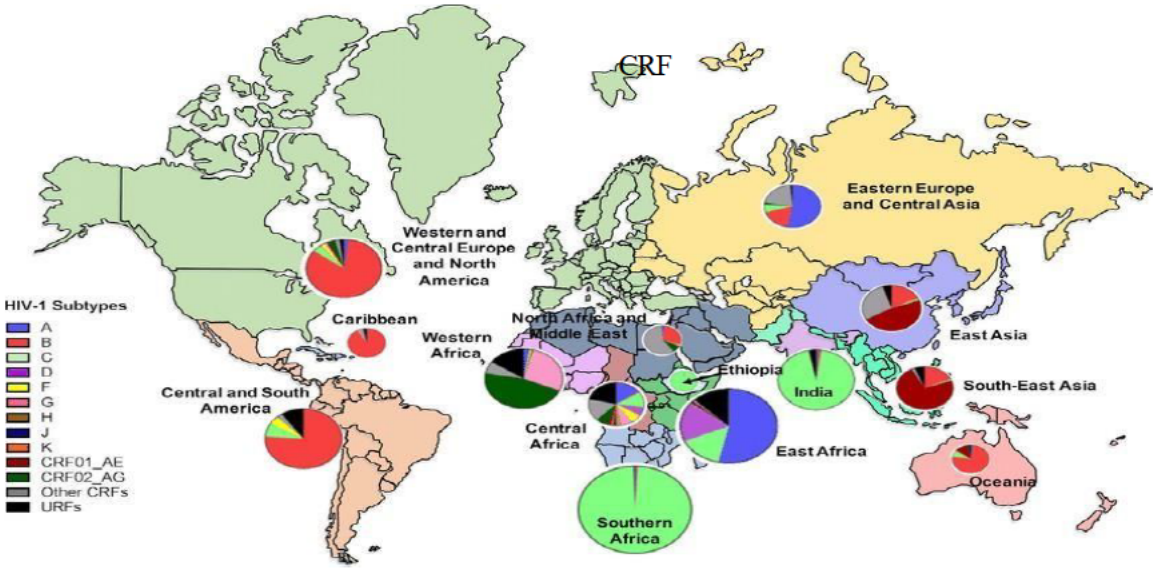

Figure 1: Global map illustrating the regional prevalence of HIV-1 subtypes

2.3 Genome and Structure of HIV-1

The HIV virion is a ~120 nm particle containing a 9750-nucleotide single-stranded RNA genome (~10 kb) enclosed within a conical capsid, surrounded by envelope proteins and a host-derived plasma membrane (Ganser-Pornillos et al., 2011; Pornillos & Ganser-Pornillos, 2019; Ratner et al., 1985; Westby & Dalgleish, 1998). The HIV-1 genome encodes for proteins such as *trans-activator of transcription factor (tat), group-specific antigen (gag), polymerase (pol),* and *envelope (env),* which are common to all retroviruses, as well as accessory proteins for viral replication, including *virion infectivity factor (vif), viral protein R (vpr), viral protein U (vpu),* and *negative factor (nef)* (Cohen et al., 1996; Cullen, 1991; Seelamgari et al., 2004). *Gag* is the primary structural protein of HIV-1, cleaved upon viral maturation to release the Capsid (CA), Matrix (MA), and Nucleocapsid (NC) proteins, which provide the structural components of the virus (Bell & Lever, 2013; Cullen & Greene, 1990; Stacey et al., 2025; Waheed & Freed, 2012). *Pol* gene functions as the second open reading frame and the second component of the *Gag/Pol* fusion protein, encoding polyproteins that comprise the precursors to the reverse transcriptase, integrase, and protease genes (Bell & Lever, 2013). Protease cleaves the *Gag* and *Gag/Pol* proteins into their separate components; integrase (IN) catalyzes the insertion of the DNA copy of the viral genetic information into the host cell genome; and reverse transcriptase (RT) copies the viral RNA into DNA (Goff, 2004; Panganiban & Temin, 1984; Wagner et al., 2015). *Env* encodes the glycoproteins gp120 and gp41, which are visible on the surface of the virus and aid in the virion's identification and fusion with the host cell (Bell & Lever, 2013; Harrison, 2005). The envelope trimer is a pivotal vaccine immunogen and serves as the exclusive target for neutralizing antibodies (Haynes et al., 2023; Seabright et al., 2019; Wyatt & Sodroski, 1998). *Tat* is expressed prior to integration of the infecting viral genome and is one of the very first genes to be expressed when infected with HIV-1 (Wu & Marsh, 2003). It encodes for regulatory proteins and boosts viral transcription and RNA elongation, thus promoting the expression of HIV-1 during the initiation and elongation of transcription (Bagashev & Sawaya, 2013; Karn & Stoltzfus, 2012; Seelamgari et al., 2004). *Rev* facilitates the nuclear export of unspliced or partially spliced mRNAs for the translation of vital viral proteins or the encapsidation of whole viral genomes (Jayaraman et al., 2019; Purcell & Martin, 1993; Truman et al., 2020). Numerous treatment strategies have been developed to disrupt *Rev* activity, primarily focusing on the interaction between *Rev* and its RNA target, RRE (Daelemans & Pannecouque, 2006). Hauber et al., 2005 demonstrated the inhibition of HIV-1 replication through the targeted restriction of HIV *Rev* activity by the indirect disruption of eukaryotic initiation factor 5A activation.

Vif enhances virus infectivity by stabilizing the virus core, preventing premature degradation of nucleoprotein complexes, and facilitating efficient DNA synthesis (Coffin et al., 1997; Öhagen & Gabuzda, 2000; Wang et al., 2014). *Vif* counteracts the action of human proteins that suppress HIV-1 infection, such as APOBEC and CEM15 (Malim, 2009; Sheehy et al., 2002). Goncalves et al., 2002 demonstrated that intracellular expression of a Vif-specific single-chain antibody neutralizes Vif

function, making cells highly resistant to HIV-inhibiting Vif-produced viral particles, preventing reverse transcription. *Vif* inhibitors, utilizing the DNA-editing cytidine deaminase A3G, a key restriction factor in macrophages and CD4+ T cells, could offer a new class of antiretroviral medications (Nathans et al., 2008; Sharkey et al., 2019). *Vpu* is essential for efficient HIV-1 replication, facilitating virus budding and the maturation of progeny virions by promoting CD4 downregulation, which enables Env trafficking to the cell surface for incorporation into assembling viral particles (Dubé et al., 2010; Strebel et al., 1988; Willey et al., 1992; Wolf Lindwasser et al., 2007). Attempts to develop anti-*Vpu* medications have led to the discovery of the compound BIT225, which was found to inhibit *Vpu* ion channel function and exhibit anti-HIV-1 action (Khoury et al., 2010). *Vpr* aids HIV-1 cell infection by transporting the pre-integration complex (PIC) into the nucleus, facilitating the nuclear entry of the HIV-1 provirus into non-dividing cells by promoting binding to importins and nucleoporins (Heinzinger et al., 1994; Kogan & Rappaport, 2011). It also inhibits the formation of chronically infected HIV-producer cell lines by inducing infected cells to arrest in the G2/M phase of the cell cycle (He et al., 1995; Stewart et al., 1997; Zhang & Bieniasz, 2020). Several natural products, including fumagillin, damnacanthal, and several isopimarane diterpenoids, have been studied for the inhibition of *Vpr* activity (González, 2017; Kamata et al., 2006; Watanabe et al., 2006; Win et al., 2016). *Nef* downregulates MHC class I and CD4 receptors, improving Env integration, particle release, and affecting CD4+ T-cell signalling pathways, potentially shielding infected cells from cytotoxic T cell killing (Chaudhuri et al., 2007; Giolo, 2009; Kwon et al., 2020; Lama et al., 1999; Witkowski & Verhasselt, 2013). By downregulating CD4 and class I MHC, Nef reduces the immune system's ability to detect the infected cell and modifies the cells to increase their capacity to produce fully infectious virus particles (Garcia & Miller, 1991; Mariani et al., 1993; O. Schwartz et al., 1996).

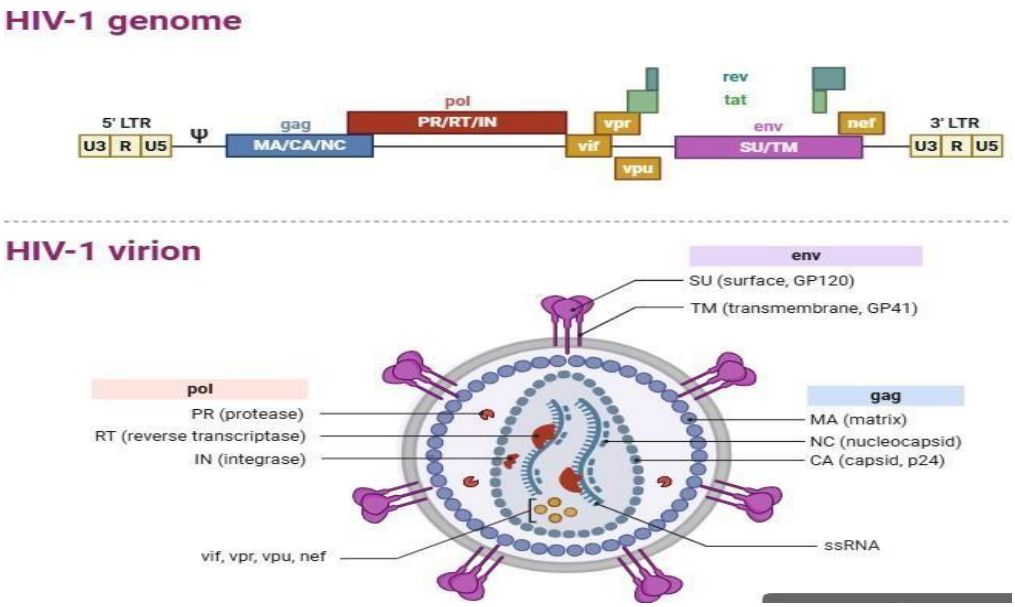

Figure 2: HIV proteins and accessory proteins. Created in https://BioRender.com

2.4 Replication Cycle of HIV-1

The HIV-1 replication cycle, which completes in approximately 24 hours, involves multiple stages that can each serve as potential targets for therapeutic intervention (Deeks et al., 2015; A. Engelman & Cherepanov, 2012; Flexner, 2007). It begins with the binding of the HIV virion to the surface of the host cell, resulting in the interaction of the HIV envelope with the CCR5 or CXCR4 receptors of the target cell (Klasse, 2012; Wilen et al., 2012). The envelope-encoded gp120 binds to the CD4 molecule found on the surface of the T lymphocytes, resulting in conformational changes and interaction between the gp120 and chemokine receptors (Doms & Moore, 2000; Gomez & Hope, 2005; Hoffman & Doms, 1998). These interactions result in fusion of the viral and host cell membranes, and the HIV molecule is injected into the host cell cytoplasm. Antiretroviral therapy targeting this stage is known as CCR5/CXCR4 and Fusion inhibitors, and it includes Enfuvirtide, Lenacapavir, and Maraviroc.

Following viral entry, the single-stranded viral RNA is reverse transcribed into double-stranded DNA (Hu & Hughes, 2012; Menéndez-Arias, 2010; Menéndez-Arias et al., 2017). This is facilitated by DNA polymerase, RNase H, and the reverse transcriptase enzymes (Basu et al., 2008). DNA polymerase duplicates the RNA, while RNase H degrades it (Bebenek & Kunkel, 2004; Rothwell & Waksman, 2005; Wahba et al., 2011). After synthesis of the viral DNA by reverse transcription in the cell cytoplasm, the DNA is associated with the integrase gene and other proteins to form a high molecular weight nucleoprotein complex, known as the pre-integration complex, which migrates into the nucleus for integration (Asante-Appiah & Skalka, 1997; Brown et al., 1987; Craigie, 2001). Zidovudine (AZT), a member of the group of medications called nucleoside reverse transcriptase inhibitors, or NRTIs, was approved as the first medication to treat HIV/AIDS in March 1987 (Kinch & Patridge, 2014).

The activity of reverse transcriptase produces linear and circular forms of HIV DNA, which are integrated into the host genome and can then be replicated together with the cells of the body (Figure 3) (Hamid et al., 2017). The integrase gene, encoded by the virus, facilitates the process of integration (Bushman et al., 1990). Despite the success of integrase inhibitors in antiretroviral therapy, resistance mutations have emerged, underscoring the need for a deeper understanding of the integration process. This research addresses this gap by investigating the integration site and the enzyme responsible, HIV-1 integrase, as new approaches to HIV treatment aim to block the integration process and the enzyme that facilitates it (Di Santo, 2014).

An HIV-1 provirus is formed in newly infected cells and acts as a template for the transcription of viral messengers by the cellular Pol II polymerase (Dehaseth et al., 1998; Ramos-Pascual, 2019). The viral promoter located in the U3 region of the 5' LTR initiates the transcription process (Mori & Valente, 2020; Roebuck & Saifuddin, 2018; Roebuck1 et al., 1999). The viral Rev protein and the rev responsive element (RRE) interact with each other to mediate the migration of partially spliced and unspliced

mRNA from the nucleus into the cytoplasm (Fernandes et al., 2012; Henderson & Percipalle, 1997). The Rev protein also interacts with the cellular export factor Crm1 to connect the viral RNA to the export machinery (Daelemans et al., 2002).

Following transcription and nuclear import, the Gag and Gag-Pol precursors, the envelope glycoproteins, the viral genomes, and the HIV accessory proteins come together to form a viral particle. These components are assembled on the host plasma cellular membranes, where the immature virion buds through the cellular membrane and is then released through the action of host cell cofactors recruited by the Gag protein (Sundquist & Kräusslich, 2012). The progeny virion is then detached from an infected cell to cross the host plasma membrane and obtain its lipid envelope (Garoff et al., 1998; Votteler & Sundquist, 2013). This process is facilitated by the p6 domain of the Gag protein and the cellular Tsg101 protein, which together enable newly formed virions to pinch off from the host cell and enter the circulation (Göttlinger, 2001; Pornillos et al., 2003). Proteolysis transforms the immature virion into a mature, infectious virus (Ganser-Pornillos et al., 2011; Konvalinka et al., 2015; Mattei et al., 2016; McGraw et al., 2024; Novikova et al., 2019; Qu et al., 2021). Protease inhibitors aim to block the viral polyprotein precursors from being cleaved into mature, functional proteins required for viral replication, they include lopinavir, tipranavir, and darunavir (Majerová & Novotný, 2021).

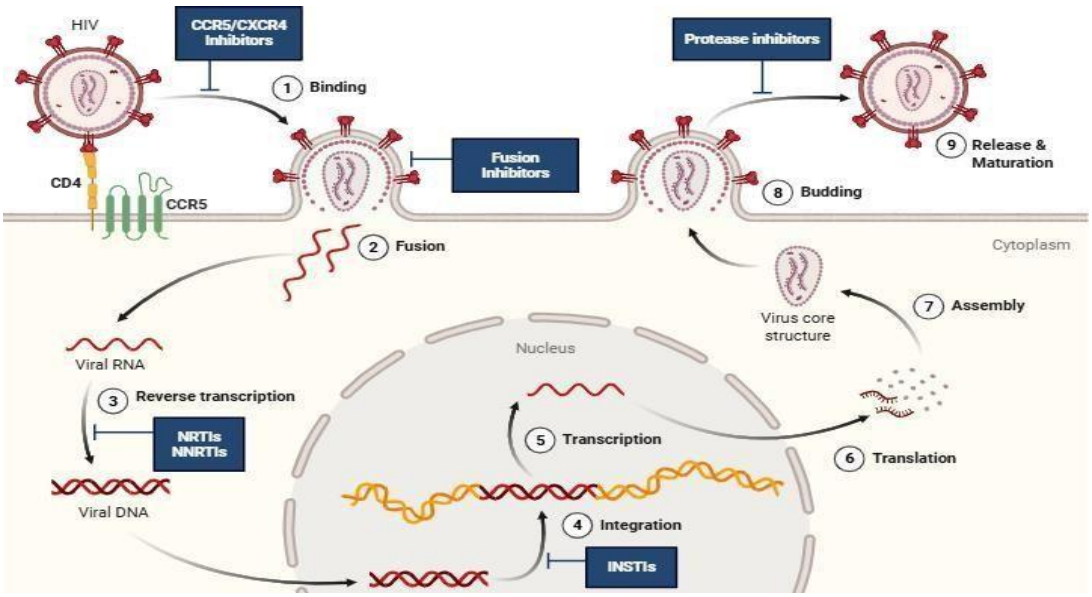

Figure 3: The HIV-1 replication cycle and antiretroviral drugs targeting each stage of replication.

Table 1**:** Antiretroviral drug classes and their mechanisms of action

Drug Class	Mechanism of Action	Examples of Commonly used Drugs
Entry Inhibitors	Impede the binding, fusion, and entry of the virus into human cells by interrupting the CCR5 and CXCR4 HIV receptor-mediated entry of the HIV virus into the host cell	Enfuvirtide (T-20); Fostemsavir (FTR); Ibalizumab (IBA); Lenacapavir (LEN); Maraviroc (MVC)
Nucleoside reverse transcriptase inhibitors	Inhibit the reverse transcription of HIV RNA into DNA by promoting chain termination following their incorporation into the viral DNA	Abacavir (ABC); Tenofovir disoproxil fumarate (TDF); Lamivudine (3TC); Emtricitabine (FTC); Tenofovir alafenamide (TAF)
Non-nucleoside reverse transcriptase inhibitors	Interrupt the HIV reverse transcriptase activity by tethering its pocket and transforming its shape	Doravirine (DOR); Efavirenz (EFV); Etravirine (ETR); Nevirapine (NVP); Rilpivirine (RPV)
Integrase strand transfer inhibitors	Interrupt the integration of the viral DNA into the host nucleus.	Bictegravir (BIC); Dolutegravir (DTG); Elvitegravir (EVG); Raltegravir (RAL); Cabotegravir (CAB)
Protease Inhibitors	Substrate analogs for the HIV protease enzyme, bound to its active site to prevent it from further activity, inhibiting maturation of the virus and preventing the creation of functional viruses.	Atazanavir (ATV); Darunavir (DRV); Darunavir/cobicistat (DRV/c); Fosamprenavir (FPV); Lopinavir (LPV)

2.5 3’ Processing and Strand Transfer

During HIV-1 integration, a series of sequential events occur to incorporate the viral genome into the host chromosome, catalyzed by the integrase gene (Brown et al., 1987; Craigie & Bushman, 2012). Integration takes place at the terminal ends of the viral DNA strand and occurs in two main stages, which are 3’ processing and strand transfer (A. Engelman et al., 1991; Engelman & Singh, 2018; Passos et al., 2021; Smith et al., 2021). During 3’ processing, two nucleotides are detached from each strand of the linear DNA, leaving behind overhanging CA_{OH} ends (Figure 4) (Asante-Appiah & Skalka, 1997; Craigie & Bushman, 2012; Pauza, 1990). During strand transfer, the 5’ ends of the target DNA are covalently joined to the 3’ ends of the processed DNA (Asante-Appiah & Skalka, 1997; Craigie & Bushman, 2012). In the third and final reaction, an integrated provirus surrounded by five-base-pair direct repeats of the target site DNA is generated by removing unpaired nucleotides from the viral 5’ ends and joining them to the target site 3’ ends. To complete the integration process, the single-strand gaps and the dinucleotide overhangs at the 5’end of the viral DNA are repaired by the cellular enzymes (Yoder & Bushman, 2000). The integrase enzyme facilitates the process of integration, in which the HIV genome is inserted into the host genome, thereby concealing the HIV DNA within the host genome (Lewinski & Bushman, 2005). One of the most promising novel approaches to anti-retroviral therapy is the use of integrase strand transfer inhibitors, a new class of HIV medications that target the HIV-1 integrase enzyme and interrupt the integration of viral DNA into the host nucleus. Raltegravir was the first integrase strand transfer inhibitor to receive approval for the treatment of HIV (Hicks & Gulick, 2009; Temesgen & Siraj, 2008). Following the development of Raltegravir, several next-generation INSTIs have been developed to enhance potency and overcome resistance. Elvitegravir, first approved in 2012, expanded treatment options but required boosting (Lampiris, 2012; Marchand, 2012; A. V. Zhao et al., 2022). In contrast, dolutegravir introduced a high genetic barrier to resistance and became a global first-line therapy (Egger et al., 2024; A. V. Zhao et al., 2022). Bictegravir was approved in 2018 and offered potent, unboosted once-daily dosing, while Cabotegravir, approved in 2020, enabled long-acting injectable regimens (Daar et al., 2018; Pinto et al., 2023; Swindells et al., 2020). Despite advances in strand-transfer inhibition, no drugs have been approved that specifically target the 3′-processing step or the nuclear import pathway (Luo & Muesing, 2010). Agents such as LEDGINs remain investigational, leaving strand transfer as the only clinically validated integrase inhibition mechanism currently employed in ART (Desimmie et al., 2013).

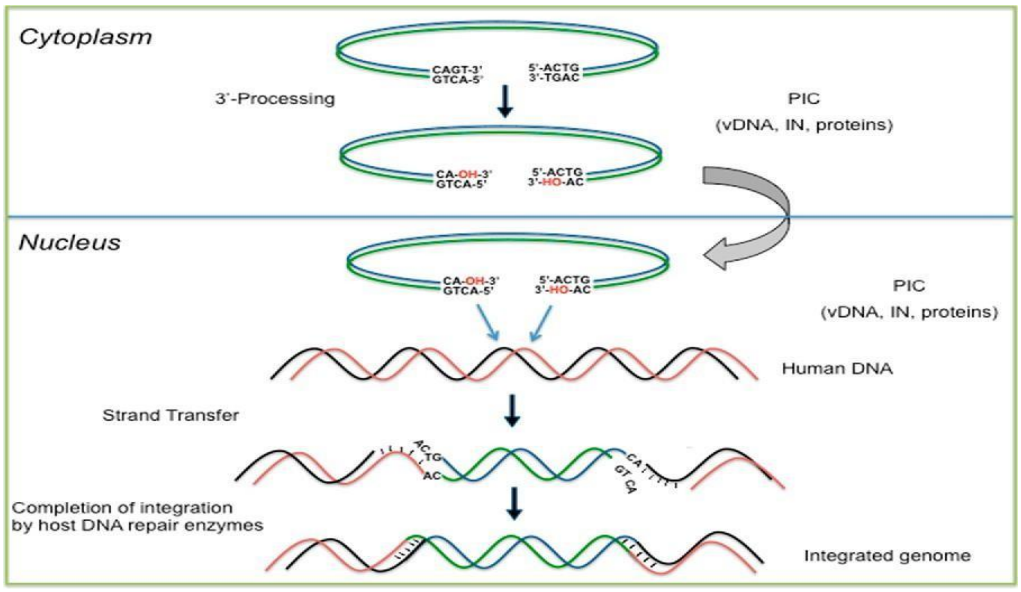

Figure 4: Sequence and outline of the process of integration

2.6 Treatment for HIV

The primary treatment for HIV is known as antiretroviral therapy, and consists of a mixture of different HIV medications taken on a daily basis to inhibit the virus and promote immune system recovery (Arts & Hazuda, 2012). Azidothymidine (Zidovudine), a nucleoside reverse transcriptase inhibitor (NRTI), was the first drug specifically designed to target and inhibit HIV replication (Broder, 2010; Fischl et al., 1987). NRTIs are analogs of endogenous 2'-deoxy-nucleosides and nucleotides, that form deoxynucleoside triphosphates (dNTPs) capable of viral inhibition; they compete for HIV RT incorporation with endogenous dNTPs and serve as chain-terminators of viral reverse transcripts (Cihlar & Ray, 2010). Currently, six groups of HIV antiretroviral drugs that target different stages of the HIV life cycle are in use (Table 1); these include the Nucleoside reverse transcriptase inhibitors (NRTIs), Non-nucleoside reverse transcriptase inhibitors (NNRTIs), Protease inhibitors (PIs), Fusion inhibitors (FIs), Co-receptor inhibitors (CRIs), and Integrase strand transfer inhibitors (INSTIs), and these medications have been shown to be effective in preventing HIV from replicating (De Clercq, 2013; Eggleton & Nagalli, 2020, 2022; Kumari & Singh, 2012).

Adhering to daily ART regimens is crucial for maintaining an undetectable viral load and preventing the virus from developing resistance to the drug. People living with HIV who take ART can keep their viral load undetectable, and are essentially at zero risk of HIV transmission to their sexual partners (Eisinger et al., 2019). Although ART is remarkably efficient, a cure for HIV remains elusive, and certain factors continue to impede the capacity of ART to eradicate HIV infection (Ndung'u et al., 2019). HIV-1 remains in a stable reservoir of latently infected resting memory CD4+ T cells, with minimal reduction observed even in individuals receiving ART (Churchill et al., 2016; Eisele & Siliciano, 2012; Mzingwane & Tiemessen, 2017). For most PLWH on present ART regimens, this latent reservoir is

adequate to ensure lifelong persistence of HIV-1, and eradicating it will not be possible until alternative strategies become available (Blankson et al., 2002; Eisele & Siliciano, 2012). People living with HIV who are on lifelong ART may experience drug-related toxicities and side effects, as long-term use of these medications can impact various organs and cause health complications over time (Palmisano & Vella, 2011; Powderly, 2002; Volberding & Deeks, 2010). In addition to causing toxicities, long-term ART can lead to the emergence of HIV drug resistance, which can develop when the virus mutates, reducing the effectiveness of ART regimens and limiting treatment options over time (SeyedAlinaghi et al., 2023). HIV drug resistance occurs as a result of changes in the HIV genome, resulting in alteration of the HIV genetic structure, impacting the capacity of antiretroviral therapy medications to hinder the replication of the virus (Shafer et al., 2000; World Health Organisation, 2024). Alterations in the sequences flanking the HIV-1 reporter gene can influence the mutations that arise within that gene (Abram et al., 2014). HIV reverse transcriptase exhibits a high error rate, introducing approximately one mutation every 2,000 nucleotides, which translates to an estimated five to ten errors per HIV-1 genome per replication cycle (Abram et al., 2014; Del Río et al., 2024). HIV replicates rapidly, generating billions of viral particles daily in untreated individuals, with plasma HIV RNA levels typically ranging from 10^3 to 10^5 copies/mL and reaching up to 10^6 copies/mL during acute infection or advanced disease (Zdanowicz, 2006). The rapid replication of HIV, coupled with its short half-life of 1–2 days and the high error rate of reverse transcriptase, drives the emergence of diverse viral variants within a single host, resulting in differential responses to antiretroviral therapy (Abram et al., 2010; Alizon et al., 1986; Perelson et al., 1996).

HIV drug resistance may also occur as a result of diminished adherence to the antiretroviral therapy by the patient, as well as an inappropriate prescription of antiretroviral drugs (Bangsberg, 2008; Sethi et al., 2003). The introduction of better-tolerated regimens has resulted in an overall decline in the frequency of drug resistance. However, drug resistance is still reported in 7-15% of patients initiating first-line ART (Cortez & Maldarelli, 2011; Hogg et al., 2006). The emergence of drug resistance limits and exhausts the number of available treatment options. This results in HIV-1 disease progression and death (Hogg et al., 2006). As HIV-1 drug resistance variants are transmitted with new infections, this threatens to reverse the progress accomplished by ART and may increase the mortality and morbidity rates (Cortez & Maldarelli, 2011). All antiretroviral medications, including those that were recently approved for treatment, could potentially become fully or partially inactive as a result of drug resistance (World Health Organisation, 2024).

2.7 Integrase Drug Resistance

The integration of viral DNA into the host genome is a critical step in the HIV-1 replication cycle, mediated by the viral integrase (IN) enzyme (Delelis et al., 2008; Elliott & Kutluay, 2020). Integrase strand transfer inhibitors (INSTIs) have revolutionized antiretroviral therapy (ART) by targeting this

essential step (Jóźwik et al., 2020; Smith et al., 2021). Currently, the approved INSTI for use in PLWH include Raltegravir (RAL), Elvitegravir (EVG), Dolutegravir (DTG), Cabotegravir (CAB), and Bictegravir (BIC) (Nguyen et al., 2011; Podany et al., 2020). First-generation INSTIs, such as RAL and EVG, and newer second-generation inhibitors like DTG and BIC, offer potent viral suppression with favourable resistance profiles (Geretti et al., 2012). However, the emergence of integrase drug resistance mutations (DRMs) threatens long-term treatment success. Similar to other antiretroviral drug resistance mutations (DRMs), integrase DRMs primarily arise due to the high error rate of the HIV reverse transcriptase enzyme, which generates a genetically diverse pool of reverse-transcribed DNA and multiple viral subpopulations (Quashie et al., 2013).

Key mutations associated with INSTI resistance have been identified at specific positions within the integrase enzyme (Abram et al., 2013). Mutations at positions 143, 148, and 155 have been frequently observed in patients experiencing virological failure while on RAL-containing regimens (Blanco et al., 2011). Specifically, mutations such as Y143R, Q148HKR, and N155H can individually reduce susceptibility to RAL by more than tenfold, particularly in experimental settings (Blanco et al., 2011). The presence of accessory mutations, such as T97A, can further contribute to INSTI resistance and improve viral fitness in the context of primary resistance mutations (George et al., 2018). The emergence of these mutations highlights the importance of understanding INSTI resistance mechanisms to inform the development of novel and effective HIV treatment strategies (Paydary et al., 2013).

In South Africa, the rise of INSTI drug resistance is a growing concern, particularly as DTG-based regimens have been adopted as the preferred first-line treatment under the national antiretroviral therapy (ART) program (McCluskey et al., 2021; Naidoo et al., 2025). Eastern and Southern Africa has one of the highest burdens of HIV globally, with an estimated 21.1 million people living with the virus, making effective long-term treatment strategies essential (UNAIDS, 2025). Although integrase resistance remains relatively rare compared to reverse transcriptase and protease inhibitor resistance, the increasing use of DTG raises the possibility of future resistance emergence, especially in cases of poor adherence or limited viral load monitoring (Mahomed et al., 2020). Some mutations in the HIV-1 integrase enzyme, such as R263K, G118R, H51Y, and E138K, have been found to cause low-level resistance to DTG, suggesting that integrase resistance could become more prevalent with expanded INSTI use (Wainberg & Han, 2015). The increasing reliance on DTG-based antiretroviral therapy in South Africa, while generally effective, necessitates alternative strategies due to the potential for DTG resistance to emerge, particularly in treatment-experienced individuals (Naidoo et al., 2025). One such strategy involves the exploration of bioactive compounds derived from medicinal plants and their associated endophytic fungi, which have shown promising antiviral potential (Manganyi & Ateba, 2020).

2.8 Medicinal Plants and Endophytic Fungi as Emerging Sources of Antiviral Agents

Medicinal plants have been used for centuries to treat a wide range of ailments, and many modern pharmaceuticals are derived from them due to their rich repertoire of natural products with therapeutic properties. These bioactive compounds exhibit antifungal, antibacterial, and antiviral activities, underscoring the continued relevance of medicinal plants in both traditional and modern healthcare systems (Bassey et al., 2023; Ingale & Hivrale, 2010). *Hypoxis hemerocallidea*, also known as African Potato, is a well-known member of the *Hypoxidaceae* family that is widely cultivated in South Africa and has long been used in traditional African medicine to treat various ailments, including colds, flu, nausea, heart weakness, infertility, mood disorders, and urinary tract infections (Nair et al., 2007; Owira & Ojewole, 2009). In a study conducted in the Eastern Cape in South Africa, *Hypoxis hemerocallidea* was found to be the most frequently traded plant among a total of 166 traded medicinal plants (Dold & Cocks, 2002). 84% of clinic attendees with moderate to severe HIV illness who were receiving ART reported using traditional medicines for the treatment of HIV, with African Potato and *Aloe vera* being the most frequently used sources of traditional medicine (Babb et al., 2007). Research conducted *in vitro* and *in vivo* has demonstrated that African potatoes and their metabolites have anti-oxidant, anti-inflammatory, antidiabetic, anticonvulsant, antibacterial, and CD4-count-lowering properties (Bassey et al., 2023). Hypoxoside, a phytochemical derived from *Hypoxis* plants, can be converted to rooperol, which has been found to have potent pharmacological activities against cancer, inflammation, as well as HIV (Coetzee et al., 1996; Nair et al., 2007).

Figure 5: *Hypoxis hemerocallidea (Hypoxidaceae),* also known as African potato[5]

Recent scientific interest has focused not only on the phytochemicals produced by medicinal plants, but also on the endophytic fungi they host, which may biosynthesize unique secondary metabolites with therapeutic potential (Tiwari & Bae, 2022). Endophytic fungi are a group of microorganisms that reside within the internal structures of plants, colonizing them without causing harm (Gunatilaka, 2006). These endophytes exist in a symbiotic relationship with the plant and are increasingly recognized as a reservoir

of novel bioactive compounds (Gakuubi et al., 2021). Endophytes produce secondary metabolites that have many biological activities; these metabolites can be used in drug discovery and have many medical applications (Fadiji & Babalola, 2020; Narayanan & Glick, 2022; Prajapati et al., 2021). Endophytes have been shown to produce secondary metabolites with antiviral, anticancer, and antimicrobial properties, including terpenes and terpenoids, sesquiterpenoids, diterpenoids, triterpenoids, meroterpenoids, steroids, polyketides, alkaloids, and other compounds, and are therefore being studied for drug discovery (Rustamova et al., 2020). Secondary metabolites derived from endophytic fungi have been shown by several studies to possess antiviral activity (Lacerda et al., 2022).

Various fungal metabolites isolated from terrestrial fungi, including diverse chemical structures such as naphthoquinones and terpenoids, were found to inhibit HIV-1 integrase with IC_{50} values ranging from 0.5 to 120 µM (Singh, Jayasuriya, et al., 2003). Cytosporic acid, a polyketide derived from the filamentous fungus *Cytospora sp.,* was found to inhibit the strand transfer reaction of HIV-1 integrase with an IC_{50} of 20 µM (Jayasuriya et al., 2003). The polyketide natural products *Xanthoviridicatins E and F* isolated from the endophytic fungus *Penicillium chrysogenum* were found to inhibit the cleavage reaction of HIV-1 integrase with IC_{50} of 6 and 5 µM, respectively (Singh, Zink, et al., 2003). Isocoumarin penicilisorin and azaphilone derivatives penicilazaphilones A and B, derived from the endophytic fungus *Penicillium sclerotiorum* PSU-A13, isolated from the leaves of *Garcinia atroviridis,* were found to show inhibitory activity against HIV-1 protease and integrase enzymes, with IC_{50} values ranging from 14.5 to 62.7 mg/ml (Arunpanichlert et al., 2010). The limitations of these studies are that *in vivo* testing of them was not done, which will qualify the further investigation in a clinical trial. Recently, the endophytic fungus *Penicillium* species *BCt* was found to produce phytochemicals such as coumarin (2H-1-benzopyran-2-one), coumaric acid (3-benzofuran-carboxylic acid), hynecromone (coumarin 4), 4-hydroxy-9-(3-methyl-2-butyl) furo (3,2-g) chloronen-7-one) that inhibited the replication of the three critical HIV-1 enzymes, reverse transcriptase, integrase, and protease (Govindappa et al., 2021). These studies demonstrate that endophytic fungi can be a potential source of inhibitors of the HIV-1 integration stage and the integrase enzyme, and should therefore be explored for the development of new and non-toxic anti-HIV agents.

Notably, members of the genus *Alternaria* have also shown inhibitory activity against HIV, such as the endophytic fungus *Alternaria tenuissima,* which produces altertoxins that demonstrate inhibitory activity against the enzyme reverse transcriptase, thereby preventing the conversion of HIV RNA to DNA (Bashyal et al., 2014). Four fractionated compounds isolated from the endophytic fungus *Alternaria tenuissima* were found to inhibit HIV-1 replication almost completely (Wellensiek et al., 2013). Secondary metabolites from *Alternaria alternata*, particularly those isolated from endophytic fungi, have shown promising antiviral activity against HIV-1, potentially inhibiting HIV-1 viral replication and attachment. *Alternaria alternata* PO4PR2 crude extracts from *Sclerocarya birrea* and *Hypoxis* plants were found to show anti-HIV-1 efficacy in TZM-bl cell lines and inhibited viral

multiplication in CD4+ T cells and PBMCs (Nzimande et al., 2022). The fractionated crude extract of *Alternaria alternata* PO4PR2 showed 100% inhibition against HIV-1 integration and 70% inhibition against HIV-1 reverse transcription (Kubheka et al., 2024). In a recent study, the crude extract of *Alternaria alternata* PO4PR2 demonstrated strong antiviral activity against various HIV-1 subtypes and integrase drug-resistant strains. Several stages of the HIV-1 life cycle were suppressed by the extract (Naidu et al., 2025). In another study, methanol extracts of *Alternaria* species were found to exhibit a high level of inhibition of HIV-RT, integrase, and protease activity (Govindappa et al., 2015). These findings highlight *Alternaria alternata* PO4PR2 as a potent source of bioactive metabolites with multi-stage antiviral activity, including inhibition of reverse transcriptase, integrase, and protease. These results warrant further investigation into the specific compounds responsible for the observed activity, their mechanisms of action, and their potential development into novel anti-HIV therapeutics, particularly as integrase inhibitor resistance continues to threaten current treatment regimens.

2.9 Principles of the assays

2.9.1 Bioinformatics and Sequence Analysis in HIV Drug Resistance Mutations

Bioinformatics has become an essential tool in HIV research, particularly for understanding viral genetic diversity, drug-resistance evolution, and the functional consequences of sequence variation (Bello et al., 2011; Blassel et al., 2021). Computational resources and curated databases facilitate rapid interpretation of viral genotypes, enabling the assessment of drug susceptibility, detection of resistance-associated mutations (RAMs), and prediction of their clinical impact (Zhang et al., 2025). These tools are invaluable for both fundamental research and the design of therapeutic interventions, such as studies exploring novel antiviral compounds, including fungal metabolites from *Alternaria alternata* (Kusmiati et al., 2024). Several databases and resources are widely used in HIV bioinformatics, each providing complementary information (Doherty et al., 2005; Kuiken et al., 2003; Sargeant et al., 2011). The Stanford HIV Drug Resistance Database (HIVDB) is the most widely used international resource for HIV drug resistance interpretation. It contains curated lists of RAMs for all major drug classes, including reverse transcriptase inhibitors, protease inhibitors, and integrase strand transfer inhibitors (INSTIs) (Blassel et al., 2021; Tang et al., 2012). HIVDB generates genotype-to-phenotype predictions by aligning query sequences to reference strains, identifying known RAMs, and applying mutation penalty scores to calculate an overall resistance score for each drug (Shafer, 2006; Shen et al., 2016). These scores are categorized into susceptibility levels, i.e., susceptible, potential low-level resistance, low-level, intermediate, or high-level resistance, allowing for standardized interpretation across studies (Harjani et al., 2022). The Los Alamos National Laboratory (LANL) HIV Sequence Database serves as a global repository for HIV sequences, providing tools for multiple sequence alignment, phylogenetic analysis, subtype identification, and recombination detection (Foley et al., 2018; Linchangco Jr et al., 2022). The LANL resource is particularly useful for studying genetic diversity, viral evolution, and population-level trends in HIV resistance (Kavuraya et al., 2025; Oluniyi et al., 2022; Yuan et al., 2020).

The IAS–USA Drug Resistance Mutation List, compiled annually by experts, provides clinically relevant RAMs that serve as a reference for validating observed mutations, though it does not generate resistance scores (Wensing et al., 2022, 2025). GenBank, maintained by NCBI, offers a comprehensive public archive for sequence retrieval, facilitating comparisons with previously published sequences and enabling the discovery of novel variants (Benson et al., 2005; H. Miller et al., 2009). Together, these databases support a comprehensive bioinformatic workflow by providing both reference sequences and curated mutation information. In the present study, these bioinformatic resources, including HIVDB, LANL, NCBI and GenBank, were systematically utilized to identify the most prevalent and clinically relevant HIV-1 integrase drug-resistance mutations across global datasets. Using the Stanford HIVDB mutation penalty scoring system, we assessed the susceptibility profiles of these mutations to current INSTIs, confirming the well-documented resistance patterns reported in previous studies that characterized key integrase mutations, such as Y143H/R, Q148H/R/K, G118R, N155H, and R263K, and their associated reductions in drug sensitivity (Maruapula et al., 2022, 2024; Seatla et al., 2021).

2.9.2 Nucleic Acid Extraction

Nucleic acid extraction (NAE) is a basic molecular biology method used to separate and purify DNA or RNA from biological samples by lysing cells, removing impurities such as proteins and lipids, and concentrating the genetic material; as the first and most crucial step in any amplification process, NAE provides pure nucleic acids that are essential for a wide range of downstream applications, including biotechnology, molecular diagnostics (such as PCR and DNA sequencing), forensic investigations, and genetic research (Shin, 2018; Sirakov, 2016). NAE is one of the most crucial processes in molecular biology, as it lays the foundation for subsequent procedures, as well as many molecular diagnostic kits (Tan & Yiap, 2009; Wink et al., 2006). Following the lysis of cells or tissues, RNA is separated from genomic deoxyribonucleic acid (DNA) and other cellular components (such as enzymes, salts, and nucleotides) using RNA purification techniques such as phenol-chloroform extraction, affinity-based methods, solid phase extraction, and magnetic separation (Göktürk et al., n.d.). RNA can be extracted in a single step by using a guanidinium thiocyanate-phenol-chloroform mixture (Ali et al., 2017; Chomczynski & Sacchi, 1987, 2006). The basic principle behind this method is the separation of RNA using an acidic solution that contains guanidinium thiocyanate, sodium acetate, phenol, and chloroform, followed by centrifugation to separate the RNA from DNA (Chomczynski & Sacchi, 2006). The majority of DNA and proteins stay in the lower organic phase or interphase in acidic environments, while complete RNA stays in the upper aqueous phase, followed by precipitation with isopropanol to recover the total RNA (Chomczynski & Sacchi, 2006). RNA was extracted using the Invitrogen PureLink RNA Mini Kit protocol, a column-based kit that isolates high-quality total RNA from a wide range of sample types in 20 minutes, utilizing common laboratory equipment (Thermo Fisher Scientific, USA). Solid-phase extraction employs a spin column driven by centrifugal force to immobilize RNA onto its particles using a chaotropic chemical, enabling rapid and efficient DNA purification while

overcoming the drawbacks of liquid extraction, such as incomplete phase separation (Shin, 2013; Tan & Yiap, 2009). Column-based RNA extraction approaches are low-cost, safe, have minimal toxicity, and can be used to extract Proteins, RNA, and DNA simultaneously from the same sample without compromising quality and yield, as well as to harvest RNA from a variety of animal cell types in a short amount of time (Niu et al., 2019; Tolosa et al., 2007). The PureLink extraction kit was found to be the most suitable for detecting mRNA by RT-PCR, as it produces high-quality, DNA-free RNA suitable for detection by RT-PCR (Rump et al., 2010). Using this kit substantially reduces the occurrence of false-positive results, enabling the rapid RT-PCR detection of mRNA (Rump et al., 2010). Once the RNA has been extracted, the RNA concentration of the sample is determined by measuring its absorbance at 260 nm (A260). The A260/A280 ratio is used as a reference to assess the purity of the RNA sample; "pure" RNA is defined as having a value of approximately 2.0 (Hashemipetroudi et al., 2018).

2.9.3 Polymerase Chain Reaction

The polymerase chain reaction (PCR) is a widely used nucleic acid amplification technique that employs the thermostable enzyme Taq polymerase to generate DNA through repeated cycles of denaturation, primer annealing, and extension; it has become a key tool in biomolecular research, enabling accurate detection and analysis of specific DNA and RNA segments (Khehra et al., 2024). The isolated RNA was used to synthesize complementary DNA (cDNA) following the Superscript III One- Step Reverse Transcriptase Polymerase Chain Reaction (RT-PCR) protocol (ThermoFisher Scientific, USA). Using RT-PCR, RNA sequences can be amplified, isolated, or identified. As the first step in RT- PCR, reverse transcriptase, a retroviral enzyme, produces a single-stranded complementary DNA copy of the RNA (Freeman et al., 1999). The reverse transcriptase enzyme transforms RNA into its complementary DNA sequence, and DNA polymerase synthesizes a second strand. Double-stranded complementary DNA is produced, which may then be amplified using PCR as usual. Due to its sensitivity and specificity, RT-PCR is particularly well-suited for detecting trace amounts of the target RNA. The components required for the reaction include DNA polymerase, primers, deoxyribonucleotides, dGTP, dATP, dCTP, and dTTP (Green & Sambrook, 2019). The two synthetic oligonucleotides making up the primers are made up of complementary sequences to the two ends of the target fragment (Freeman et al., 1999). They serve as the attachment sites necessary for DNA polymerase to function after annealing to their target sites on opposing strands of the template DNA, defining the region to be amplified (Marmiroli & Maestri, 2007).

A repetitive-sampling *Alu*-*gag* PCR was used for the quantification of HIV-1 integration (Liszewski, Jianqing, et al., 2009a; Mavigner et al., 2016a). This PCR test detects and quantifies integrated HIV DNA in two steps using an integration standard (IS) cell line (Liszewski et al., 2009). In the human genome, the repeat element *Alu* appears roughly every 5,000 base pairs, while the structural elements

of the virion particle are encoded by the HIV *Gag* gene (Jelinek & Schmid, 1982; Mighell et al., 1997). Once HIV has integrated into the human genome, the DNA target template will contain binding sites for *Alu* and *Gag* primers, with the gag primer acting as an anchor in the HIV genome, while the Alu primer acts as an anchor in the human genome (Liszewski, Yu, et al., 2009). To measure the number of *Alu-gag* amplicons generated in the initial PCR round, a second, HIV-specific, real-time PCR step was performed following the first PCR, and quantification was accomplished by contrasting the resulting signals with those acquired by using the IS (Liszewski, Yu, et al., 2009). To quantify integrated HIV-1 DNA**,** viral integration can also be analysed using *Alu*-LTR Real-Time PCR (Brussel et al., 2005; Brussel & Sonigo, 2003; Mbisa et al., 2009). It may be possible to precisely evaluate how HIV-1 DNA or HIV-1-based lentiviral vectors integrate using this real-time nested PCR technique, which may potentially be a useful tool for testing prospective integration inhibitors and detecting integrated HIV-1 DNA in patients (Brussel et al., 2005). Conventional PCR tests identify PCR products at the point in each PCR reaction where exponential amplification is no longer possible, resulting in varying amounts of the final product, or amplicon. On the other hand, quantitative or real-time PCR quantifies the amplicon during exponential amplification, where each cycle should theoretically double the amount of product produced, rather than at the conclusion of the reaction (Denman & McSweeney, 2005). The cycle threshold (Ct) for real-time PCR is the cycle at which fluorescence is thought to be observable above the background during the exponential phase. The Ct values are utilized for quantification (Denman & McSweeney, 2005).

2.9.4 Site-Directed Mutagenesis

Site-directed mutagenesis (SDM) is an *in vitro* technique that introduces a desired mutation in a double-stranded DNA plasmid using custom-created oligonucleotide primers (Kunkel, 1985). Through SDM, a gene's nucleotide sequence can be altered at a specified location (Walker, 2016). The New England Biolabs Q5 site-directed mutagenesis kit was used to introduce the integrase drug-resistant mutations N155H, Y143R, Q148R, G118R, Q148K, and R263K into the HIV-1 integrase. To introduce the point mutation of interest, forward and reverse primers were designed using the NEBaseChanger primer design tool (https://nebasechanger.neb.com/). To generate full-length amplicons of the plasmid with the desired mutation, the plasmid containing the gene of interest was PCR amplified using the NEB Q5 High-Fidelity 2X Master Mix, which contains the Q5 High-Fidelity DNA Polymerase. The NEB T_m Calculator was used to determine the optimal annealing temperature when using this enzyme. The PCR products were analysed using the Bio-Rad ChemiDoc Gel Imaging Systems (CA, USA), and the parental plasmid DNA was then digested using the Kinase, Ligase, and DpnI (KLD) enzyme mix, which contains a unique blend of enzymes in a buffer that preserves the activity of the enzymes, and the amplicon was ligated to produce the new mutant plasmid. The generated plasmids were then transformed into competent *Escherichia coli (E. coli)* (Yang et al., 2022). Following transformation, the

cells were plated on LB agar plates with 100 mg/l of ampicillin, and colonies were picked for plasmid extraction using the GeneJET Plasmid Miniprep Kit (Thermofisher Scientific, USA) (Yang et al., 2022).

Alternative methods of introducing mutations include the Quickchange site-directed mutagenesis protocol, the Unrestricted Mutagenesis and Cloning (URMAC) method, and the recently reported P3 site-directed mutagenesis (Mousavi et al., 2025). The QuickChange site-directed mutagenesis technique incorporates two complementary mutagenic primers into nascent, synthesized DNA, enabling site-specific mutations. Afterward, the successful amplification of double-stranded mutant DNA can be confirmed through agarose gel electrophoresis (Loke & Sim, 2001). The QuickChange method is a fast and accurate site-directed mutagenesis technique that utilizes two overlapping complementary primers to amplify an entire plasmid-based gene template in a single PCR reaction, enabling the rapid introduction of point mutations into the sequence of interest (Braman et al., 1996; W. Wang & Malcolm, 1999). P3 site-directed mutagenesis is a method that efficiently uses primer pairs with 3'-overhangs to introduce specific alterations into DNA sequences (Mousavi et al., 2025). In order to replicate the original DNA containing the desired mutation(s), URMAC uses the property of DNA ligation to transform a linear PCR product made from a plasmid template into circular DNA that can be opened at a second site by amplification with primers containing the desired mutation(s), circularized by ligation again, and amplified with the original primers (Hallak et al., 2017). While methods such as the QuickChange protocol, URMAC, and P3 site-directed mutagenesis described above are efficient in their own right, the NEB Q5 SDM kit used in this study offers a simpler and more cost-effective alternative that is well-suited for mutating large plasmids and enables direct transformation of overlapping fragments (Yang et al., 2022).

2.9.5 Sanger Sequencing

Sanger sequencing is a rapid and straightforward technique for identifying nucleotide sequences in single-stranded DNA, utilizing E. coli DNA polymerase I and DNA polymerase from bacteriophage T4 in the presence of various limiting nucleoside triphosphates (Sanger & Coulson, 1975). Sanger sequencing was used to confirm the presence of the introduced mutations, utilizing the BigDye Terminator v3.1 Cycle Sequencing Kit (Waltham, Massachusetts, United States of America). It involves determining a DNA sequence by synthesizing a complementary DNA strand using a DNA polymerase enzyme, a template DNA strand, and a primer. The process involves the integration of chain-terminating dideoxynucleotides and subsequent capillary electrophoresis to determine the nucleotide sequence of the DNA template (Al-Shuhaib & Hashim, 2023). The linear order of nucleotides is determined by separating randomly terminated DNA fragments through electrophoresis. Fluorescently labelled terminators, capillary electrophoresis, and automated laser-based detection systems are utilized to enhance the accuracy of nucleotide sequence identification (Rizzo & Buck, 2012). Notwithstanding the availability of more sophisticated techniques, Sanger sequencing remains the most accurate and

accessible sequencing technique available today, due to its precisely defined chemistry, and it continues to serve as the benchmark in numerous applications owing to its dependability (Al-Shuhaib & Hashim, 2023; Rizzo & Buck, 2012). Novel sequencing techniques include next-generation sequencing (NGS), a high-throughput DNA technology that simultaneously sequences numerous distinct DNA sequences in a single reaction by tracking the successive insertion of nucleotides into spatially arranged, immobilized DNA templates (Pinxten & Howard, 2014; Rizzo & Buck, 2012). NGS can be used to sequence complete genomes or be restricted to particular regions of interest, such as a limited number of individual genes or all 22,000 coding genes that make up a whole exome (Behjati & Tarpey, 2013; Pinxten & Howard, 2014). Oxford Nanopore sequencers utilize flow cells with a variety of nanopores in an electro-resistant membrane to uniquely disrupt the current by each DNA or RNA molecule that flows through one of the nanopores, enabling the identification of the molecule and its corresponding DNA or RNA sequence (Deamer et al., 2016). For large-scale sequencing needs, Illumina utilizes second-generation sequencing technology, which involves generating clusters on flow cells and sequencing by synthesis with reversible terminator chemistry, employing fluorescently tagged nucleotides to produce up to 10 million single-molecule clusters per square centimeter (Satam et al., 2023).

2.9.6 Antiviral Assays

A variety of antiviral assays are routinely employed to quantify HIV-1 infection and evaluate the inhibitory activity of natural or synthetic compounds, such as the Galactosidase assay, Luciferase assay, and the P24 ELISA. Together, these assays provide complementary insights into viral entry, replication, and integration events, forming the methodological foundation for evaluating antiviral activity. Among the most widely used are the β-galactosidase (β-gal) reporter assay, the luciferase reporter assay, and the p24 antigen ELISA (Miyamoto et al., 2015; Sutthent et al., 2003; Xing et al., 2016). In this work, we assessed the ability of the *Alternaria alternata* (PO4PR2) fungal crude extracts to suppress HIV-1 replication using a Luciferase TZM-bl cell-based assay. The TZM-bl cell line (also known as JC57BL13), derived from HeLa cells and controlled by the HIV-1 LTR, is designed to express CD4, CCR5, and CXCR4 (Montefiori, 2004; Platt et al., 1998). It contains integrated reporter genes for β-galactosidase and firefly luciferase, both driven by the HIV-1 promoter, which allow sensitive and precise measurement of HIV-1 infection (Naldini et al., 1996; Wei et al., 2002). After HIV-1 infects a TZM-bl cell, the viral protein Tat activates the HIV-1 promoter, triggering the production of luciferase, which can then be measured by luminescence as an indicator of viral infection (Xing et al., 2016). The majority of HIV strains, including primary HIV-1 isolates and molecularly cloned Env-pseudotyped viruses, are able to infect these cell lines with ease (Sarzotti-Kelsoe et al., 2014).

The luciferase assay, which measures luciferase activity, is a rapid, single-round infectivity test frequently used to evaluate the activity of substances, particularly those derived from microbial natural products (such as fungal extracts), in HIV-1 strains (Nzimande et al., 2022, 2023). It is reproducible,

highly sensitive, and offers a dynamic range, versatility, and ease of use, which can significantly enhance assay performance by providing 10- to 1,000-fold higher assay sensitivity than fluorescent reporters like GFP (Assa, 2008; Brasier & Ron, 1992). The luciferase assay is incredibly quick, easy, reasonably priced, sensitive, and has a wide linear range (Smale, 2010). The light generated by the chemical reaction is measured by a luminometer apparatus and is expressed in relative light units (RLU) (Iyer et al., 2019). Relative light units quantify the light emitted during a bioluminescent reaction when ATP interacts with the luciferase; because ATP is found in all living cells, RLU readings provide a relative indicator of biological activity on a surface or in a liquid sample (Lundin, 2000; Morciano et al., 2017; Shama & Malik, 2013). When measuring HIV-1 inhibition, infected, untreated cells are expected to produce high RLU values, reflecting active viral replication, while uninfected cells or those treated with a potent inhibitor, such as the control drugs used in the assay, should yield very low RLU values, representing background luminescence; test samples typically fall between these positive and negative controls, with RLU values decreasing as the inhibitor concentration increases (Maphanga et al., 2017). To calculate percent inhibition, the RLU values from the treated wells are compared to the RLU values of the uninhibited, infected control wells, which gives a percentage of inhibition. By testing a range of inhibitor concentrations, we can then plot a dose-response curve to determine the IC_{50}, the concentration of a substance needed to inhibit a biological process by 50% (Srinivasan & Lloyd, 2024).

The β-gal assay relies on cell lines engineered to carry the bacterial lacZ reporter gene (encoding β-galactosidase) under control of the HIV-1 long terminal repeat (LTR) (Bártolo et al., 2018; Charnay et al., 2009; Hoffmann et al., 2011). Upon HIV infection and Tat-mediated transcriptional activation of the LTR, β-galactosidase is expressed (Charnay et al., 2009; Ruiz et al., 2014). β-gal indicator cell lines offer a straightforward, quantitative bioassay for infectious HIV-1, suitable for plaque assays, neutralization studies, and screening antiviral agents (Miyamoto et al., 2015). The β-gal assay offers a lower-cost, simpler alternative with adequate sensitivity for many applications, although its dynamic range is more limited and it is less sensitive than luciferase (Y. Huang et al., 2022). Luciferase reporter assays using the widely adopted cell line TZM-bl, build on a similar concept but generally offer much greater sensitivity and dynamic range (Sarzotti-Kelsoe et al., 2014; Xing et al., 2016). In TZM-bl cells, firefly luciferase is integrated under the HIV LTR; upon infection, Tat-triggered transcription leads to luciferase expression, which can be rapidly and quantitatively measured through luminescence (Morozov et al., 2022). The advantages of the luciferase assay include high sensitivity (even low-level infection can be detected), broad dynamic range, fast readout, and compatibility with high-throughput formats (K. Y. Chen et al., 2022). The luciferase assay is particularly suited for detecting early infection events, entry efficiency, and transactivation of viral LTR, making it ideal for screening compounds that may block entry or early transcription (Kolokoltsov & Davey, 2004). The p24 antigen ELISA, in contrast, does not depend on reporter-gene expression but directly measures the viral capsid protein (p24) present in the supernatant or lysate (Hermle et al., 2010). Using capture and detection antibodies

specific for p24, the assay quantifies the amount of viral protein produced, which reflects the degree of virus replication and particle release (Ozono et al., 2020). p24 ELISA measures the output of the replication cycle and the amount of viral capsid produced, and is therefore particularly relevant for assessing virus production, release, or the effects of inhibitors on later stages of the viral life cycle (assembly, release, maturation) (Richter et al., 2025). Because p24 reflects actual viral protein levels, it offers a readout more directly related to viral yield compared to reporter-based assays (Passaes et al., 2017). Conventional p24 ELISA has a narrower dynamic range and lower sensitivity compared to luciferase assays, and typically does not distinguish between infectious and non-infectious virions (Harper et al., 2015).

The CEM-GXR assay is a sensitive fluorescence-based system used to quantify HIV-1 infection and evaluate antiviral activity in a physiologically relevant T-cell context (Brockman et al., 2006; Dahal et al., 2021; Ojwach et al., 2022). CEM-GXR cells are derived from the human CEM T-lymphoblastoid line and engineered to express a fluorescent reporter, such as GFP or RFP, under the control of the HIV-1 LTR (Brockman et al., 2006b; Miki et al., 2022). Unlike enzymatic reporters that require cell lysis or substrate addition, the CEM-GXR platform provides a live-cell, non-destructive readout, enabling real-time monitoring of viral replication kinetics and simultaneous assessment of cell viability or drug-induced toxicity within the same population (Gervaix et al., 1997; Tietjen et al., 2015). Because each fluorescent cell represents a single infected event, the assay offers high-resolution, single-cell sensitivity and can detect subtle differences in infection levels, even at low viral inputs or partial inhibition.

The use of the luciferase assay in TZM-bl cells can sensitively detect the inhibition of early infection/entry or transcriptional activation by fungal metabolites. Follow-up with p24 ELISA would then show whether the extract suppresses the production or release of viral particles over time, indicating an effect on later stages of the replication cycle. The inclusion of the β-gal assay, although perhaps less sensitive, could provide corroborative evidence, especially in resource-limited settings, and strengthen confidence in observed effects by demonstrating consistency across different assay platforms. Their combined use enhances the reliability of antiviral evaluation, for instance, against natural-product extracts like *A. alternata* PO4PR2 fraction, by reducing assay-specific biases and covering multiple points in the HIV replication cycle.

2.9.7 Integrase Activity Assay

The integration of viral DNA into the host DNA is catalyzed by the HIV integrase gene. Recombinant HIV-integrase can be used to assess the impact of natural items on HIV-1 integrase (Rege et al., 2015). To test for the inhibition of the crude extract against HIV-1 integrase, the HIV-1 integrase assay kit (XpressBio, Frederick, MD) was used to conduct the strand transfer assay (Park et al., 2021; Sayyed et al., 2023). Using a colorimetric enzyme immunoassay, the HIV-1 integrase assay was developed to investigate inhibitors for HIV-1 integrase (Figure 6). These colorimetric enzyme assays quantify the

capacity of the integrase enzyme to incorporate viral DNA into host DNA through two main enzymatic steps, 3'-processing and strand transfer, using short DNA oligonucleotides that mimic the viral DNA's long terminal repeat (LTR) sequence as substrates, typically labelled with a radioactive isotope or a fluorescent dye (Han et al., 2012). When interacting drugs are present, the suppression of HIV-1 strand transfer activity can be quantitatively measured using this technique. Detection of enzymatic activity can then be performed using fluorescently labelled products measured with plate readers, or through ELISA-type approaches that rely on avidin–alkaline phosphatase binding (Chang et al., 1996). The strand transfer inhibition assay can also be modified to validate the 3′-processing inhibitory activity of *Alternaria alternata* (Park et al., 2021; Sayyed et al., 2023).

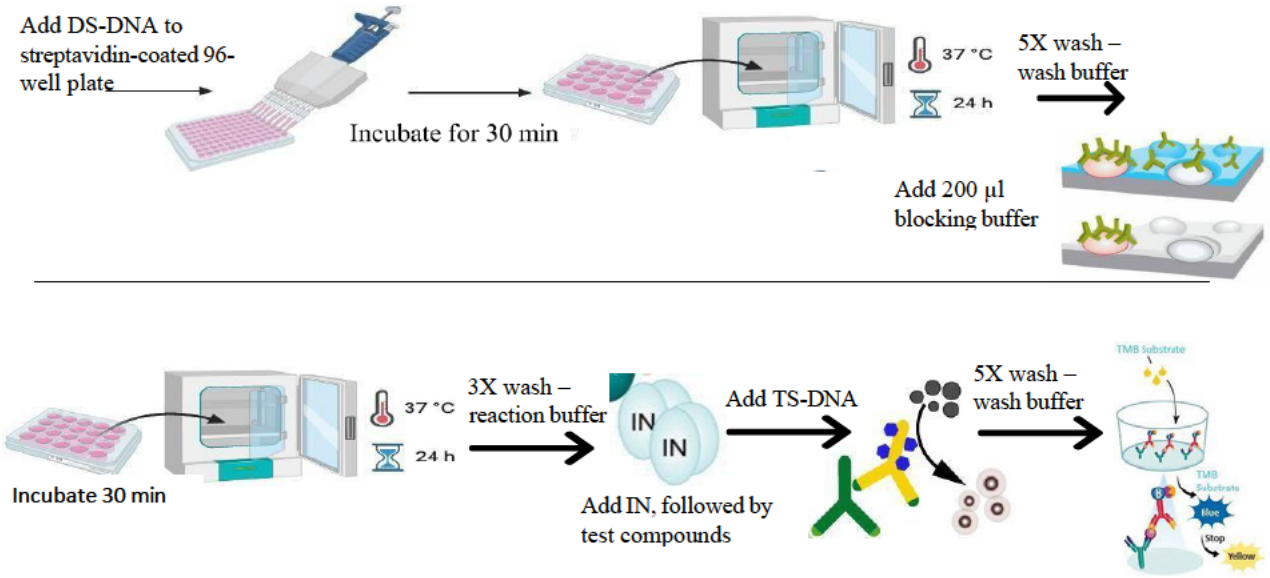

Figure 6: Workflow of the HIV-1 Integrase Activity Assay

2.9.8 Molecular docking studies

Molecular docking is gaining traction as a key computational tool in antiviral drug development, providing insights into how small molecules or natural compounds interact with viral and host proteins (Agu et al., 2023). By predicting the most favourable binding orientation of a ligand within a protein's active or allosteric site, docking helps identify compounds with potential antiviral activity before expensive laboratory testing is performed (Pinzi & Rastelli, 2019). Early docking approaches in the 1980s relied on rigid models; however, the availability of high-resolution viral protein structures and advances in computational algorithms have led to the incorporation of flexible docking and induced-fit

models (Gioia et al., 2017; Salmaso & Moro, 2018). These developments have improved predictive accuracy, particularly for structurally dynamic viral enzymes, such as HIV reverse transcriptase, integrase, and proteases.

Molecular docking involves target and ligand preparation, identification of the binding site, simulation of binding poses, scoring, and validation. Viral or host protein structures are typically retrieved from the Protein Data Bank (PDB) and prepared by removing non-essential components, adjusting protonation states, and minimizing energy to optimize geometry (Pettersen et al., 2004). Ligands undergo similar preparation to ensure the correct stereochemistry, tautomerism, and charge normalization (Hanwell et al., 2018).

Binding sites are identified using crystallographic data, literature, homologous protein structures and computational cavity detection (Clark et al., 2020; Goodsell et al., 2020; Muhammed & Aki-Yalcin, 2019). Docking simulations position ligands within these sites using rigid or flexible docking algorithms. Rigid docking is efficient for preliminary screening, whereas flexible docking captures conformational changes that are important for the accurate modelling of viral proteins. Widely used software includes AutoDock, AutoDock Vina, AutoDock Gold, Dock, Glide, and FlexX (Agu et al., 2023).

Scoring functions estimate the binding affinity by evaluating hydrogen bonding, hydrophobic interactions, electrostatics, and desolvation energies (Mohanty & Mohanty, 2023). The predicted poses are then assessed for mechanistic relevance using molecular visualization tools such as PyMOL, UCSF Chimera, or Discovery Studio (Oduro-Kwateng & Soliman, 2024; Pettersen et al., 2004; Seeliger & De Groot, 2010). Experimental validation through enzyme assays, viral inhibition studies, or structure–activity relationship (SAR) analyses is essential to confirm computational predictions (Naidu et al., 2025b; Temml & Kutil, 2021).

Docking contributes to antiviral discovery by enabling high-throughput virtual screening of compound libraries, elucidating the mechanisms of viral enzyme inhibition, exploring resistance-associated mutations, and guiding rational ligand optimization (Blanes-Mira et al., 2023). It supports fragment-based drug design and helps predict off-target effects and potential interactions within host–virus systems (Alzain et al., 2022; Issa et al., 2019). These applications make docking highly valuable for identifying and refining candidates targeting viruses such as HIV, SARS-CoV-2, influenza, and hepatitis viruses (Naidu et al., 2025; Oduro-Kwateng & Soliman, 2024; Shaikh et al., 2022; Terefe & Ghosh, 2022).

Despite its usefulness in the early phases of drug design, molecular docking has limitations, particularly in accurately accounting for protein flexibility, solvent effects, and entropic contributions. Docking results represent static snapshots and may not fully reflect the dynamic behaviour of viral proteins (S. Y. Huang & Zou, 2010). Integrating docking with molecular dynamics (MD) simulations improves the

accuracy of binding predictions by incorporating conformational flexibility and solvent interactions (Santos et al., 2019).

Emerging developments, such as machine learning (ML)–assisted scoring, artificial intelligence (AI)-driven docking algorithms, quantum mechanics–based energy calculations, and enhanced sampling techniques, are expected to further improve reliability (T. Chen et al., 2023; Crampon et al., 2022). As computational and structural approaches evolve with time, molecular docking will remain an essential tool for rational antiviral drug design and mechanistic virology research.

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CHAPTER 3: Methodology

3.1 Isolation and identification of endophytic fungi

Medicinal plants *Sclerocarya birrea* (voucher no. 18234) and *Hypoxis* (voucher no. 18233) species were collected from the South Coast region of Durban, KwaZulu-Natal, and voucher specimens were deposited at the UKZN School of Life Sciences Herbarium for taxonomic confirmation (Nzimande et al., 2022). Endophytic fungi were isolated from sterilised roots, leaves, and stems using standard surface-sterilisation and plating techniques on PDA and MEA media supplemented with ampicillin to prevent bacterial contamination. A total of fifteen fungal isolates were obtained and purified. Based on preliminary anti-HIV screening, the three isolates (PO4PR1, PO4PR2, and PO2PL1) were selected for molecular identification. Genomic DNA was extracted and the internal transcribed spacer (ITS) region was amplified using ITS1 and ITS4 primers (Nzimande et al., 2022). The PCR products were purified, sequenced, and analysed using BLAST against the NCBI GenBank database. Sequence similarity analysis identified the isolates as *Alternaria alternata*, and the sequences were deposited in GenBank under accession numbers OP648259, OP648254, and OP648252 (Nzimande et al., 2022).

3.1.1 Selection of Drug Resistance Mutations from the Stanford Drug Resistance Database

To assess the prevalence of integrase drug-resistance mutations in HIV-1 subtypes, the integrase mutation sequences were analysed in the Stanford drug resistance database. The 2021 HIV sequence compendium was obtained from the Los Alamos HIV Sequence Database. HIV sequences were downloaded from the HIV-1/SIV cpz complete genome set. The HXB2 sequence (B.FR.83.HXB2) was used as a reference sequence, and the complete genome was trimmed using the Jalview application to obtain the integrase sequence. A total of 122 sequences were analysed in the National Centre for Biotechnology Information (NCBI). The analysed sequences were then downloaded in FASTA format. All 122 sequences were compiled into one text document and aligned using ClustalW. The aligned sequences were trimmed using Jalview to obtain the integrase region of each sequence. The aligned and trimmed sequences were then analysed in the Stanford Drug Resistance Database HIVDB Program, and the drug resistance mutations were obtained and documented. To identify mutations specific to the integrase gene and the integrase stage, a targeted search was conducted using Google Scholar with the following keywords: Southern Africa, HIV integrase sequence. Three studies were selected, and their reported sequences were analysed, using their reported accession numbers (Seatla et al., 2021; Semengue et al., 2021; Steegen et al., 2019). Forty HIV-1C integrase sequences, deposited into the National Center for Biotechnology Information (NCBI) GenBank, were analyzed from the first study, with the following accession numbers:

MW690052-MW690089, MG989443.1, and MG989444.1. For the second study, 73 integrase sequences were analysed, with the following accession numbers: MW328641-713. For the third and final study, 43 sequences were analysed, with the following accession numbers: MK899435 to MK899477. A total of 156 sequences were analysed, and all sequences were downloaded from the NCBI in FASTA format. The sequences were then combined into a single text document and analyzed in the Stanford Drug Resistance Database HIVDB Program. The HIV integrase mutations were obtained and recorded. To estimate the level of resistance to a drug, the penalty scores associated with each of the DRMs present in a submitted sequence are added up, and the total score is calculated. The estimated level of resistance can be interpreted as follows: Susceptible: Total score 0 to 9; Potential low-level resistance: Total score 10 to 14; Low-level resistance: Total score 15 to 29; Intermediate resistance: Total score 30 to 59; High-level resistance: Total score >= 60 (WHO, 2012, 2019; Shafer, 2006). Susceptible indicates that no evidence of reduced ARV susceptibility has been observed compared with the wild-type virus. Potential low- level resistance indicates that the sequence contains mutations resulting from exposure to the ARV in question or may contain mutations associated with drug resistance. Low-level resistance means that the virus encoded by the submitted sequence has reduced *in vitro* ARV susceptibility. Intermediate resistance indicates a likelihood that the drug's activity will be reduced, but it will likely retain significant residual antiviral activity. Finally, high-level resistance means that the predicted level of resistance is similar to that observed in viruses with the highest levels of *in vitro* drug resistance, or that clinical data demonstrate that patients infected with viruses carrying these mutations have a reduced or no virological response to treatment with the ARV (WHO, 2012, 2019; Shafer, 2006). The N155H, Y143R, Q148R, G118R, and E138A/E138K mutations showed reduced susceptibility, particularly to EVG and RAL, while Q148R and G118R conferred broad cross-resistance. In contrast, L74I, T97A, and G140A maintained susceptibility across all drugs.

Table 2: Resistance levels of HIV-1 integrase mutations against five Integrase Strand Transfer Inhibitors

DRM	BIC	CAB	DTG	EVG	RAL
N155H	10	30	10	60	60
Y143R	0	10	0	10	60
T97A	0	0	0	10	10
L74I	0	10	0	0	0
E138K	10	15	10	15	15
Q148R	25	60	25	60	60
S147G	10	15	10	60	10
G140A	10	15	10	30	30
T97TA	0	0	0	10	10
L74M	0	10	0	0	0
N155NH	10	30	10	60	60
E157EQ	0	0	0	10	0
D232N	0	0	0	10	10
G118R	45	60	60	60	60
T66A	0	0	0	60	15
L74LM	0	10	0	0	0
E157Q	0	0	0	10	10
G163R	0	0	0	15	15
D232DN	0	0	0	10	10
L74M	0	10	0	0	0
V75AT	0	10	0	0	0
Q148K	30	60	30	60	60

3.1.2 Isolation and preparation of *Alternaria alternata* PO4PR2 crude extract

Nzimande et al. (2022) isolated and identified fifteen endophytic fungi from the roots, stems, and leaves of *Hypoxis* species (voucher no. 18233) and *S. birrea* (voucher no. 18234), collected from the South Coast region of the Durban Municipality, KwaZulu-Natal. Of the fifteen endophytic fungal isolates selected for molecular identification, three (PO4PR1, PO4PR2, and PO2PL1) were identified as *Alternaria alternata* (Nzimande et al., 2022). To prepare the endophytic fungal crude extracts, three small fungal plugs (1 cm^2) from 5-day-old Malt Extract Agar (MEA, Neogen, United States) were excised and inoculated into a 10-milliliter McCartney bottle of either Potato Dextrose Agar (PDA) (PDB, Neogen, United States), or Malt extract agar, and the bottles were then incubated for 14 days at 25 ºC in the dark. Following incubation, an identical volume of absolute methanol (ChemLab Supplies, South Africa) was added to each culture, and the cultures were subsequently placed on a rotatory shaker (Reflecta Laboratory Supplies, Glassware and Chemicals, South Africa) to shake overnight at 150 rpm and 25 °C. Gauze was used as a filter to separate the extracts from the mycelia on culture flasks that were weighed beforehand. The mycelia were then discarded, leaving the supernatants to dry at 40 °C. The dried methanol crude extracts were stored at -80°C and reconstituted in DMSO to a concentration of 400 µg/mL before each application.

3.1.3 Cell culture maintenance

TZM-bl cells generated from HeLa cells and modified to produce CD4, CCR5, and CXCR4, combined with firefly Luciferase reporter genes, were grown in a monolayer in sterile 75 cm^2 culture flasks, using Dulbecco's Modified Eagle Medium (DMEM) (Thermo Fisher Scientific, Waltham, United States of America), enhanced with 10% fetal 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 (also known as JC53BL-13), is modified to express CD4, CCR5, and CXCR4 as well as integrated reporter genes for E. coli β-galactosidase and firefly luciferase, which are controlled by an HIV-1 long terminal repeat, allowing for precise and sensitive infection measurements and efficient replication of most HIV, SIV, and SHIV strains, including native or molecularly cloned viral isolates and molecularly cloned Env-pseudotyped viruses (Morozov et al., 2022; Platt et al., 1998; Sarzotti-Kelsoe et al., 2014; Wei et al., 2002). To achieve confluency, the TZM-bl and HEK 293T cell lines were cultured at 37 °C with 5% CO_2. Phosphate-Buffered Saline (PBS) (Thermo Fisher Scientific, Waltham, United States of America) was used to wash the cells at confluency. 0.25% Trypsin-EDTA (Thermo Fisher Scientific, Waltham, United States of America) was used to

trypsinize cell monolayers, and 0.4% trypan blue dye was used to count the trypsinized cells, after which the cells were subcultured until confluency was achieved. Trypan blue was used to quantify the number of cells and their viability since living cells do not absorb the dye, whereas dead cells do.

3.1.4 Site-Directed Mutagenesis

3.1.4.1 Design of primers for site-directed mutagenesis

The HIV Drug Resistance Mutations (DRM) were analysed from the Stanford Drug Resistance Database HIVDB Programme, and mutations that confer resistance to Integrase Strand Transfer Inhibitors (INSTI) were analysed. The DRM sequences were downloaded from the National Centre for Biotechnology Information (NCBI) and analysed. Using the aforementioned criteria, integrase inhibitor drug-resistance mutations G118R, N155H, R263K, and Y143R were selected for further study. Primers were designed using the NEBaseChanger (New England Biolabs, Massachusetts, United States of America) primer design tool, and the corresponding mutations were introduced into the HIV-1 molecular clone pNL4.3 using the Q5 site-directed mutagenesis kit (New England Biolabs, Massachusetts, United States of America).

Table 3: Drug-resistant mutations designed with the NEBaseChanger primer design tool

Primer Name	Sequence (5'- 3')
N155H: FWD	AGAATCTATG**CAT**AAAGAATTAAAGAAAATTATAG
N155H: REV	ACT**ACT**CCTTGACTTTGG
Y143R: FWD	TGGAATTCCC**CGC**AATCCCCAAAG
Y143R: REV	AATTCCT**GCT**TGATTCC
G118R: FWD	TACTGACAAT**CGC**AGCAATTTCAC
G118R: REV	TGTATTGTTTTTACT**GGC**C
R263K: FWD	AGTACCAAGGAAA**AAA**GTAAAAATC
R263K: REV	ACC**TTT**ATGTCACTGTTATCTTGTATTAC

3.1.4.2 PCR Reaction

For each individual mutation, an exponential amplification polymerase chain reaction (PCR) was performed according to the manufacturer's protocol for the Q5 site-directed mutagenesis kit (New England Biolabs, Massachusetts, United States of America), using the pNL4.3 plasmid as the template for site-directed mutagenesis. Briefly, 12.5 µL of Q5 hot start high-fidelity (New England Biolabs, Massachusetts, United States of America) master mix, 1 µL of pNL4.3 DNA template at 1–25 ng/µL, 9 µL of deionised water, and 1.24 µL of corresponding forward and reverse primers (G118R_FWD: TACTGACAATCGCAGCAATTTCAC; G118R_REV: TGTATTGTTTTTACTGGCC; ; N155H_FWD AGAATCTATGCATAAAGAATTAAAGAAAATTATAG; N155H_REV ACTACTCCTTGACTTTGG; R263K_FWD: AGTACCAAGGAAAAAAGTAAAAATC; R263K_REV: ACCTTTATGTCACTGTTATCTTGTATTAC; Y143R_FWD TGGAATTCCCCGCAATCCCCAAAG; Y143R: REV AATTCCTGCTTGATTCC) were added into each individual thin-walled PCR tube to make a 25 µL reaction. Each reaction was spun on a vortex to mix, and the individual tubes were transferred to a thermal cycler and thermocycled on the Applied Biosystems mini Amp thermal cycler (Thermo Fisher Scientific, Waltham, United States of America) using the following conditions: Initial denaturation was run at 98 °C for 30 seconds, followed by annealing at 98 °C for 5 seconds then 55-60 °C for 10 seconds for 25 cycles and finally 72 °C for 1 minute 40 seconds. Final extension was run at 72 °C for 2 minutes and the hold temperature was 4 °C. Following PCR amplification, the parental DNA was digested to ligate the PCR product using the kinase, ligase, and DpnI digestion (KLD) enzyme mix (New England Biolabs, Massachusetts, United States of America). 1 µL of the PCR products produced in the previous PCR reaction step was transferred to a clean thin-walled PCR tube, together with 5 µL of 2X KLD reaction buffer, 1 µL 10X KLD enzyme mix and 3 µL of nuclease-free water to a final volume of 10 µL. The reaction was mixed by gently pipetting up and then incubated at room temperature (25°C) for 5 minutes.

3.1.4.3 Transformation of competent *Escherichia coli* cells

5 µL of the KLD mix was added to 50 µL of chemically competent *Escherichia coli (E. coli)* cells and then incubated on ice for 30 minutes. After 30 minutes, the cells were heat-shocked by placing them in a prewarmed water bath at 42°C for 30 seconds, followed by an additional 5 minutes of incubation on ice. 950 µL of Super Optimal broth with Catabolite repression (SOC medium) was added, and the cells were gently shaken on a shaking incubator at 37°C for 1 hour. After 1 hour, 100 µL of each reaction was spread onto LB agar plates with 100 mg/L of ampicillin, which were then incubated overnight at 37°C. The next day, colonies were picked for plasmid extraction. A sterile plastic pipette tip was used to pick colonies from the transformation of competent E. coli, which were then transferred to a 15 mL centrifuge tube with 11 mL of LB media supplemented with 10 µL of ampicillin. The infected LB media was shaken at 250 rpm overnight at 37°C. Afterward, the plasmid DNA was extracted using the GeneJET Plasmid Miniprep Kit (Thermofisher Scientific, Waltham, Massachusetts, United States of

America) according to the manufacturer's protocol. The quality and purity of the extracted plasmid DNA were assessed using 1% gel electrophoresis and Nanodrop quantification.

3.1.5 Confirmation of the inserted mutations by Sequencing

3.1.5.1 PCR Amplification of the integrase gene

The integrase gene of the selected mutants was PCR amplified using the HotStarTaq Master Mix (QIAGEN, Hulsterweg, Netherlands) according to the manufacturer's protocol. 25 µL of HotStarTaq,1 µL 4155F- GTACCAGCACACAAAGGRATTG forward primer, 1 µL 5264R- CCTGTATGCAGACCCCAATATGTT reverse primer, 1 µL DNA template and nuclease-free water were added into a thin-walled PCR tube and vortexed to mix. The reaction was run in the Applied Biosystems miniamp thermal cycler (ThermoFisher Scientific, Waltham, United States of America) under the following conditions: 1 cycle at 95 °C for 5 minutes, 1 cycle at 94 °C for 1 minute, then 55 °C for 1 minute, then 72 °C for 3 minutes and 30 seconds for 30 cycles, and finally 1 cycle at 72 °C for 10 minutes. Amplification of the amplicons for the integrase gene was confirmed using Nanodrop quantification and a 1% gel electrophoresis.

3.1.5.2 Cycle Sequencing reaction

A master mix reaction was prepared using the BigDye Terminator v3.1 cycle sequencing kit (Thermo Fisher Scientific, Waltham, United States of America), according to the protocol by Naidu, 2025, consisting of the following components: 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 3.4 µL of deionised water to makeup the reaction volume to 10 µL. A MicroAmp Optical 96-well reaction PCR plate (Thermo Fischer Scientific, Waltham, United States of America) was aliquoted with 10 µL of the reaction mixture and mixed. The forward primer M13- TGTAAAACGACGGCCAGT was used as the positive control and PGEM was used as the template. The reaction was run on a Applied Biosystems mini amp thermal cycler (ThermoFischer Scientific, Waltham, United States of America) under the following conditions: 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.

3.1.5.3 Cycle-sequencing reaction clean-up

1 µL 125 mM EDTA (pH 8.0) was added to the corresponding wells of the 96-well reaction PCR plate and mixed, followed by the addition of 26 µL of 3 M NaOAc (pH 5.2) and 100% ethanol solution to each corresponding well. The 96-well reaction plate was sealed with an adhesive cover, and the reaction was incubated at room temperature for 10 minutes. This was followed by centrifugation at 3000 g for 20 minutes. The 96-well plate was then covered with sterile paper and centrifuged again at 150 g for 5 minutes. 35 µL of 70% cold Ethanol was then added to each well, and the plate was centrifuged for an additional 5 minutes at 3000 g. The plate was inverted over a sterile paper towel and centrifuged for an

additional 1 minute at 150 g. Afterward, the sample was dried at 50 °C, covered with an adhesive foil cover, and stored at -20 °C. The products were sequenced using capillary electrophoresis on the 3500 Genetic Analyzer (Applied Biosystems, Waltham, United States of America). Next, using the Contig Assembly Tool version 3, the full-length integrase gene sequences were assembled and aligned in Unipro UGENE (version 50.0, Unipro).

3.1.6 Plasmid DNA Preparation and Purification

100 µL each of the glycerol stocks of the G118R, N155H, R263K, and Y143R plasmids were cultured in 100 mL of prewarmed LB (Luria-Bertani) broth supplemented with 100 µL 1% ampicillin and shaken overnight at 37°C in a shaking incubator at 230 RPM. Plasmid DNA was subsequently produced and purified using the QIAGEN Plasmid Maxi Kit (QIAGEN, Hulsterweg, Netherlands) according to the manufacturer's guidelines. The Nanodrop One^{TM} and 1% gel electrophoresis were used to evaluate the quality and purity of plasmid DNA.

3.1.7 Evaluation of the cytotoxicity and cell viability of *Alternaria alternata* PO4PR2 crude extract using the MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) assay

The MTT (3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide) cell viability experiment was performed using the CYQUANT MTT cell proliferation kit (Thermo Fisher Scientific, Waltham, United States of America) to evaluate the viability and cytotoxicity of the *Alternaria alternata* PO4PR2 crude extract. 15,000 TZM-bl cells with 200 µL of DMEM were seeded in each well of a 96-well culture plate and incubated at 37 ºC with 5% CO_2. 10 µL of the drug control azidothymidine (AZT) and 10 µL of the crude extract of *Alternaria alternata* PO4PR2 were added to different wells and serially diluted five times, and the plate was incubated for 48 hours at 37°C with 5% CO_2 and 95% humidity. Control wells without the addition of either substance were also included. Following the 48 hours, 10 µL of 12 mM MTT solution was added to each well, and the plate was incubated for 4 hours at 37°C with 5% CO_2 and 95% humidity. After 4 hours, 50 µL of 100% DMSO was added to each well and incubated for 10 minutes. The absorbance was then read at a wavelength of 540 nm using the Victor Nivo microplate reader (PerkinElmer, Waltham, United States of America). The following formula was then used to calculate the percentage of cell viability:

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

GraphPad Prism software (Version 5) was then used to calculate the drug control azidothymidine and *Alternaria alternata* PO4PR2 crude extract CC_{50} (50% cytotoxic concentration) using a dose-response curve.

3.1.8 Generation of HIV-1 Pseudovirus by Transfection

To prepare the HIV-1 pseudovirus, 10 million HEK293T cells were transfected in 10 ml growth medium (made up of DMEM supplemented with 10% heat-inactivated fetal bovine serum, 50 µg/mL

Gentamycin, and 25 mM HEPES buffer) in a T75 flask with 12 µL of backbone recombinant plasmids (pCMV and pVSV-g) using FUGENE Transfection Reagent (Promega, Wisconsin, United States of America) following the manufacturer's protocol. Briefly, 3×10^6 HEK 293T cells were seeded in a T-75 culture flask and incubated overnight at 37°C with 5% CO_2 and 95% humidity. 12 µL of the backbone recombinant plasmids was added to a sterile 15 mL tube together with 88 µL of DMEM, such that the mixture made up a volume of 100 µL, and to another sterile 15 mL tube, 150 µL of DMEM was added together with 36 µL of the FUGENE transfection reagent (Promega, Wisconsin, United States of America). The contents of the first tube were transferred to the second tube with the FUGENE solution, and the combined mixture was vortexed to mix and then incubated at room temperature for 30 minutes to allow the complex to form. The entire contents of the transfection complex were then transferred to the T75 flask of HEK293T cells seeded the day before, and the flask was gently swirled to allow uniform distribution of the complexes, after which it was incubated for 48 hours at 37°C, 5% CO_2, and 95% humidity. After 48 hours, the supernatants containing the virus were harvested by collecting the medium from the flask using a pipette and filtered through a 0.45 µm filter. 1 mL aliquots of the virus were transferred to 2 mL cryovials, and stored at -80 °C.

3.1.9 Titration of HIV-1 viruses in TZM-bl cell lines (TCID Assay)

The 50% Tissue culture infectious dose ($TCID_{50}$) of each virus stock was determined in a single-infection assay in TZM-bl cells. Briefly, 100 µL of growth medium (GM) was aliquoted to all wells of a 96-well cell culture plate. 25 µL of the transfected viral stock was transferred to four wells in row one of the 96-well plate and was serially diluted. In four repetitions, four wells in row one received 25 µL of the transfected viral stock, which was serially diluted four times. 100 µL of TZM-bl cells containing 37.5 µg/mL DEAE-dextran (Diethylaminoethyl-dextran) (10000 cells/ 100 µL GM) were added to all wells and incubated for 48 hours at 37°C with 5% CO_2 and 95% humidity. After 48 hours, 100 µL of cell culture media was extracted and 100 µL of Bright Glo™ luciferase reagent was added to each well without illumination with the exception of the control in row 12. The plate was incubated at room temperature for 2 minutes to allow complete cell lysis. The contents of each well were mixed by pipetting and 150 µL was transferred to a corresponding 96-well black plate. The plate was then immediately read on a luminometer at 540 nm (PerkinElmer, United States of America) to quantify the Relative light units (RLU). The $TCID_{50}$ was estimated from an exhibited curve by choosing the dilution concentration that produced a minimum of 15,000 and a maximum of 50,000 RLUs.

3.1.10 Luciferase-based antiviral assay

The viruses produced by transfection were used to test the HIV-1 suppression of fungal extracts using a luciferase-based antiviral assay (Kubheka et al., 2024; Naidu et al., 2025; Nzimande et al., 2022). 150 µL, 100 µL and 140 µL of DMEM supplemented with 10% FBS, 25 mM HEPES buffer and 1% Penicillin-Streptomycin were added to a Corning Costar 96-well culture plate (Corning, New York, United States of America) as a cell control, virus control, and sample, respectively, followed by the

addition of 11 µL of the drug controls (Azidothymidine or Raltegravir and Dolutegravir) and *Alternaria alternata* PO4PR2 crude extract. A 3-fold dilution was performed. The experimental controls were infected (virus control) and uninfected TZM-bl cell lines (cell control). Then 50 µL of the G118R, N155H, R263K, and Y143R viruses at $TCID_{50}$ were added to the 96-well cell culture plate and incubated for 1 hour. Ten thousand TZM-bl cells per well, supplemented with 37.5 µg/mL DEAE-dextran, were added to each well of the plate, and the culture was maintained for 48 hours at 37°C, 5% CO_2, and 95% humidity. Following 48 hours of incubation, 150 µL of medium was decanted and replaced with 100 µL of Bright Glo luciferase reagent (Promega, Anatech, South Africa) in the absence of light. The luminance was immediately measured using the Victor Nivo microplate reader at 540 nm (PerkinElmer, Waltham, United States of America). The percentage of viral inhibition was calculated using the following formula:

$$\%\ \text{HIV inhibition} = \frac{\text{Average sample} - \text{average control}}{1 - (\text{Average viral control} - \text{Average control})} \times 100$$

A dose-response curve was used to calculate the IC_{50} (inhibitory concentration) using the GraphPad Prism software (Version 5).

3.1.11 HIV Integrase assay

The anti-HIV-1 integrase activity of the *Alternaria alternata* PO4PR2 crude extract was determined using the HIV Integrase Assay Kit EZ-1700 (Express Biotech International, United States of America). According to the manufacturer's instructions, 100 µL of 1X double-stranded (DS) DNA solution was added to each well of a streptavidin-coated 96-well plate and incubated for 30 min in a 37ºC incubator. Following the incubation, the liquid from the plate wells was aspirated, and the plate was washed 5 times with 300 µL of 1X wash buffer. 200 µL of blocking buffer was added to each well, and the plate was incubated for an additional 30 minutes at 37 °C. Following the incubation, the liquid from the plate wells was aspirated, and the plate was washed 3 times with 200 µL reaction buffer. A 100 µL integrase enzyme solution was added to each well, and the plate was incubated for 30 minutes at 37 °C. Reaction buffer only replicates (without integrase) were added as a no-enzyme negative control. 50 µL per well of Sodium Azide, Raltergravir, and the *Alternaria alternata* PO4PR2 crude extract in reaction buffer was added to two lanes each, and the plate was incubated for 5 min at room temperature. 50 µL of 1X target substrate (TS) DNA solution was then added directly to the buffer and test articles present in each well. The reactions were then mixed by tapping the plate gently against a stationary hand 5 times and the plate was incubated for 30 min at 37ºC. The liquid from the plate wells was then aspirated and washed five times with 300 µL wash solution. 100 µL of HRP antibody solution was added to each well and incubated for 30 minutes at 37ºC. The liquid from the plate wells was aspirated again and washed five times with 300 µL of wash solution. Then, 100 µL of TMB peroxidase substrate solution was added to each well and incubated for 10 minutes at room temperature. 100 µL of TMB stop solution was added

directly to the wells containing the TMB substrate, and the absorbance of the wells was read using a plate reader set at 450 nm.

3.1.12 Quantification of integrated HIV-1 DNA using Real-Time PCR

Peripheral blood mononuclear cells (PBMCs) from HIV-1-uninfected participants were sourced from the Female Rising through Education, Support and Health (FRESH) cohort, an ongoing prospective study in KwaZulu-Natal, South Africa (Ndung'u et al., 2018). The FRESH programme focuses on early detection of HIV infection among young women at high risk and routinely collects blood and mucosal samples to investigate HIV pathogenesis, acquisition risk factors, and to support vaccine and cure research initiatives (Dong et al., 2018; Ndung'u et al., 2018). PBMCs that had been previously cryopreserved were retrieved from liquid nitrogen storage and thawed on the day of experimentation. Vials were rapidly warmed in a 37 °C water bath until partially thawed, then transferred into 8 mL of pre-warmed R10 medium (RPMI-1640, Gibco, supplemented with 10% FBS, 1% L-glutamine, 1% penicillin-streptomycin and 1% HEPES) in a 15 mL tube. Cells were centrifuged at 1500 rpm for 10 minutes at room temperature. The supernatant was then removed, and the pellet was resuspended in 10 mL of fresh R10. A second centrifugation step was performed, after which the final pellet was resuspended in 2 mL R10 and allowed to rest for 2 hours at 37 °C in a 5% CO_2 incubator.

For experimental treatments, PBMCs from 20 HIV-negative donors were exposed to the *Alternaria alternata* PO4PR2 crude extract or DTG as a positive antiviral control. Untreated PBMCs served as the negative control. Cells were plated at 1×10^5 cells per well in a 24-well plate containing RPMI 1640 medium (R10). Each treatment group received 20 µL of the appropriate compound. Subsequently, 1500 µL of the PBMC suspension was added to each well, followed by 100 µL of HIV-1 NL4.3 ($TCID_{50}$) for all groups except the uninfected control. Three days post-infection, RNA was isolated from the infected and uninfected PBMCs using the PureLink RNA Mini Kit (ThermoFisher Scientific, USA) according to the manufacturer's protocol. Briefly, 600 µL of Lysis Buffer with 1% 2-mercaptoethanol was added to a previously centrifuged cell pellet, and the mixture was vortexed until the pellet was dispersed and the cells were lysed. 600 µL of 70% ethanol was then added to the cell lysate and vortexed to mix. 700 µL of the sample was then transferred to a spin cartridge with an attached collection tube. The sample was then centrifuged at 12,000 g for 15 seconds, the flow-through was discarded, and the spin cartridge was reinserted into the same collection tube. This process was repeated 3-4 times until the entire sample had been processed. 700 µL of Wash Buffer I was added to the spin cartridge and centrifuged at 12,000 g for 15 seconds. The collection tube with the flow-through was discarded, and a new spin cartridge was inserted into a collection tube. 500 µL of Wash Buffer II with ethanol was added to the spin cartridge, and it was centrifuged at 12,000g for 15 seconds. The flow-through was then discarded. The second step was repeated, and the sample was centrifuged at 12,000 g for 2 minutes to dry the membrane with bound RNA. The collection tube was discarded, and the spin cartridge was inserted into a recovery tube. 100 µL of RNase-free water was then added to the centre of the spin cartridge, and the sample

was incubated for 1 minute at room temperature. The spin cartridge was then centrifuged at 12,000 g for 2 minutes to elute the RNA into the recovery tube, and the spin cartridge was discarded. The extracted RNA was stored at -20ºC until use.

The extracted RNA was used for complementary DNA (cDNA) synthesis, which was performed using the Superscript III Reverse Transcriptase kit (Thermofischer, California, USA) according to the manufacturer's protocol. The reverse transcription reaction consisted of 1 µL *Alu-gag* primers, 5 µL total RNA, 1 µL 10 mM each dNTP mix, and distilled water, all added to a final reaction volume of 12 µL. The mixture was heated to 65°C for 5 minutes to denature any secondary structures of the primers, and then quickly chilled on ice to prevent these structures from re-forming. The contents of the tube were collected by brief centrifugation, and the following were added: 1X First-Strand Buffer, 0.1 M DTT, and 1 µL RNaseOUT. The contents of the tube were mixed and incubated at 25°C for 2 min. Following this, 1 µL of SuperScript II RT (Thermofisher Scientific, USA) was added and mixed gently by pipetting up and down. Sterile, distilled water was then added to a final volume of 20 µL. The tubes were incubated at 25°C for 10 minutes and then at 42°C for 50 minutes, followed by inactivation at 70°C for 15 minutes. The synthesized cDNA was used immediately as a template to measure *Alu-gag* expression or stored at -20°C until use.

Alu-gag PCR was performed as follows: The first round PCR reaction was prepared on ice using 1X PCR buffer (ThermoFisher), 1.5 mM MgSO4 (ThermoFisher), 0.5 mM dNTPs (ThermoFisher), 1 µM *Alu* forward primer (5′- GCC TCC CAA AGT GCT GGG ATT ACA G- 3′), 6 µM *Gag* reverse primer (5′- TCG CTT TCA GGT CCC TGT TCG- 3′), 2.5 U of Platinum Taq polymerase (ThermoFisher), and 150 ng of genomic DNA. Cycling conditions were as follows: initial denaturation of 95 ◦C for 2 minutes, followed by 14 cycles of denaturation at 95 ◦C for 30 seconds; annealing at 50 ◦C for 30 seconds and extension at 72 ◦C for 210 seconds. The nested PCR reaction was then prepared on ice using 1X PCR buffer (ThermoFisher), 1.5 mM MgSO4 (ThermoFisher), 0.5 mM dNTPs (ThermoFisher), 0.5 µM *AluGag* forward primer (5′- GGT GCG AGA GCG TCA GTA T- 3′) and 0.5 µM *AluGag* reverse primer (5′-AGC TCC CTG CTT GCC CAT A-3′), 0.15 µM *AluGag* probe (6FAM- AAA ATT CGG TTA AGG CCA GGG GGA AAG AA-QSY7), 1 U of Platinum Taq polymerase (ThermoFisher), and 2 µL of *Alu-gag* PCR product. Cycling conditions were as follows: 95 ◦C for 5 minutes, followed by 50 cycles of 95 ◦C for 10 seconds and 60 ◦C for 30 seconds. Human GAPDH was used for normalization (ThermoFisher). The experiment was performed in three independent replicates.

3.1.13 Computational Systems Preparation

The crystal structure of the wild-type HIV-1 integrase (IN) complexed with double-stranded viral DNA, two Mg^{2+} ions, and dolutegravir (PDB ID: 8FN7) was retrieved from the RCSB Protein Data Bank (https://www.rcsb.org/) (Goodsell et al., 2020). Missing residues were modelled using MODELLER 10.6, and the best model was selected based on the lowest discrete optimized protein energy (zDOPE)

score (Yang et al., 2012). Drug-resistant variants (G118R, N155H, Y143R, and R263K) were generated from the wild-type template using the Dunbrack Rotamer Library 2010, implemented in UCSF Chimera 1.19 (Shapovalov & Dunbrack, 2011). All systems were prepared by retaining the viral DNA and Mg^{2+} ions, as these cofactors are essential for maintaining the active site conformation of IN (Li et al., 2023). Protein–DNA complexes were prepared by removing crystallographic water molecules, adding polar hydrogens, and assigning atomic charges using the AMBER force field (Butt et al., 2020).

The three-dimensional (3D) structures of 14 secondary metabolites from *Alternaria alternata* PO4PR2 (previously characterized experimentally) (Naidu et al., 2025) and the reference inhibitors Dolutegravir (DTG) and Raltegravir (RAL) were obtained from PubChem (https://pubchem.ncbi.nlm.nih.gov/) (Kim et al., 2016). Ligands were geometrically optimized in Avogadro 1.2.0 using the Generalized Amber Force Field (GAFF), followed by final energy minimization in UCSF Chimera 1.19, which employed 100 steps of steepest descent and 10 steps of conjugate gradient minimization. Hydrogens were added, Gasteiger charges were assigned, and all prepared structures were saved in the PDBQT format for docking (Butt et al., 2020).

3.1.14 Molecular Docking Calculations and Analysis

Solid-phase extraction and GC–MS analysis were performed by Nzimande, (2022), and several secondary metabolites were identified, including cyclotrisiloxane, octamethyl (22.92%), propanenitrile (16.67%), pyrrolo[1,2-a]pyrazine-1,4-dione derivatives (10.42%), coumarin derivatives (13.7%), silane derivatives, and 1,2-cyclobutanedicarbonitrile. Many of these compounds have previously been reported to have antimicrobial, antioxidant, anti-inflammatory, and antiviral properties. Given that the crude extracts demonstrated measurable HIV-1 inhibition and that these compounds were among the predominant constituents identified, they represent plausible bioactive candidates responsible for the observed antiviral activity. Therefore, these metabolites were selected for in silico molecular docking studies to evaluate their potential interactions with HIV-1 integrase and assess their possible role in inhibiting viral integration, particularly in drug-resistant variants.

Molecular docking calculations were performed using AutoDock Vina 1.1.2 (Trott & Olson, 2010). The grid box was centered within the IN catalytic core–DNA interface at coordinates (x = 55.13, y = 72.57, z = 83.56) with dimensions (x = 24.53 Å, y = 18.71 Å, z = 22.83 Å). The docking parameters were set as follows: number of modes = 10, exhaustiveness = 16, and energy range = 3 kcal/mol. All ligands were docked against the wild-type and mutant HIV-1 IN–DNA–Mg^{2+} complexes. The lowest-energy poses were selected based on the binding affinity (kcal/mol) and inspected for key interactions with the catalytic residues (ASP64, ASP116, AND GLU152), Mg^{2+} ions, and DNA bases. Protein–ligand complexes were analyzed using BIOVIA Discovery Studio Visualizer 2024, highlighting hydrogen bonds, hydrophobic contacts, van der Waals interactions, and metal coordination interactions (Naidu et al., 2025). Comparative interaction profiling between the reference inhibitors and *Alternaria alternata* PO4PR2 metabolites provided structural insights into their potential mechanisms of inhibition of HIV-

1 IN.

3.2 Statistical Analysis

The average of three separate trials served as the basis for all of the experiments' findings. A non-linear curve-fitting tool in GraphPad Prism was used to analyse concentration-response data in order to calculate the IC_{50} values.

CHAPTER 4: Results and Discussion

Alternaria alternata PO4PR2 crude extracts inhibit the integrase drug-resistance mutant by INSTI

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Abstract

Background: Natural products derived from endophytic fungi have emerged as promising sources of bioactive compounds with antiviral potential. *Alternaria alternata* PO4PR2 has previously been reported to exhibit anti-HIV activity by targeting multiple stages of HIV-1 replication. Therefore, this study investigates the anti-HIV-1 activity of the *A. alternata* PO4PR2 crude extract against INSTI drug-resistant viruses and its inhibitory effect on the integration stage of HIV replication.

Materials and Methods: Major drug-resistant mutations (G118R, N155H, R263K, and Y143R) affecting HIV-1 integrase were identified using the Stanford HIV Drug Resistance Database**.** These were inserted into the HIV-1 pNL4.3 molecular clone using site-directed mutagenesis. Sanger sequencing verified the successful insertion of these mutations. HEK293T cells were transfected to generate integrase drug-resistant HIV-1 variants, and TZM-bl cell lines were used to test their infectivity. The 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide (MTT) assay was used to test the cytotoxicity of the *Alternaria alternata* PO4PR2 crude extracts in TZM-bl cell lines. The anti-HIV-1 activity of *Alternaria alternata* PO4PR2 was measured using a luciferase-based antiviral assay, and its impact on the integration phase was evaluated using an HIV-1 integrase activity assay. Integrated HIV-1 DNA was quantified using a two-step *Alu-gag* nested PCR assay normalized to human GAPDH. 14 previously characterized *A. alternata* metabolites were docked against wild-type and drug-resistant HIV-1 integrase–DNA–Mg^{2+} complexes to predict binding affinities and interaction profiles.

Results: The drug-resistant mutant viruses (G118R, N155H, R263K, and Y143R) were strongly inhibited by the *Alternaria alternata* PO4PR2 crude extract, with IC_{50} values of 0.1044 ± 0.023 µg/mL, 0.1032 ± 0.025 µg/mL, 0.02971 ± 0.003 µg/mL, and 0.1652 ± 0.027 µg/mL, respectively. A positive control, Raltegravir (IC_{50} = 0.0134 ± 0.002 µg/mL), was used. The *Alternaria alternata* PO4PR2 crude extract was found to be non-toxic against TZM-bl cell lines, maintaining over 80% cell viability, and a CC_{50} of 300 ± 1.84 µg/mL. The mechanism of action of the *Alternaria alternata* PO4PR2 crude extract was confirmed by an HIV-1 integrase activity assay, which demonstrated 67% inhibition of integrase activity, comparable to the 67% inhibition observed with the licensed drug Raltegravir, specifically

blocking the strand-transfer step of HIV-1 DNA integration. Peripheral blood mononuclear cells treated with the extract exhibited a measurable reduction in integrated HIV-1 DNA compared to untreated controls. Docking analyses supported these findings, revealing that several metabolites, particularly coumarin, 3,4-dihydro-4,5,7-trimethyl- and 2,3-2H-benzofuran-2-one, and 3,3,4,6-tetramethyl displayed strong and mutation-tolerant binding affinities (−5.6 to −6.2 kcal/mol) within the catalytic pocket of HIV-1 integrase.

Discussion and Conclusion: These findings support *Alternaria alternata* PO4PR2 as a promising source of antiretroviral compounds targeting resistant HIV-1 integrase, bolstering its potential as a source of innovative antiviral medicines aimed at resistant HIV-1 integrase.

Keywords: HIV-1 integrase inhibition; Endophytic fungi; *Alternaria alternata*; Drug-resistant HIV variants; Integration site analysis (*Alu*-PCR); Natural antiviral agents

4.1 Introduction

Although major strides have been made in the management of Human Immunodeficiency virus (HIV) infection, the virus continues to pose significant public health challenges, affecting 40.8 million people globally as of 2025 (UNAIDS, 2025). The burden of HIV remains disproportionately high in sub-Saharan Africa, with Southern Africa bearing the greatest share of the global epidemic; in South Africa alone, an estimated 7.6 million people are currently living with HIV (UNAIDS, 2025). Following the discovery of HIV, substantial progress has been achieved in understanding the virus and developing effective therapeutic strategies, particularly the introduction of antiretroviral therapy (ART), which has transformed HIV from a fatal illness into a manageable chronic condition (Kemnic & Gulick, 2018; Lange & Ananworanich, 2014; Sharp & Hahn, 2011).

The widespread use of ART has significantly reduced the burden of HIV by reducing viral loads to undetectable levels (Eggleton & Nagalli, 2020). This not only improves the health and longevity of people living with HIV (PLWH) by reducing HIV-related morbidity, but also effectively prevents sexual transmission of the virus, a principle known as Undetectable = Untransmittable (U=U) (Eggleton & Nagalli, 2020; Eisinger et al., 2019). However, despite the remarkable successes of ART, several challenges persist, including the inability to eliminate latent viral reservoirs, drug-related toxicities, virological failure, and the emergence of HIV drug resistance (Chun et al., 2015; Foka & Mufhandu, 2023; Ho & Siliciano, 2015; Palmisano & Vella, 2011; Powderly, 2002). These limitations highlight the urgent need for novel therapeutic strategies that are better tolerated by PLWH and capable of effectively targeting drug-resistant HIV-1 strains (Appiedu-Addo et al., 2025).

HIV-1 integration is a critical and irreversible step in the viral replication cycle, consisting of two enzymatic reactions catalyzed by the viral integrase enzyme: 3’ processing, where the viral DNA ends are prepared for insertion, and strand transfer, which covalently inserts the viral DNA into the host

genome (Elliott & Kutluay, 2020). Integrase cleaves two nucleotides from each 3' end of the viral DNA at a conserved CA dinucleotide, exposing reactive 3'-hydroxyl (OH) groups (Lesbats et al., 2016). This processing step prepares the viral DNA ends for integration into the host genome. The strand transfer process takes place in the nucleus after the pre-integration complex (PIC) is transported into the host cell nucleus (Piller et al., 2003). The integrase enzyme catalyzes the insertion of the processed 3' viral DNA ends into the host DNA. The exposed 3'-OH groups attack the phosphodiester backbone of host DNA, integrating both ends of the viral DNA into the host genome (Craigie, 2012). This results in gaps and overhangs at the integration site, which are later repaired by host cellular machinery (Skalka & Katz, 2005). These two steps are crucial for the stable integration of the HIV-1 genome into host DNA, which contributes to viral latency and persistence, and are therefore important targets for antiretroviral drugs. Most integrase inhibitors, such as Raltegravir and Dolutegravir, target the strand transfer step. Understanding how bioactive compounds affect these specific integration steps, especially in drug-resistant viral strains, is crucial for the development of new therapeutic agents.

In the search for alternative and more effective treatment strategies, endophytic fungi have emerged as promising sources of novel bioactive compounds with therapeutic potential against HIV (Omomowo et al., 2023; Wen et al., 2022). These symbiotic microorganisms reside within plant tissues without causing harm and are known to produce a diverse array of secondary metabolites, some of which have demonstrated antiviral activity, including the inhibition of HIV (Ebadi & Ebadi, 2024; Hyde & Soytong, 2008). Members of the genus *Alternaria* have demonstrated significant inhibitory activity against HIV, highlighting their potential as a source of novel antiviral compounds for further investigation (Roy, 2017). Wellensiek et al (2013) successfully isolated and identified four compounds from the endophytic fungus *Alternaria tenuissima*, which completely inhibited HIV-1 replication in T-lymphocytes. Similarly, altertoxins isolated from *Alternaria tenuissima* QUE1Se demonstrated complete inhibition of HIV-1 replication in T-cells at low micromolar concentrations (Bashyal et al., 2014). Additionally, methanol extracts of *Alternaria* species isolated from Calophyllum inophyllum were found to exhibit strong inhibitory effects against HIV-1 reverse transcriptase, integrase, and protease, outperforming other fungal extracts and standard antiretroviral drugs. (Govindappa et al., 2015)

In another study, crude extracts of *Alternaria alternata* PO4PR2 showed anti-HIV-1 activity in the TZM-bl cell line at inhibitory concentration (IC_{50}) values ranging from 0.017 to 1.170 µg/mL, maintaining the virus replication inhibition profile on both peripheral blood mononuclear cells (PBMCs) and $CD4^+$ T cells at concentrations ranging from 0.3 to 50.2 ng/mL (Nzimande et al., 2022). Recently, it was found that fractionated crude extracts of *Alternaria alternata* PO4PR2 demonstrated potent anti-HIV activity by inhibiting multiple stages of the viral life cycle, most notably integration, while showing minimal $CD4^+$ T cell activation, indicating both antiviral and immunomodulatory properties (Kubheka et al., 2024). Furthermore, Naidu et al. (2025) showed that crude extracts of *Alternaria alternata* PO4PR2 demonstrated potent anti-HIV activity against multiple HIV-1 subtypes and integrase-resistant strains, acting at several stages of the viral life cycle and involving bioactive compounds with strong

integrase-binding affinity. Using GC-MS and molecular docking analyses, this study identified several secondary metabolites in the *Alternaria alternata* PO4PR2 crude extract, such as benzofuran, N-acetyl-diazabicyclo, coumarin, cyclopropanecarboxamide, and pyrrolo-pyrazine derivatives, as key contributors to its broad anti-HIV activity (Naidu et al., 2025).

While *Alternaria alternata* PO4PR2 crude extracts have shown promising anti-HIV activity, the detailed mechanisms by which their bioactive compounds inhibit the HIV-1 integration process, particularly against integrase drug-resistant variants, remain poorly understood. Elucidating these interactions at the molecular level is critical for developing targeted therapies that can effectively block viral integration and overcome resistance. Therefore, this study aims to elucidate the antiviral effects of secondary metabolites produced by the *Alternaria alternata* PO4PR2 on HIV-1 integration, with particular focus on their activity against integrase drug-resistant variants.

4.2 Results

This study evaluated the anti-HIV activity of *Alternaria alternata* PO4PR2 crude extract against HIV-1 integrase drug-resistant variants. Major INSTI-resistance mutations (G118R, N155H, R263K, and Y143R) were selected from the Stanford Drug Resistance database and introduced into the pNL4.3 plasmid using site-directed mutagenesis, and confirmed by Sanger sequencing. The cytotoxicity of the crude extract was assessed using the MTT assay, and the infectivity of the mutant viruses was quantified using the $TCID_{50}$ assay. A luciferase-based antiviral assay was used to measure the antiviral activity of the crude extract against TZM-bl cell lines, and an integrase strand-transfer inhibition assay was used to determine whether the extract directly inhibited HIV-1 integrase activity.

4.2.1 Identification and Selection of HIV-1 Integrase Drug-Resistant Mutants from the Stanford Drug Resistance Database

Drug-resistant HIV-1 integrase mutations were identified by screening integrase sequences derived from the Los Alamos HIV Sequence Database and analysing them using the Stanford HIV Drug Resistance Database (HIVDB). Major integrase strand-transfer inhibitor (INSTI)-associated drug-resistance mutations were prioritised based on their clinical relevance, frequency across HIV-1 subtypes, and documented contributions to resistance against first- and second-generation INSTIs, including Raltegravir, Elvitegravir, and Dolutegravir. Four key mutations, G118R, N155H, R263K, and Y143R, representing distinct INSTI resistance pathways, were selected for further evaluation. These mutations were individually introduced into the HIV-1 pNL4.3 molecular clone using site-directed mutagenesis and successfully confirmed by Sanger sequencing. The resulting mutant viruses were subsequently used to assess the ability of the *Alternaria alternata* PO4PR2 crude extract to inhibit HIV-1 variants harbouring clinically relevant integrase resistance substitutions.

4.2.2 Sequence alignment of integrase G118R, N155H, R263K, and Y143R mutations

Figure 7 illustrates the sequence alignment of the full-length HIV-1 integrase gene from the site-directed mutant constructs compared with the wild-type integrase sequence. The purpose of this analysis was to confirm the successful introduction of specific integrase drug-resistance mutations generated through site-directed mutagenesis. The assembled nucleotide sequences obtained from Sanger sequencing were aligned against the wild-type integrase reference sequence to identify and verify the presence of the introduced point mutations. Sequence alignment was performed using *BioEdit* software, allowing for a direct, base-by-base comparison between the mutant and wild-type integrase genes. Regions corresponding to the known integrase drug-resistance mutations, G118R, N155H, R263K, and Y143R, are shown, with nucleotide differences highlighted by red boxes. These highlighted regions indicate the precise nucleotide substitutions responsible for the corresponding amino acid changes within the integrase coding region.

For each mutant construct, the expected nucleotide change was observed at the correct genomic position, while the surrounding sequences remained identical to the wild-type integrase gene. This confirms that the mutations were correctly introduced without additional alterations elsewhere in the integrase sequence. Further confirmation of the sequence alignments and mutation positions was performed using *Jalview* software, which provided additional visual validation of the conserved regions and mutation sites. These analyses confirm the successful generation of the G118R, N155H, R263K, and Y143R integrase drug-resistant mutants, validating their use in subsequent experiments assessing susceptibility to the *Alternaria alternata* PO4PR2 crude extract and reference integrase inhibitor.

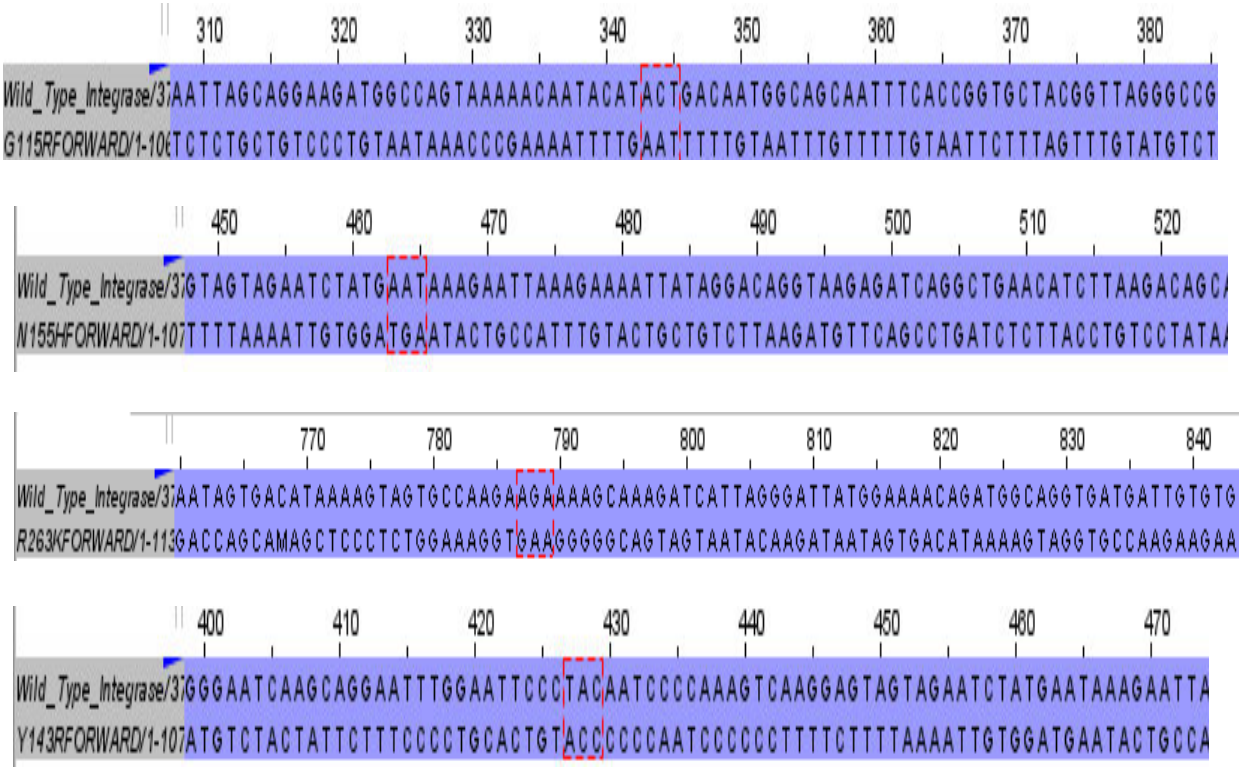

Figure 7: Sequence alignment of integrase mutants against the wild-type integrase gene.

4.2.3 Cytotoxicity and cell viability of *Alternaria alternata* PO4PR2 crude extract using MTT assay

The dose-response curve shows the percentage of cell cytotoxicity and was used to determine the CC_{50} of the *Alternaria alternata* PO4PR2 crude extract. The blue line represents the *Alternaria alternata* PO4PR2 crude extract (CC_{50} = 300 ± 1.84 µg/mL), while the red line represents the Azidothymidine (CC_{50} of 385 ± 6.74 µg/mL). The y-axis of the dose-response curve shows the percent cell cytotoxicity, and the x-axis shows the log concentration of the *Alternaria alternata* PO4PR2 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 was applied. Figure 8A shows that the *Alternaria alternata* PO4PR2 crude extract maintained TZM-bl cell viability above 80% in the MTT assay, indicating low cytotoxicity at the tested concentration (300 µg/mL). Similarly, the drug control Azidothymidine (AZT) also demonstrated cell viability above 80% at the tested concentration (300 µg/mL). The dose–response curve in Figure 8B was used to determine the CC_{50} values for both treatments. The crude extract showed a CC_{50} of 300 ± 1.84 µg/mL, while AZT exhibited a CC_{50} of 385 ± 6.74 µg/mL, confirming that the extract is comparable in safety to the reference drug. The y-axis of Figure 8B represents percent cytotoxicity, while the x-axis displays the log-transformed concentrations (300 to 0.00003 µg/mL). Overall, both AZT and the crude extract showed low cytotoxicity and high cell viability, demonstrating that the *Alternaria alternata* PO4PR2 crude extract is non-toxic to TZM-bl cells and is as safe as AZT within the tested concentration range.

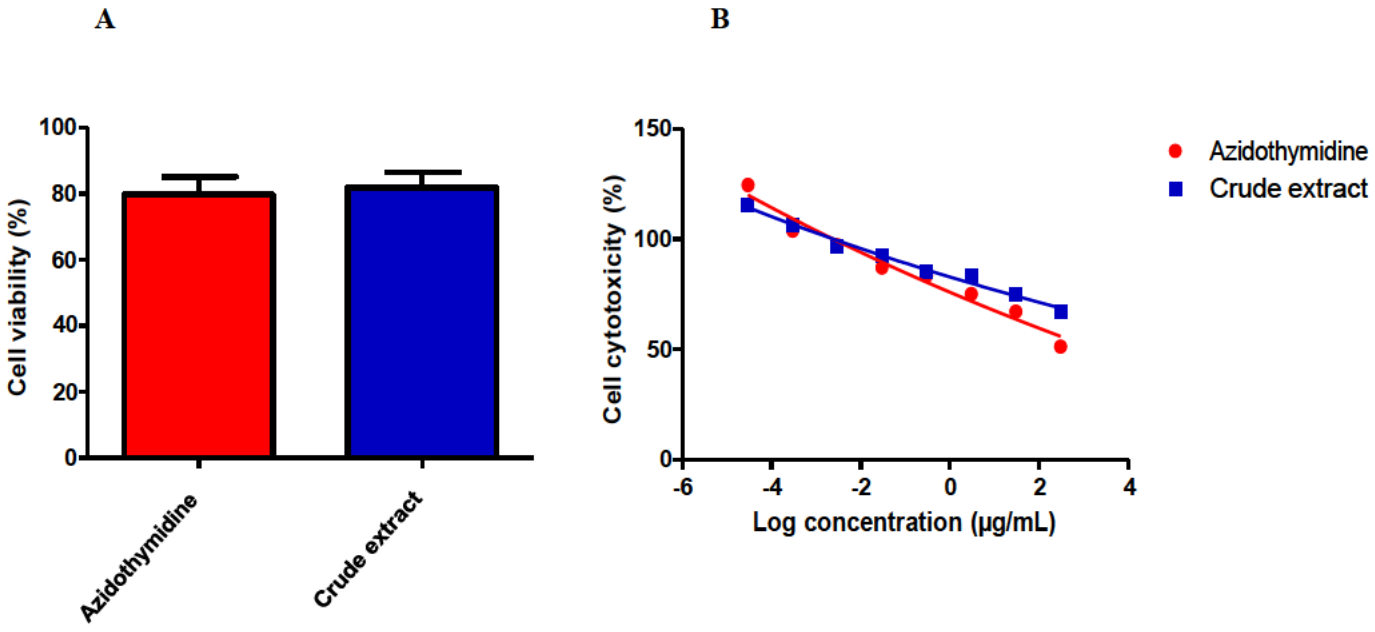

Figure 8: Cell Viability (A) and cytotoxicity (B) of the *A. alternata* crude extract against TZM-Bl cells

In Figure 9 the Tissue Culture Infectious Dose (TCID) assay was used to evaluate the infectivity in relative light units (RLUs) of the HIV-1 integrase drug-resistant mutants G118R, N155H, R263K, and Y143R in TZM-bl cells against the wild-type virus pNL4.3. The dilutions required to infect 50% of cultured cells ($TCID_{50}$) for each virus were determined, providing a quantitative measure of infectious virus particles. TZM-bl cells were exposed to serial dilutions of the virus, and infection was measured in RLUs using a luminometer. The integrase drug-resistant HIV strains were deemed infectious if they could generate at least 15000 RLUs. Due to their production of over 15,000 RLUs, the integrase drug-resistant HIV strains G118R, N155H, R263K, and Y143R were deemed infectious. All four tested drug-resistant mutants produced RLU values above 80,000, which exceeds the 15,000 RLU threshold for infectivity, indicating that they remain capable of infecting TZM-bl cells. The wild-type virus, pNL4.3, generated a significantly higher RLU (~270,000), reflecting its higher baseline infectivity compared to the drug-resistant mutants. This suggests that while the mutants retain infectivity, their replication efficiency in TZM-bl cells is lower than that of the wild-type virus.

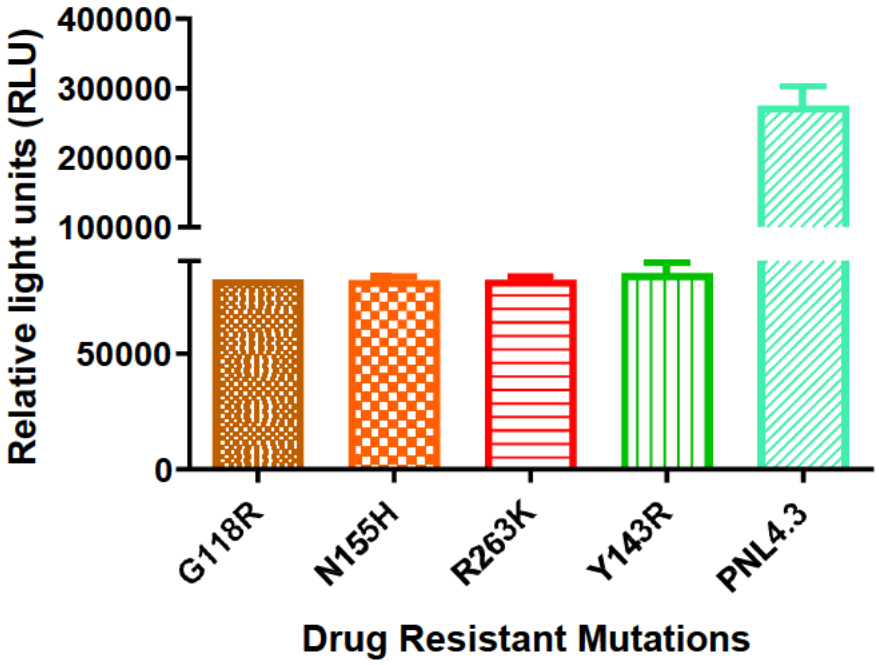

Figure 9: Infectivity of the integrase drug-resistant mutants to the wild-type pNL4.3 virus

4.2.4 Antiviral activity of *Alternaria alternata* PO4PR2 crude extract against integrase drug-resistant mutants

A luciferase-based assay was used to assess the antiviral efficacy of *Alternaria alternata* PO4PR2 crude extract and integrase inhibitor drug controls Dolutegravir and Raltegravir against the integrase inhibitor drug-resistant strains G118R, N155H, R263K, and Y143R (Figure 10). The HIV replication of drug-resistant strains G118R, N155H, R263K, and Y143R was completely inhibited at 300 µg/mL of Raltegravir, Dolutegravir, and 300 µg/mL of the *Alternaria alternata* PO4PR2 crude extract. An IC_{50} of less than 10 µg/mL suggests that the extract was an extremely potent HIV inhibitor. The IC_{50} values of G118R, N155H, R263K, and Y143R (0.1044 ± 0.023 µg/mL, 0.1032 ± 0.025 µg/mL, 0.02971 ± 0.033 µg/mL
and 0.1652 ± 0.027 µg/mL, respectively) treated with the crude extract of *Alternaria alternata* PO4PR2 show that it was very effective against the integrase drug-resistant strains.

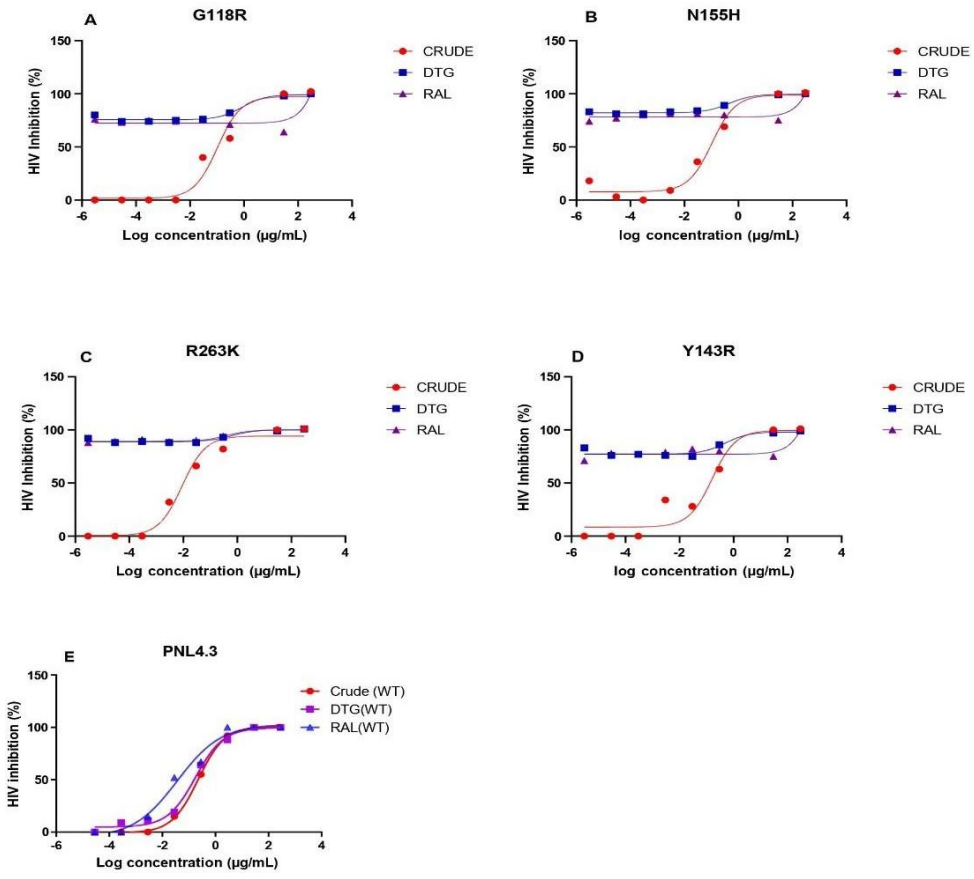

Figure 10: Percentage inhibition of HIV-1 integrase mutants G118R (A), N155H (B), R263K (C), and Y143R (D) by the *A. alternata* crude extract. The approved integrase strand transfer inhibitors Raltegravir (RAL) and Dolutegravir (DTG) were used as drug controls.

The IC_{50} values of HIV-1 integrase mutant viruses G118R, N155H, R263K, and Y143R (0.1044 ± 0.023 µg/mL, 0.1032 ± 0.025 µg/mL, 0.02971 ± 0.003 µg/mL and 0.1652 ± 0.027 µg/mL, respectively), were compared by expressing them as fold changes relative to the IC_{50} of the reference virus pNL4.3, which is known to be infectious (Figure 11). The fold change was calculated using the ratio: virus IC_{50} / pNL4.3 IC_{50}. A fold change below 1 indicates higher sensitivity to the *Alternaria alternata* PO4PR2 crude extract compared to pNL4.3, while values above 1 reflect reduced susceptibility (Table 4). The fold changes for each of the drug-resistant mutants (G118R, N155H, R263K, and Y143R) were less than 1, indicating that the mutant viruses require a lower concentration of the *Alternaria alternata* PO4PR2 crude extract to achieve the same inhibitory effect as pNL4.3, meaning that these viruses are more susceptible or less resistant to the *Alternaria alternata* PO4PR2 crude extract. This indicates that the virus is inhibited at a lower concentration than the pNL4.3 virus, suggesting greater susceptibility or hypersensitivity to the *A. alternata* PO4PR2 crude extract. The integrase drug-resistant mutants exhibited fold change values lower than 1, indicating they were more sensitive to the extract than the pNL4.3 virus.

Table 4: IC_{50} and fold changes of integrase mutant viruses relative to pNL4.3.

Virus	IC_{50}	Fold Change	Fold Change Interpretation
G118R	0.1044 ± 0.023 µg/mL	0.46	Increased Susceptibility (FC < 1)
N155H	0.1032 ± 0.025 µg/mL	0.464	Increased Susceptibility (FC < 1)
R263K	0.02971 ± 0.033 µg/mL	0.134	Increased Susceptibility (FC < 1)
Y143R	0.1652 ± 0.027 µg/mL	0.74	Increased Susceptibility (FC < 1)

4.2.5 Analysis of IC_{50} Values and Selectivity Indexes for *Alternaria alternata* PO4PR2 Crude Extract Against HIV-1 Drug-Resistant Mutations

To evaluate whether the *Alternaria alternata* PO4PR2 crude extract demonstrated meaningful antiviral activity relative to its cytotoxicity, a selectivity index (SI) was calculated using the ratio CC_{50}/IC_{50} (Table 5). This value, also known as the therapeutic index, helps indicate whether a compound should be prioritised for additional investigation. In this study, compounds with an SI of at least 2 were classified as showing antiviral activity, while those with SI values of 10 or higher were regarded as highly active (Mogatle et al., 2008; Russo et al., 2021; Stamets, 2014). The half-maximal inhibitory concentrations (IC_{50}) and selectivity indexes obtained for the *Alternaria alternata* PO4PR2 crude extract were compared with those of the Dolutegravir drug control across a panel of the HIV-1 drug-resistant mutants. This analysis provided an initial indication of whether the *Alternaria alternata* PO4PR2 extract demonstrates favourable safety and efficacy profiles across different

HIV-1 drug-resistant mutants.

The selectivity index analysis demonstrated that the *Alternaria alternata* PO4PR2 crude extract exhibited strong antiviral selectivity across all tested HIV-1 integrase drug-resistant mutants. All of the SI values for the extract were substantially greater than the threshold of 10, indicating high antiviral activity coupled with low cytotoxicity. The extract showed exceptionally high SI values against the R263K mutant, reflecting favourable antiviral selectivity against this clinically relevant resistance mutation. Although the IC_{50} values of the mutants were higher than that of the pNL4.3 control, indicating reduced susceptibility, the high SI values suggest that effective viral inhibition was still achieved at concentrations below cytotoxic levels.

When compared with the Dolutegravir control, the *A. alternata* PO4PR2 extract consistently displayed higher SI values for most mutants, highlighting a broad safety margin despite the higher IC_{50} values. These findings indicate that the *A. alternata* PO4PR2 crude extract retains potent antiviral activity against multiple HIV-1 drug-resistant mutants, supporting its potential as a promising source of anti- HIV compounds for further investigation.

Table 5: IC_{50} and selectivity indexes of the *A. alternata* crude extract against HIV-1 mutants

Virus	**Subtype**	**IC_{50} *Alternaria alternata,* PO4PR2 (**µg/mL)	**IC_{50} Dolutegravir (**µg/mL)	**SI *Alternaria alternata* PO4PR2**	**SI Dolutegravir**
G118R	C	0.1044 ± 0.023	0.8549 ± 0.003	2873.56	350.92
N155H	C	0.1032 ± 0.025	0.4431± 0.003	2906.98	677.05
R263K	C	0.02971 ± 0.003	0.6192 ± 0.004	10097.61	484.50
Y143R	C	0.1652 ± 0.027	0.5032 ± 0.003	1846.15	596.18
pNL4.3	B	0.2223 ± 0.015	0.1770 ± 0.002	1349.53	1694.92

4.2.1 Relative Fold Change in IC_{50} of Drug-resistant mutants against pNL4.3 virus

The IC_{50} values of the G118R, N155H, R263K, and Y143R mutant viruses were expressed as fold changes in IC_{50} relative to the IC_{50} of the pNL4.3 infectious control virus (0.2223 ± 0.015 µg/mL) (Table 4 and Figure 11). Fold change was calculated as the ratio of the mutant virus IC_{50} to the pNL4.3 control IC_{50} (mutant virus IC_{50}/ pNL4.3 IC_{50}). The comparative analysis of the IC_{50} values revealed variable, but generally high, susceptibility among the HIV-1 integrase drug-resistant mutants following treatment with the *Alternaria alternata* PO4PR2 crude extract. A fold change in IC_{50} less than 1 indicates a decrease in IC_{50} relative to control pNL4.3, demonstrating greater susceptibility (hypersensitivity) relative to pNL4.3. All four of the tested mutants (G118R, N155H, R263K, and Y143R) exhibited fold change values less than 1, indicating that the drug-resistant mutants were more susceptible to the *Alternaria alternata* PO4PR2 crude extract than the pNL4.3 wild-type virus. Specifically, the R263K mutant demonstrated the lowest fold change (0.134), indicating that it was the most sensitive to the extract and could be inhibited at the

lowest concentrations, reflecting enhanced susceptibility. This finding suggests that the *Alternaria alternata* PO4PR2 crude extract retains potent antiviral activity against key drug-resistant mutations and exhibits a favourable activity profile against these INSTI drug- resistant mutations, making it a promising source for compounds that can overcome the current resistance challenges.

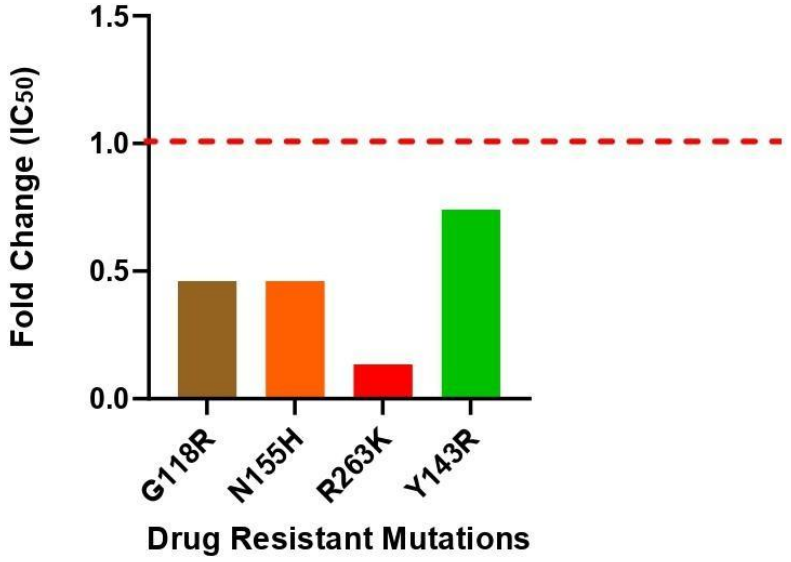

Figure 11: Fold change in IC50 for mutant viruses after treatment with the *A. alternata* crude extract

4.2.2 The effects of *Alternaria alternata* PO4PR2, Sodium azide, and Raltegravir on HIV-1 integrase activity

Figure 12 shows the inhibitory effects of three compounds, Sodium azide (A), Raltegravir (B), and the crude extract from *Alternaria alternata* PO4PR2 (C) on HIV-1 integrase activity. Raltegravir, a clinically approved integrase inhibitor, exhibited the highest potency with an IC_{50} of 0.03477 µg/mL, confirming its strong antiviral activity. Sodium azide demonstrated moderate inhibition with an IC_{50} of 0.7654 µg/mL, indicating potential integrase-targeting properties, but at higher concentrations. The *Alternaria alternata* PO4PR2 crude extract also exhibited dose-dependent inhibition, with an IC_{50} of 2.85 µg/mL, indicating the presence of bioactive compounds that warrant further purification and characterization. These findings support the potential of *Alternaria alternata* PO4PR2 as a promising source of natural product-based HIV-1 integrase inhibitors, warranting further investigation into its active constituents and mechanisms of action.

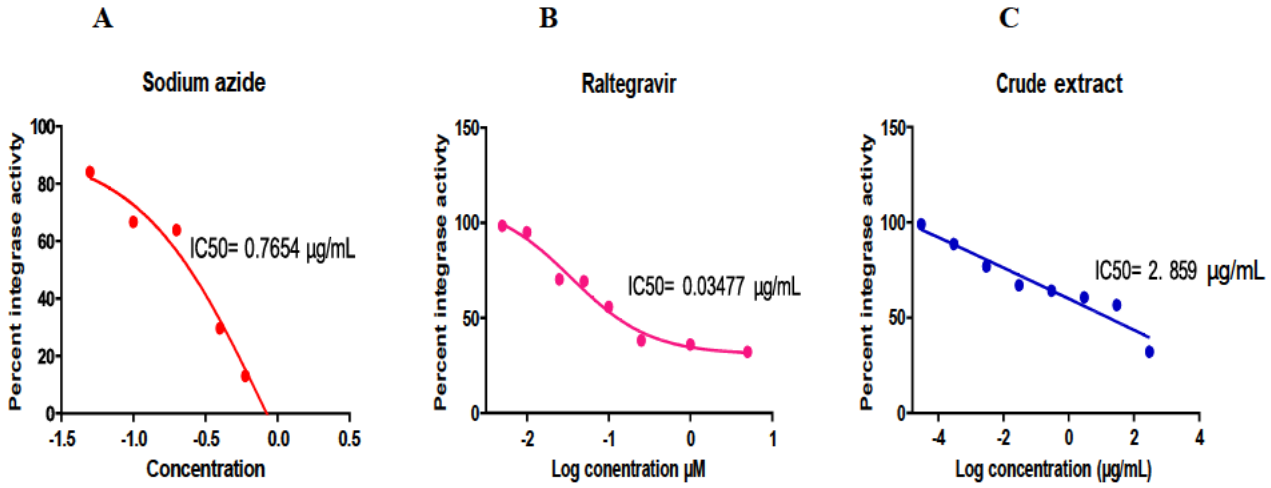

Figure 12: Inhibition of HIV-1 integrase by Sodium azide (A), Raltegravir (B), and *A. alternata* crude extract (C)

4.2.3 Real-Time PCR Analysis

Peripheral blood mononuclear cells were obtained from HIV-negative participants enrolled in the FRESH cohort in KwaZulu-Natal, South Africa. Cryopreserved PBMCs from 20 donors were thawed and used for experimentation, where cells were infected with HIV-1 viruses containing one of four integrase drug-resistant mutations (G118R, N155H, R263K, or Y143R) and then treated with Raltegravir, Dolutegravir, or the *Alternaria alternata* PO4PR2 crude extract to assess inhibition of HIV-1 integration, with untreated cells included as the control. The untreated infected cells were included as controls to determine baseline integration levels. Integrated HIV-1 DNA was quantified to compare the effect of the *A. alternata* PO4PR2 treatment across the different drug-resistant viral strains. Quantification of integrated HIV-1 DNA was performed by extracting DNA from $\mathbf{2 \times 10^6}$ PBMCs, and a two-step *Alu-gag* nested PCR assay was used to measure proviral integration, normalized to human GAPDH. Treatment of PBMCs infected with 300 µg/mL of *A. alternata* PO4PR2 extract resulted in a marked reduction in integrated HIV-1 DNA across all tested integrase drug-resistant mutants (G118R, N155H, R263K, and Y143R) (Figure 13A). In untreated cells, each mutation demonstrated relatively high levels of integration (approximately 0.50–0.55 copies), whereas treatment with the *A. alternata* PO4PR2 crude extract consistently reduced integration to approximately 0.23–0.30 copies. Statistical analysis confirmed that these differences were highly significant for all of the tested mutations ($p = 0.0011$). Treatment with 300 µg/mL of the clinically approved integrase inhibitor Raltegravir resulted in the greatest reduction in integrated HIV-1 DNA levels compared to the untreated control, while Dolutegravir and the *Alternaria alternata* PO4PR2 extract also reduced integration levels. The *Alternaria alternata* PO4PR2 extract demonstrated a measurable decrease in integrated viral DNA relative to the untreated group (Figure 13B), indicating that the crude extract possesses anti-HIV activity targeting the integration step of viral replication. The extract performed comparably to Dolutegravir,

suggesting that naturally derived metabolites from *Alternaria alternata* PO4PR2 may inhibit HIV-1 integration with an efficacy approaching that of the licensed antiretroviral drugs. These findings highlight the potential value of the *Alternaria alternata* PO4PR2 crude extracts as promising sources of integrase-inhibiting compounds and support the need for further characterization, optimization, and mechanistic studies.

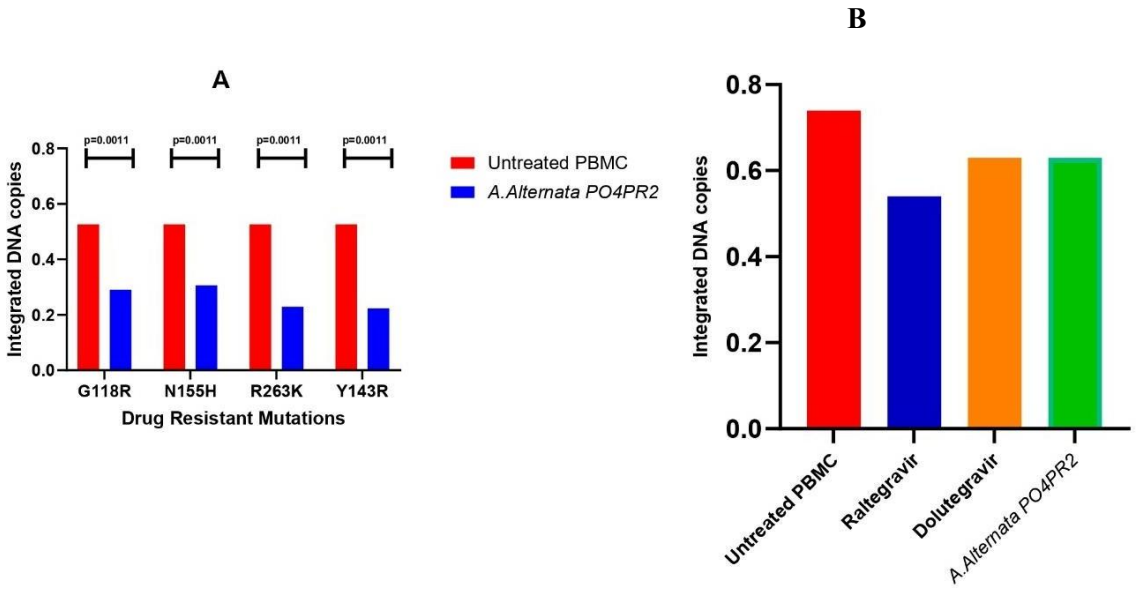

Figure 13: Integrated DNA copies post-treatment with the *A. alternata* crude extract (A) and INSTI-resistant mutants and PBMCs infected with wild-type pNL4.3 (B)

4.2.4 Docking Calculations of the Bioactive Compounds of *Alternaria alternata* PO4PR2 Against HIV-1 Integrase Drug-Resistant Variants

4.2.4.1 Binding Affinity Analysis

Molecular docking simulations were performed against the catalytic core domains of HIV-1 integrase, including the wild-type and four clinically relevant resistant mutants (G118R, N155H, Y143R, and R263K). The binding poses of the metabolites were visualized and compared with those of the reference inhibitors, Raltegravir and Dolutegravir, across both wild-type and mutant integrase structures (Figure 14). All metabolites occupied the catalytic pocket, aligning closely with the binding orientations of the reference drugs, indicating potential engagement with key residues essential for integrase inhibition. The docking results were compared with those of approved INSTIs to assess the relative binding strengths and resistance tolerance. More negative docking scores correspond to higher predicted binding affinities, indicating stronger ligand–protein interactions (Figure 15). Among the screened metabolites, coumarin, 3,4-dihydro-4,5,7-trimethyl- exhibited the most favourable binding across all IN variants, with docking scores ranging from −5.9 to −6.2 kcal/mol, suggesting a robust and mutation-resilient affinity toward the active site of the enzyme. 2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl- and

pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl) followed closely, showing consistently strong binding energies of −5.6 to −6.1 kcal/mol and −5.0 to −5.6 kcal/mol, respectively. These comparable affinities across resistant IN variants indicate that these compounds could maintain their inhibitory activity even in drug-resistant viral strains. Conversely, hexamethylcyclotrisiloxane and octamethylcyclotetrasiloxane displayed the weakest binding affinities (−3.2 to −3.8 kcal/mol), suggesting a limited potential for IN inhibition. Similarly, citraconic anhydride and 2-acetylcyclopentanone exhibited moderate binding energies (−4.2 to −4.9 kcal/mol), implying less stable enzyme–ligand interactions.

From a comparative standpoint, the control drugs, Raltegravir and Dolutegravir, demonstrated docking scores between −7.0 and −7.9 kcal/mol across all variants, consistent with their known high binding affinity and clinical efficacy against IN-mediated viral integration. While none of the secondary metabolites surpassed the binding strengths of the INSTIs, coumarin, 3,4-dihydro-4,5,7-trimethyl- and 2,3-2H-benzofuran-2-one, 3,3,4,6-tetramethyl- showed promising affinities approaching those of the reference.

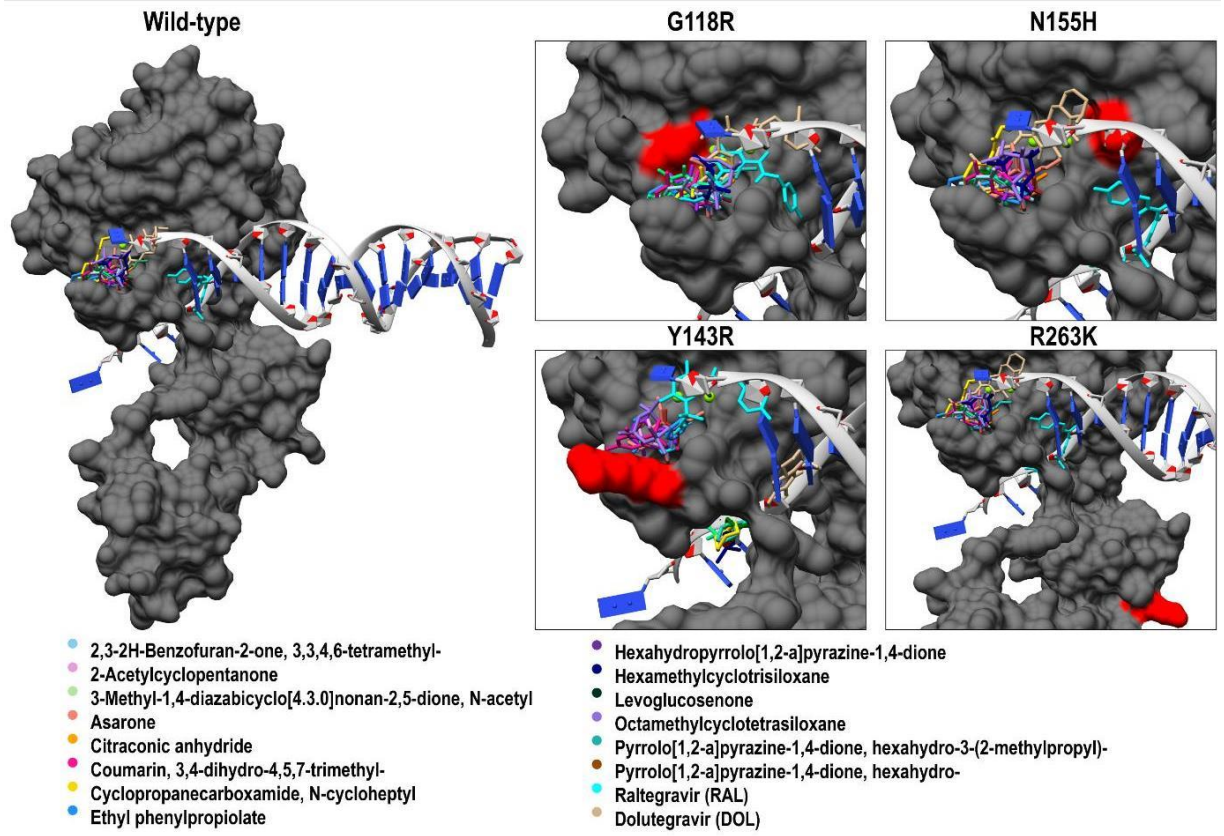

Figure 14: Binding conformations of *A. alternata* within the integrase–DNA–Mg^{2+} catalytic complex.

	Wildtype	G118R	N155H	Y143R	R263K
2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl-	-5.8	-6.1	-5.8	-5.6	-5.8
2-Acetylcyclopentanone	-4.6	-4.9	-4.6	-4.4	-4.6
3-Methyl-1,4-diazabicyclo[4.3.0]nonan-2,5-dione, N-acetyl	-5.2	-5.7	-5.3	-5.1	-5.2
Asarone	-5.3	-5.4	-5.1	-4.9	-5.3
Citraconic anhydride	-4.4	-4.5	-4.4	-4.2	-4.4
Coumarin, 3,4-dihydro-4,5,7-trimethyl-	-5.9	-6.2	-5.9	-5.0	-5.9
Cyclopropanecarboxamide, N-cycloheptyl	-4.5	-4.9	-4.6	-4.4	-4.5
Ethyl phenylpropiolate	-5.4	-5.4	-5.4	-4.4	-5.4
Hexahydropyrrolo[1,2-a]pyrazine-1,4-dione	-4.9	-5.1	-4.9	-4.7	-4.9
Hexamethylcyclotrisiloxane	-3.3	-3.3	-3.3	-3.2	-3.3
Levoglucosenone	-4.1	-4.4	-4.1	-3.9	-4.1
Octamethylcyclotetrasiloxane	-3.8	-4.0	-3.8	-3.8	-3.8
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl)-	-5.3	-5.6	-5.3	-5.0	-5.3
Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-	-5.0	-5.3	-5.0	-4.8	-5.0
Raltegravir (RAL)	-7.6	-7.7	-7.9	-7.0	-7.6
Dolutegravir (DTG)	-7.7	-7.8	-7.8	-7.3	-7.7

Figure 15: Molecular docking scores of *A. alternata* against HIV-1 IN and HIV-1 mutant strains[18]

4.2.4.2 Protein–Ligand Interaction Analysis

Comparative protein–ligand interaction profiling was performed for the *Alternaria alternata* metabolites 2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl- and Coumarin, 3,4-dihydro-4,5,7-trimethyl- against wild-type and four clinically relevant HIV-1 IN variants (G118R, N155H, Y143R, and R263K). Reference inhibitors, Dolutegravir (DTG) and Raltegravir (RAL), were included to delineate the impact of resistance-associated mutations on ligand recognition and metal coordination within the IN–DNA–Mg^{2+} catalytic complex (Figure 14). In the wild-type IN–DNA landscape, both *Alternaria alternata* PO4PR2 metabolites were stably anchored within the catalytic pocket at the IN–DNA interface. 2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl- established hydrogen bonds with ASN144 and TYR143, supplemented by π–π stacking and π–alkyl interactions with TYR143. van der Waals contacts involving ASP116, ASN117, PRO142, PRO145, GLN148, and the Mg^{2+} ion (Mg301) further stabilized the complex.

Likewise, coumarin, 3,4-dihydro-4,5,7-trimethyl- formed hydrogen bonds, π–π stacking, and π–alkyl interactions with TYR143, alongside a π–sigma contact with the DNA base DA F:21. The persistence of π–π stacking with TYR143 across both ligands underscores the central role of this residue in ligand anchoring at the active site. In contrast, the reference inhibitors exhibited distinct binding characteristics. Raltegravir formed hydrogen bonds with GLN53, GLN146, SER147, and SER153, along with π–alkyl interactions involving VAL54 and HIS114 but lacked direct metal coordination. Additional hydrogen bonding, hydrophobic, and van der Waals contacts with DNA base pairs further stabilized the binding within the IN–DNA complex. However, Dolutegravir demonstrated stronger metal–acceptor coordination with Mg^{2+} (Mg302) and formed hydrogen bonds with THR66, HIS67, GLY118, TYR143, and GLU152, complemented by π–anion, π–π stacking, and halogen (fluorine) interactions (Figure 16A). These extensive polar and coordination contacts reflect the deeper and more stable engagement of Dolutegravir within the catalytic triad compared to that of Raltegravir.

Upon introduction of the G118R mutation, both metabolites retained their key aromatic stacking interactions with TYR143, indicating structural tolerance to perturbations near the catalytic core. 2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl- gained additional hydrogen bonds with PRO145 and GLN148, while coumarin, 3,4-dihydro-4,5,7-trimethyl- maintained TYR143 binding and formed new hydrogen bonds with ASN144 alongside alkyl contacts with PRO142. These compensatory interactions suggest adaptive stabilization, despite the steric influence of ARG118. In comparison, Raltegravir exhibited extended coordination with both Mg^{2+} ions, forming hydrogen bonds with ARG118, TYR143, PRO142, and GLN146, as well as π–π stacking with DC F:20, whereas dolutegravir preserved its canonical halogen, hydrogen bonding (THR66, HIS67, GLU152), and π–anion contacts (Figure 16B). For the N155H variant, both *Alternaria alternata* PO4PR2 metabolites maintained conserved hydrogen

bonding with ASN144 (2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl-) or TYR143 (coumarin, 3,4-dihydro-4,5,7-trimethyl-) and retained π–π stacking with TYR143. Their interaction networks were largely unaltered compared to those of the wild type, indicating minimal disruption by the N155H substitution. Correspondingly, Raltegravir and dolutegravir sustained strong coordination with DNA bases and Mg^{2+} ions, confirming that this mutation exerts limited influence on ligand positioning at the catalytic triad (Figure 16C). In the Y143R variant, a marked conformational reorganization occurred around the active site loop. 2,3-2H-Benzofuran-2-one, 3,3,4,6-tetramethyl- shifted its π–π stacking from TYR143 to HIS114, forming hydrogen bonds with ASP55 and SER147 and hydrophobic contacts with ILE60 and VAL79. Conversely, coumarin, 3,4-dihydro-4,5,7-trimethyl- displayed weakened binding, restricted to π–alkyl interactions with ARG143 and van der Waals contacts within the catalytic loop. In comparison, Raltegravir maintained robust hydrogen bonding and metal coordination, whereas dolutegravir formed multiple stabilizing contacts with DNA bases (Figure 16D). For the R263K variant, both metabolites recapitulated the wild-type interaction profiles, maintaining hydrogen bonds and π–π stacking with TYR143 and van der Waals interactions with PRO142, PRO145, and GLN148. The persistence of TYR143-mediated interactions signifies a strong conformational conservation around the DNA-binding groove. Raltegravir retained its DNA base contacts, whereas dolutegravir continued to exhibit robust Mg^{2+} coordination (Figure 16E).

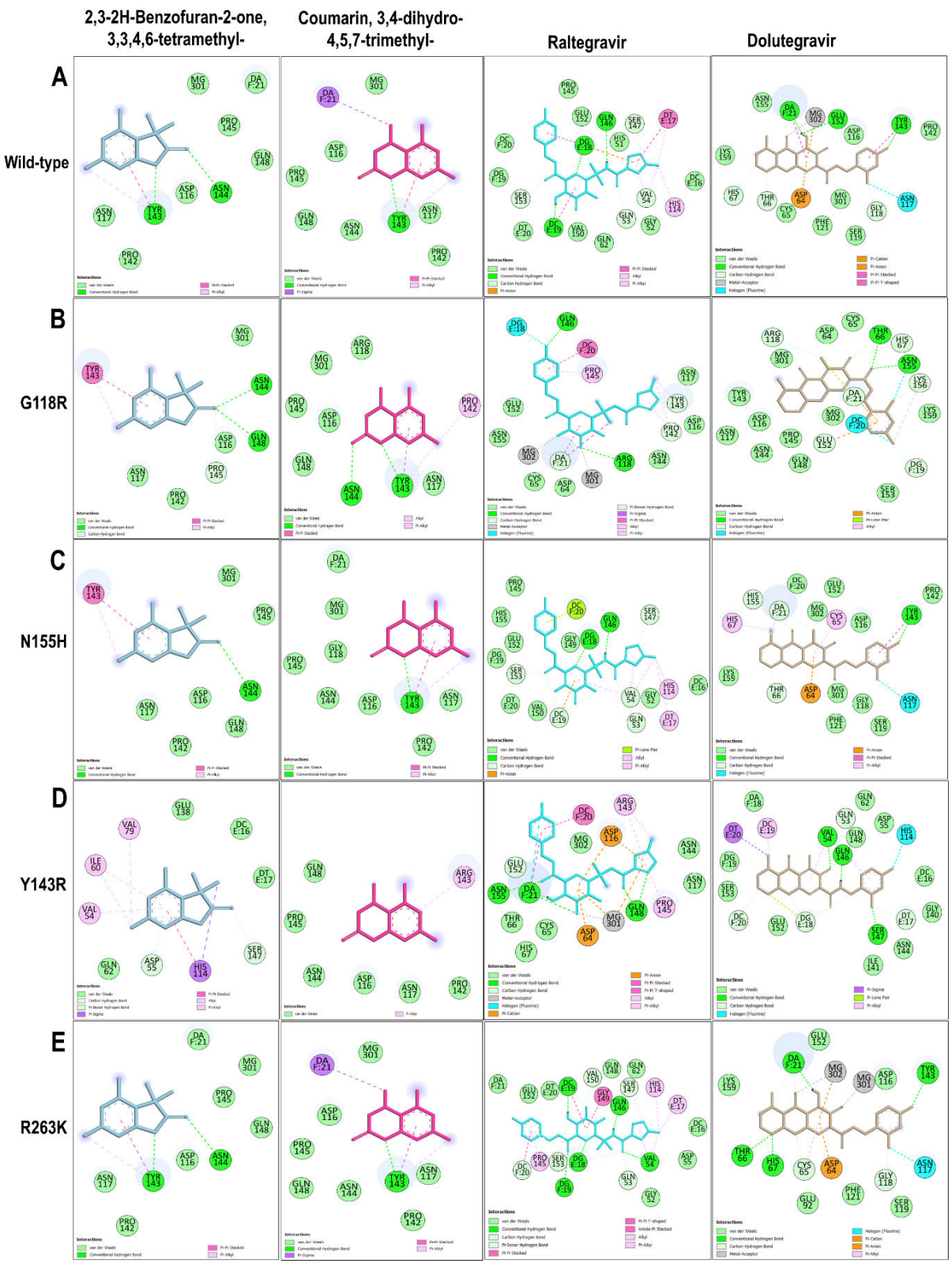

Figure 16**:** Interaction maps of *A. alternata* metabolites within the active site of wild-type and mutant IN–DNA–Mg^{2+} complexes.

4.3 Discussion

Alternaria species produce a diverse array of secondary metabolites, including polyketides, terpenoids, and alkaloids, many of which exhibit significant biological activities. Crude extracts of *Alternaria alternata* PO4PR2 isolated from the roots, stems, and leaves of *Sclerocarya birrea* and *Hypoxis* plants, were found to inhibit HIV-1 replication at various stages (Nzimande et al., 2022). Previous studies have shown that *Alternaria* species produce bioactive secondary metabolites with notable anti-HIV activity, particularly by interfering with critical stages of the HIV replication cycle, such as reverse transcriptase, nuclear import, and viral integration, thereby preventing the establishment of infection Bashyal et al., 2014; Nzimande et al., 2023; Wellensiek et al., 2013). Recent studies demonstrated that *Alternaria alternata* PO4PR2 crude extracts exhibit anti-HIV activity, with complete inhibition of the integration stage of the viral replication cycle (Kubheka et al., 2024; Naidu et al., 2025). However, these studies did not elucidate the precise mechanism of action underlying the observed anti-HIV activity and the specific bioactive compounds responsible for inhibiting viral integration. Therefore, this study investigated the anti-HIV activity of *Alternaria alternata* PO4PR2 crude extract, with a particular focus on its effects against wild-type and integrase inhibitor-resistant HIV-1 variants. Our results demonstrated that the *Alternaria alternata* PO4PR2 crude extract exhibited notable inhibitory activity against both wild-type and drug-resistant HIV-1 integrase mutants, indicating that *A. alternata* PO4PR2 produces bioactive metabolites capable of targeting the viral integration stage and offering promise for the development of new antiretroviral agents.

Analysis from the Stanford Drug Resistance Database (HIVDB) identified G118R, N155H, R263K, and Y143R as the most clinically significant HIV-1 integrase mutations. These are well-established resistance-associated mutations that compromise the efficacy of first- and second-generation integrase strand transfer inhibitors (INSTIs) and have been reported in clinical isolates from patients experiencing virological failure on Raltegravir (RAL) and Dolutegravir (DTG) (Ferrer et al., 2025; Hassounah et al., 2014). The N155H and Y143R mutations are classic Raltegravir pathway substitutions, conferring reduced susceptibility to RAL and, to a lesser extent, elvitegravir (EVG) (Wainberg et al., 2012b; Xiao et al., 2023). The G118R and R263K mutations are characteristic of Dolutegravir resistance pathways and are often associated with moderate to high-level resistance to DTG, Bictegravir (BIC), and Cabotegravir (CAB) (Brenner et al., 2016; Ngoufack Jagni Semengue et al., 2022; Xiao et al., 2023). The R263K mutation has been shown to reduce the enzymatic efficiency of HIV-1 integrase and the viral replication capacity (Wares et al., 2014). Analysis of integrase gene sequences obtained from the Los Alamos HIV Sequence Database and NCBI further confirmed the presence of these clinically relevant mutations across multiple HIV-1 subtypes, including subtype C, which predominates in Southern Africa (Hemelaar et al., 2011; Khan et al., 2023; Lihana et al., 2012; Musyoki et al., 2015). Similar mutation profiles were observed in previous studies, indicating that these resistance-associated variants are circulating in the region and may compromise the long-term efficacy of INSTI-based regimens (Ndashimye et al., 2020; Ngoufack Jagni Semengue et al., 2022; Seatla et al., 2021; Steegen

et al., 2019). The consistent detection of these mutations across independent datasets underscores their clinical significance and highlights the urgent need to identify alternative therapeutic agents that can retain activity against such drug-resistant variants. The ability of the *A. alternata* PO4PR2 crude extract to inhibit these clinically significant integrase mutants suggests that its secondary metabolites may offer a new avenue for developing broad-spectrum inhibitors with potential to overcome current resistance barriers in HIV-1 therapy.

For a medicinal plant extract to be considered safe or non-toxic, it should have a high CC_{50} value (50% cytotoxic concentration), ideally above 20 µg/mL or significantly higher, indicating that a much larger concentration is required to kill 50% of live cells, and therefore, the extract poses lower toxicity to mammalian cells (Njeru & Muema, 2021). The anti-HIV-1 effects of the *Alternaria alternata* PO4PR2 crude extract were observed at concentrations that did not induce cytotoxicity in host cells, demonstrating a favourable selectivity index (Figure 8). The cytotoxicity evaluation of the *Alternaria alternata* PO4PR2 crude extract using the MTT assay revealed that the extract exhibited low toxicity toward TZM-bl cells, maintaining over 80% cell viability across the tested concentration range. The calculated CC_{50} value of 300 ± 1.84 µg/mL supports its low cytotoxic profile and falls within an acceptable range for plant-derived extracts used in preliminary antiviral screening, which are classified as highly toxic if the CC_{50} is less or equal to 20 µg/mL, slightly toxic if the CC_{50} is between 21–200 µg/mL, and none toxic if the CC_{50} is between 201–500 µg/mL (Anywar et al., 2022; Njeru & Muema, 2021). The observed cytotoxicity profile of the *A. alternata* PO4PR2 crude extract aligns with previous reports describing similarly low cytotoxicity for the *A. alternata* PO4PR2 extract-derived compounds in mammalian cell models (Kubheka et al., 2024; Naidu et al., 2025; Nzimande et al., 2022). This balance between efficacy and low toxicity is crucial for the development of safe therapeutic agents, as compounds that are highly toxic to host cells are generally unsuitable for clinical use. These findings indicate that the bioactive metabolites in the extract do not adversely affect cell metabolism or membrane integrity at therapeutic concentrations, suggesting that the *Alternaria alternata* PO4PR2 crude extract is non-toxic and safe for *in vitro* use.

The G118R, N155H, R263K, and Y143R integrase drug-resistance mutants were introduced into the pNL4.3 plasmid by site-directed mutagenesis (Figure 7), and the single-cycle infectious clones were generated by transfection. The infectivity assessment using the Tissue Culture Infectious Dose ($TCID_{50}$) assay confirmed that all tested HIV-1 integrase drug-resistant mutants (G118R, N155H, R263K, and Y143R) remained infectious in TZM-bl cells, each producing relative light unit values exceeding 80,000, well above the 15,000 RLU threshold for infectivity (Figure 9). Although these mutants demonstrated robust infectivity, their replication efficiency was lower than that of the wild-type pNL4.3 virus, indicating that the wild-type virus retains superior replicative fitness compared to the integrase-resistant variants. Despite the modest reduction in infectivity compared to the pNL4.3 variant, the ability of all four integrase mutants to achieve high RLU values confirms that they remain replication-

competent and capable of productive infection *in vitro*. These findings further support those of Naidu et al. (2025), who similarly reported that integrase drug-resistant mutations such as T66K and S230R retained measurable infectivity in TZM-bl cells, producing RLUs above the infectious threshold (Coll et al., 2003).

Previous studies have reported findings consistent with our observations, showing that integrase drug-resistant mutations, such as G118R, Y143R, N155H, and R263K, as well as other relevant integrase drug-resistant mutations introduced via site-directed mutagenesis, can generate infectious viruses following transfection (Hassounah et al., 2014). Consistent with our findings, these integrase drug-resistant mutants exhibited reduced single-cycle infectivity and diminished replication capacity compared to the wild-type virus (Hassounah et al., 2014). Together, these results indicate that HIV-1 integrase mutations preserve sufficient viral fitness to sustain infection, reinforcing the importance of identifying antiviral agents, such as the *A. alternata* PO4PR2 crude extract, that remain effective even against infectious, drug-resistant HIV-1 variants.

A luciferase-based assay was used to assess the anti-HIV-1 efficacy of the *Alternaria alternata* PO4PR2 crude extract against the G118R, N155H, R263K, and Y143R mutant viruses, quantifying inhibition as a percentage and determining the IC_{50} (Figure 10). All samples were tested in triplicate, and the resulting IC_{50} values were combined and reported as geometric means with corresponding standard deviations. Across the range of concentrations tested, the *Alternaria alternata* PO4PR2 crude extract consistently demonstrated strong antiviral activity, with inhibition percentages remaining above 50% (Figure 10). This observation is consistent with previous studies, which report that *Alternaria* species are capable of inhibiting HIV replication across a range of concentrations (Bashyal et al., 2014). The value of the IC_{50} serves as an indicator of an extract's potency, and it is correlated to the amount of drug necessary to produce the desired effect - the lower the IC_{50} value, the more potent the drug (Berrouet et al., 2020). An extract is regarded as highly active when its IC_{50} value falls below 10 µg/mL (Irabor et al., 2021). The IC_{50} values of the HIV-1 G118R, N155H, R263K, and Y143R mutant viruses treated with the *A. alternata* PO4PR2 crude extract were below the 10 µg/mL threshold, indicating that the extract exhibited strong antiviral activity against the mutant strains (Irabor et al., 2021). This finding is in agreement with previous reports demonstrating that *Alternaria alternata* PO4PR2 extracts possess broad-spectrum antiviral activity across HIV-1 subtypes and HIV-1 integrase drug-resistant mutations, and maintain efficacy against integrase-resistant mutants (Naidu et al., 2025).

The half-maximal inhibitory concentrations of the G118R, N155H, R263K, and Y143R mutant viruses were expressed as a fold change in IC_{50} relative to the IC_{50} of the pNL4.3 (0.2223 ± 0.015 µg/mL) (Table 5). The comparative analysis of the IC_{50} values revealed variable susceptibility among the HIV-1 integrase drug-resistant mutants following treatment with the *Alternaria alternata* PO4PR2 crude extract. The fold change in IC_{50} relative to control pNL4.3 was calculated as a ratio of the mutant virus IC_{50}/ pNL4.3 IC_{50} (Clutter et al., 2016). A fold change in IC_{50} less than 1 indicates a decrease in IC_{50}

relative to control pNL4.3, indicating greater susceptibility relative to pNL4.3 (Perno & Bertoli, 2006). When expressed as fold changes relative to the reference strain pNL4.3, the G118R, N155H, R263K and Y143R mutants exhibited fold change values less than 1, indicating that these variants required lower concentrations of the extract to achieve comparable inhibition. This suggests a degree of increased susceptibility or to the *Alternaria alternata* PO4PR2 extract compared with the pNL4.3 virus. The pNL4.3 reference strain, a well-characterized, highly infectious wild-type HIV-1 subtype B virus, is typically used as a drug-sensitive control because it lacks resistance mutations and responds efficiently to integrase inhibitors (Abad et al., 2004; Hassounah et al., 2017; Rezaei et al., 2007). Similar findings were observed for the N155H and G118R mutants, both of which exhibited a fold change lower than 1, relative to the wild-type virus, indicating that they are more sensitive to the extract than the wild-type virus and could be inhibited at lower concentrations, reflecting enhanced susceptibility (Kobayashi et al., 2008).

The HIV-1 integrase inhibition assay serves as a crucial tool for evaluating compounds that interfere with the integration of viral DNA into the host genome, a defining and irreversible step in the HIV replication cycle (Craigie, 2012; Lesbats et al., 2016; Sayyed et al., 2023). Because this assay specifically measures the integrase-mediated strand-transfer reaction, the stage in which processed viral DNA ends are covalently inserted into host chromosomal DNA, it enables the direct assessment of agents that block the strand-transfer step of HIV replication (Sayyed et al., 2023). By assessing the ability of test agents to disrupt this enzymatic process, the assay plays a crucial role in the development of antiretroviral therapies specifically designed to block integration and prevent the establishment of a productive infection (Sayyed et al., 2023, 2024). *Alternaria alternata* PO4PR2 is explored in this study for its inhibition of the integrase allosteric site. Its mechanism involves inhibiting the strand transfer stage of HIV-1 replication and the activity of HIV-1 integrase, offering a novel approach to modulating integrase function. The *A. alternata* PO4PR2 crude extract demonstrated a clear dose-dependent inhibition of HIV-1 integrase activity, indicating that its secondary metabolites are capable of effectively targeting the HIV-1 integrase catalytic activity (Figure 12). Even at low concentrations, the extract exhibited measurable suppression of enzymatic function, with inhibitory activity increasing progressively as the concentration was elevated. The calculated IC_{50} indicates a potent inhibitory effect, suggesting that the compounds within the *A. alternata* PO4PR2 crude extract have a strong affinity for the integrase enzyme and can interfere with integrase-mediated processing steps required for viral replication. This observed potency is consistent with previous reports demonstrating that naturally derived compounds from fungal metabolites can inhibit HIV-1 integrase activity, particularly by targeting the 3' processing and strand-transfer stages of the integration process (Chaniad et al., 2016; Reinke et al., 2004; Serna-Arbeláez et al., 2021). Similarly, other natural products have also shown the capacity to interfere with HIV-1 integrase function, highlighting the growing evidence that bioactive molecules from natural sources can target HIV-1 integrase and may serve as valuable scaffolds for future antiretroviral drug development. (Gu et al., 2014; Rotich et al., 2022).

The *Alu-gag* PCR assay remains one of the most widely used techniques for quantifying integrated HIV-1 DNA and provides a crucial measure of the proviral reservoir during infection and after antiviral intervention (Brady et al., 2013; Mavigner et al., 2016; Pinzone & O'Doherty, 2018). In this study, the *Alu-gag* PCR was employed to determine whether treatment with the *Alternaria alternata* PO4PR2 crude extract had an effect on the number of integrated proviruses within infected cells. The *Alu-gag* PCR assay is particularly valuable for assessing inhibitors that target the integration step of the viral replication cycle, as it distinguishes integrated HIV-1 DNA from unintegrated episomal forms, thereby providing a sensitive and specific readout of integrase-dependent inhibition (Liszewski, Jianqing, et al., 2009). Our results demonstrated that treatment with *Alternaria alternata* PO4PR2 crude extracts led to a marked reduction in integrated proviral DNA, as shown by a decrease in the amplification of the *Alu-gag* product relative to untreated controls (Figure 13). This reduction indicates that the compounds interfered with the successful integration of viral DNA into the host genome, consistent with the mode of action of compounds that inhibit integrase catalytic activity or disrupt pre-integration complex formation.

HIV-1 integration proceeds through two enzymatic steps catalysed by the viral integrase: 3′-processing, during which IN removes two nucleotides from the viral cDNA ends to generate recessed 3′-OH groups, and strand transfer, where IN inserts these processed viral DNA ends into the host genome (Craigie, 2012a; Craigie & Bushman, 2012; Passos et al., 2021). A reduction in integrated DNA strongly aligns with interference at the strand-transfer step, as this reaction is responsible for generating the covalently integrated proviral DNA detected in integration assays (Goff, 2024; Lesbats et al., 2016; Senavirathne et al., 2023). Inhibition of 3′-processing produces intact viral cDNA; however, the untrimmed DNA ends cannot be efficiently joined to host DNA and are susceptible to aberrant repair or degradation, resulting in the accumulation of blunt-ended viral DNA (Li & Craigie, 2005). When strand transfer is inhibited, the viral cDNA cannot be inserted into host chromatin and instead accumulates in unintegrated forms such as linear viral DNA or episomal 2-LTR circles (Richetta et al., 2022; Trinité et al., 2013; Wu, 2023). This pattern of reduced integration with preserved reverse transcription mirrors the phenotype observed with established strand-transfer inhibitors such as Raltegravir, Dolutegravir, and Bictegravir, which prevent IN from catalysing the joining of viral DNA to the host genome (Mbisa et al., 2011; A. V. Zhao et al., 2022). Therefore, when this characteristic decrease in integrated DNA is observed, it is consistent with inhibition occurring at the strand-transfer stage of the integration process.

These findings align with previous studies, which have reported that natural metabolites, plant extracts, and small-molecule integrase inhibitors significantly reduce proviral integration when assessed using *Alu*-based PCR assays (Mediouni et al., 2018; Vandegraaff et al., 2001). The dose-dependent decrease in integrated HIV-1 DNA observed in this study further supports the hypothesis that the *Alternaria alternata* PO4PR2 crude extracts exhibit potent anti-integrase activity. The marked reduction in integrated proviral DNA, combined with direct suppression of integrase enzymatic activity observed from the integrase activity assay, is characteristic of compounds that block the strand-transfer phase of integration, aligning the behaviour of the *Alternaria alternata* PO4PR2 crude extracts with that of established INSTIs (Mbisa et al., 2011; A. V. Zhao et al., 2022). Taken together, the integrase activity assay and the *Alu-gag* real-time PCR results provide convergent evidence that the *Alternaria alternata* PO4PR2 crude extracts predominantly interfere with the strand-transfer step of HIV-1 integration. Even at lower concentrations, measurable inhibition was detected, indicating a strong affinity for viral or cellular components required for integration. This behaviour mirrors findings from earlier reports describing fungal-derived and other naturally occurring compounds capable of suppressing 3′ processing or strand transfer, thereby preventing completion of the integration step (Sayyed et al., 2023, 2024; Serna-Arbeláez et al., 2021).

The observed anti-HIV-1 activity can be potentially attributed to the diverse secondary metabolites produced by *Alternaria* species, including polyketides, depsidones, and phenolic compounds, which have previously been reported to possess antiviral, antimicrobial, and anticancer properties (Lou et al., 2013; Youssef et al., 2025; S. Zhao et al., 2023). Chemical profiling and antiviral findings of previous studies reported various compounds such as cyclotrisiloxane, octamethyl, propanenitrile, pyrrolo[1,2-a]pyrazine-1,4-dione derivatives, coumarin derivatives, silane derivatives, and 1,2-cyclobutanedicarbonitrile, as major constituents of *A. alternata* extracts exhibiting anti-HIV-1 activity, thereby supporting their further investigation through molecular docking analyses (Nzimande, 2022). In this study, docking analysis highlighted the lead compounds coumarin, 3,4-dihydro-4,5,7-trimethyl-, and 2,3,2H-benzofuran-2-one, 3,3,4,6-tetramethyl-, as being responsible for the antiviral activity, given their competitive binding affinities towards the wild-type and mutant IN strains. These compounds exhibited the most favourable docking scores (–6.2 to –6.8 kcal/mol), suggesting that they may directly contribute to the *Alternaria alternata* PO4PR2 crude extract's ability to disrupt the integration stage of the HIV-1 life cycle. Notably, 2,3-2H- benzofuran-2-one showed the strongest predicted interaction, indicating that it may play a particularly important role in mediating the observed integrase-inhibitory effects. The present study provides new computational evidence identifying several *A. alternata* PO4PR2 metabolites with strong and mutation- tolerant binding affinities toward the HIV-1 integrase catalytic pocket.

Across the wild-type and four drug-resistant variants (G118R, N155H, Y143R, and R263K), coumarin,

3,4-dihydro-4,5,7-trimethyl- consistently showed the strongest binding in my docking analyses, with scores ranging from −5.9 to −6.2 kcal/mol. This compound displayed a stable interaction network centred around conserved catalytic-site residues, particularly TYR143 and ASN144, indicating its ability to maintain inhibitory contact even in the presence of drug-resistance mutations. 2,3-2H-benzofuran-2-one, 3,3,4,6- tetramethyl-, also demonstrated robust binding (−5.6 to −6.1 kcal/mol), forming persistent hydrogen bonds, π–π stacking, and Mg^{2+}-associated contacts across nearly all IN variants. A third molecule, pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methylpropyl), exhibited moderate but consistent binding (−5.0 to −5.6 kcal/mol), suggesting potential complementary inhibitory effects.

These findings highlight coumarin, 3,4-dihydro-4,5,7-trimethyl- and 2,3-2H-benzofuran-2-one, 3,3,4,6-tetramethyl- as the key contributors to the *Alternaria alternata* PO4PR2 crude extract's ability to interfere with the integration stage of the HIV-1 replication cycle. In previous studies, GCMS analysis of *Alternaria alternata* PO4PR2 crude extracts revealed the presence of similar metabolites with known antimicrobial, antioxidant, and anti-inflammatory properties, including Cyclotrisiloxane octamethyl; Propaninitrile; Pyrrolol[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-methyl propyl); Silane, diethylethoxy (2-ethoxyethyloxy); Coumarin, 3,4-dihydro-4,5,7-trimethyl-, 4,5,7-Trimethyl-2-chromanone and 1,2-Cyclobutanedicarbonitrile (Nzimande et al., 2022). These findings support those of a recent study, where molecular docking analysis also identified several of these metabolites, particularly 2,3-2H-benzofuran-2-one, 3,4-dihydro-4,5,7-trimethyl-coumarin, hexahydro-3-(2-methylpropyl)-pyrrolo[1,2-a]pyrazine-1,4-dione, N-acetyl-diazabicyclononanedione, and N-cycloheptyl-cyclopropanecarboxamide, as having strong and stable binding affinities to HIV-1 integrase, including the drug-resistant T66K and S230R variants (Naidu et al., 2025).

Docking analyses showed that the *A. alternata* PO4PR2 metabolites occupy the catalytic core domain and coordinate with the Mg^{2+} cofactors in the same orientation typically exploited by strand-transfer inhibitors, further supporting inhibition specifically at the strand-transfer step. Collectively, these results suggest that selected *Alternaria alternata* PO4PR2 secondary metabolites may serve as potential scaffolds for designing next-generation integrase inhibitors, targeting the strand transfer process of HIV-1 integration inhibition with improved resilience to resistance-associated mutations. The combined docking, enzymatic, and integration assays strongly indicate that *A. alternata* PO4PR2 metabolites function as strand-transfer inhibitors, retaining activity against multiple integrase drug- resistant variants. This study contributes to the growing body of evidence that endophytic fungi, such as *Alternaria alternata* PO4PR2*,* are valuable reservoirs of bioactive metabolites with the potential to address the challenge of HIV-1 drug resistance. By advancing the discovery and development of fungal-derived integrase inhibitors, this research lays the groundwork for innovative strategies in antiretroviral therapy that could complement or improve upon existing treatment options.

Conclusion

Alternaria alternata remains largely unexplored as a source of anti-HIV bioactive compounds; yet, the findings of this study provide compelling evidence that it harbours metabolites capable of inhibiting multiple stages of the HIV-1 life cycle. The identification of integrase-binding compounds, together with the demonstrated antiviral activity *in vitro*, highlights their significant potential for contributing to novel antiviral drug discovery and reinforces the value of investigating underexplored fungal species as reservoirs of new antiviral chemotypes.

The activity of the *Alternaria alternata* PO4PR2 extract against HIV-1 suggests that natural fungal metabolites can serve as promising scaffolds for the development of new antiretroviral drugs. Given the ongoing emergence of drug-resistant HIV-1 strains and the limitations of current therapies, these findings provide a compelling rationale for further investigation into the bioactive constituents of *Alternaria alternata* PO4PR2. Subsequent studies could focus on isolating, characterizing, and optimizing these compounds to enhance their potency and pharmacological properties, thereby potentially contributing to the development of next-generation HIV-1 therapeutics.

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Chapter 4: SYNTHESIS

This study successfully demonstrated the potent anti-HIV-1 activity of the *Alternaria alternata* PO4PR2 crude extract and provided mechanistic insight into its inhibitory effects at the critical stage of viral integration. The *A. alternata* crude extract exhibite d a favourable *in vitro* safety profile, maintaining high cell viability and a low cytotoxicity against TZM-Bl cell lines, resulting in a promising therapeutic index. The crude extract also displayed robust, dose-dependent antiviral activity against both the wild-type HIV-1 strain (pNL4.3) and key integrase strand transfer inhibitor drug-resistant variants, including G118R, N155H, R263K, and Y143R. All of the tested integrase drug-resistant mutants demonstrated higher susceptibility to inhibition by the *A. alternata* crude extract than the wild-type virus, revealing a consistent pattern of hypersensitivity that positions this extract as a compelling candidate for overcoming current integrase drug resistance challenges.

The HIV-1 integrase activity assay demonstrated that the crude extract inhibits integrase enzymatic function, with inhibitory effects comparable to those observed for the licensed integrase strand transfer inhibitors such as Raltegravir and Dolutegravir. The *Alu-gag* nested PCR further revealed a marked reduction in integrated proviral DNA in treated peripheral blood mononuclear cells. These findings strongly indicate that the *A. alternata* crude extract primarily interferes with the strand-transfer step of viral integration, aligning its antiviral profile with that of the clinically approved INSTIs.

Molecular docking analyses demonstrated that multiple secondary metabolites within the *A. alternata* PO4PR2 extract are predicted to bind within the catalytic pocket of the HIV-1 integrase–DNA–Mg^{2+} complex. Among these, coumarin, 3,4-dihydro-4,5,7-trimethyl-, and 2,3-2H-benzofuran-2-one, 3,3,4,6-tetramethyl-, exhibited the most favourable and consistent binding affinities across both wild-type and resistant integrase variants. These secondary metabolites maintained stable interaction networks with conserved catalytic residues, metal cofactors, and DNA components even in the presence of resistance-associated mutations. Therefore, this study successfully establishes *Alternaria alternata* PO4PR2 as a promising source of natural compounds with INSTI-like properties capable of retaining activity against key integrase drug-resistant HIV-1 variants. While further work is required to isolate, characterize, and validate the active constituents in vivo, these findings provide strong proof of concept for the development of fungal-derived integrase inhibitors and reinforce the value of endophytic fungi as a reservoir for novel antiretroviral drug discovery.

Limitations and future directions

The study utilised crude extracts of *Alternaria alternata* PO4PR2, which contain a mixture of metabolites. Because the individual active compounds were not isolated or quantified, it is not possible to determine which specific metabolites are responsible for the observed antiviral effects or whether synergistic interactions contributed to the activity. All experiments were conducted *in vitro*

using TZM-bl cells and biochemical integrase assays. Although these systems are well-validated, they cannot fully replicate the complexity of in vivo HIV infection, pharmacokinetics, metabolism, or immune responses. Further in vivo studies are required before therapeutic relevance can be inferred. Only four clinically relevant integrase drug-resistant mutants (G118R, N155H, R263K, and Y143R) and a single laboratory-adapted subtype B reference strain (pNL4.3) were tested. Since HIV-1 subtype C predominates in Southern Africa, testing against a wider range of subtypes and naturally occurring resistant variants would improve generalisability. Although integrase inhibition was demonstrated, the exact molecular mechanism, whether allosteric disruption, catalytic site binding, or interference with host-factor interactions, was not experimentally validated. While cytotoxicity was assessed, other critical parameters such as metabolic stability, solubility, membrane permeability, and potential off-target effects were not evaluated. These factors are essential for assessing drug-likeness and clinical translation.

Future work should prioritise isolating and characterising the individual metabolites within the *Alternaria alternata* PO4PR2 extract to identify the specific compounds responsible for the observed antiviral activity and to determine whether synergistic interactions contribute to integrase inhibition. Additionally, expanding beyond *in vitro* assays to include in vivo or ex vivo models will be crucial for evaluating the physiological relevance, pharmacokinetics, and immunological effects of these metabolites. Broader screening against a wider range of HIV-1 subtypes and clinically relevant integrase-resistant variants is also needed to establish the breadth of antiviral activity and the potential applicability of these compounds across diverse viral backgrounds. Furthermore, comprehensive pharmacological profiling, including assessments of metabolic stability, solubility, permeability, and off-target effects, will be critical for determining whether these fungal metabolites have realistic potential as lead scaffolds for future integrase inhibitor development.

APPENDICES

Ethics

UNIVERSITY OF KWAZULU-NATAL
INYUVESI YAKWAZULU-NATALI

25 August 2025

Miss Ndzalo Mashabele (223023260)
School of Laboratory Medicine & Medical Science
Medical School

Dear Miss Mashabele,

Protocol reference number: BREC/00007158/2024
Project title: The antiviral effects of endophytic fungi secondary metabolites on integration stage and integrase drug-resistant variants
Degree: MMedSci

RECERTIFICATION APPLICATION APPROVAL NOTICE

Approved:	**03 July 2025**
Expiration of Ethical Approval:	**02 July 2026**

I wish to advise you that your application for recertification for the above study has been **noted and approved** by a subcommittee of the Biomedical Research Ethics Committee (BREC). 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 September 2025.

Yours sincerely

[redacted]

Ms A Marimuthu
(for) Prof S Singh
Chair: Biomedical Research Ethics Committee

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