Anti-HIV and Immunomodulatory properties of the fractionated crude extracts isolated from *Alternaria alternata*

By

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PREFACE

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

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DEDICATION

This thesis is dedicated to God, my parent and my family for their endless love, encouragement, and support throughout my master's degree.

"What is impossible with men is possible with God".
Luke18:27

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LIST OF ABBREVIATIONS

AIDS	Acquired Immunodeficiency syndrome
ARV	Antiretroviral
BBB	Blood-brain barrier
BREC	Biomedical Research Ethics Administration
cART	Combined antiretroviral therapy
CCR5	C-C chemokine receptor type 5
CC_{50}	Cell cytotoxicity at 50%
CCM	Complete culture media
$CD3^+$ T cell	Cluster of Differentiation 3
$CD4^+$ T cell	Cluster of Differentiation 4
$CD8^+$ T cell	Cluster of Differentiation 8
$CD38^+$ T cell	Cluster of Differentiation 38
$CD56^+$ T cell	Cluster of Differentiation 56
CO_2	Carbon dioxide
DC	Dendritic cells
DHHS	Department of Health and Human Services
DMEM	Dulbecco's modified eagle's medium
DMSO	Dimethyl sulfoxide
CXCR4	C-X-C Chemokine Receptor 4
DNA	Deoxyribonucleic acid
ELISA	Enzyme-linked immunosorbent assay
FBS	Fetal Bovine Serum
FDA	Food and drug administrator
FRESH	Females rising through education, support, and health.
GC-MS	Gas chromatograph- mass spectrometer
GFAHP	Group fuzzy analytic hierarchy process
GXT	Gliotoxin
HAART	Highly active retroviral therapy
HLA-DR	Human leukocyte antigen- DR isotype
HEPES	(4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid)
HIV	Human Immunodeficiency virus
HLB	Hydrophilic-lipophilic balance
HPLC	High-performance liquid chromatography
IC_{50}	Inhibitory concentration at 50%
IL-10	Interleukin-10
INSTI	Integrase strand transfer inhibitors
IT	Integrase inhibitor
LC-MS	Liquid chromatograph- mass spectrometer
LRT	Long terminal repeat
MAX	Mixed mode, strong anion-eXchange
MCX	Mixed mode, strong cation-eXchange
MTT	(3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide)
NMR	Nuclear magnetic resonance
NNRTIs	Non-nucleoside reverse transcriptase inhibitor
NRTIs	Nucleoside reverse transcriptase inhibitor.
NS	Non/ not significant
PBMC	Human peripheral blood mononuclear cells

PD-1	Programmed death 1
PI	Protease inhibitor
RLU	Relative luminescence units
RNA	Ribonucleic acid
RT	Reverse transcriptase
SA	South Africa
SI	Selective index
SPE	Solid phase extraction
SV40	Simian virus 40
$TCID_{50}$	50% Tissue culture infective doses
TGF-β	Transforming growth factor-beta
TILs	Tumour-infiltrating lymphocytes
TOA	Time of addition
UKZN	University of KwaZulu Natal
UNAIDS	Joint United Nations Programme on HIV/AIDS
WHO	World Health Organisation

ABSTRACT

Background

The Human Immunodeficiency Virus (HIV) continues to be a major global health problem despite intense efforts in international and local initiatives to address the pandemic. Antiretroviral drugs have reduced the morbidity of people living with AIDS to clinically manageable chronic diseases. However, the development of HIV drug resistance, viral reservoirs, and drug toxicity hinders the HIV cure. Therefore, there is an urgent need to develop novel anti-HIV drug candidates with improved resistance profiles and reduced drug toxicity or that target new sites to be able to eradicate the virus. Here, we investigate the fractionated crude extracts from endophytic fungi *A. alternata* as a potential anti-HIV and immunomodulatory agent.

Material and methods

Alternaria alternata PO_4PR_2 was fractionated using Solid Phase Extraction, proving MAX (Mixed mode, strong Anion-eXchange), MCX (Mixed-mode, strong Cation-eXchange) and HLB (Hydrophilic-Lipophilic Balance) columns. Then, MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay was used to determine the cell viability and cytotoxicity in the TZM-bl cells treated with fractionated fractions and crude extracts of *A. alternata*, followed by the generation of the infectious virus by transfecting 293T cells with NL4.3 plasmid DNA. Then, the antiviral activity of the fractionated crude extract from *A. alternata* PO_4PR_2 was evaluated using a Luciferase-based antiviral assay on the TZM-bl cell line. To determine the stage of the HIV-1 replication cycle with which the fractionated crude extracts from *A. alternata* PO_4PR_2 interfere, a time-of-addition experiment was carried out using ELISA p24 time-based assay (to measure p24 titer) and Luciferase time-bases assay (to determine the percentage inhibition). Then, to evaluate the immunomodulatory effect of the fractionated crude extracts from *A. alternata* on CD4+ T cell, flow cytometry was performed by infecting the PBMC and staining with fluorochrome labelled monoclonal antibodies for activation (CD38 and HLA-DR) and exhaustion (PD-1).

Results

The cell viability for crude extract and the fractionated fractions was above 80%, and the cell cytotoxicity at 50% (CC_{50}) was 285 µg/mL for the crude extract and ranging between 35.31-62,31 µg/mL for the fractionated crude extracts. The fraction fractionated with 5% methanol obtained through MCX, which involves basic compounds and weak and strong cation exchangers, demonstrated higher anti-HIV inhibition than other columns when eluted without methanol. It exhibited an IC_{50} of 0.03262 µg/mL and 86% inhibition, surpassing HLB (with 32-73% inhibition and an IC_{50} range of 0.1474 to 5.146 µg/mL) and MAX (with 40-62% inhibition and an IC_{50} range of 0.3313 to 5.240 µg/mL). Time-of-addition results indicate that the crude extract and the MCX fraction inhibit the virus at various stages, including viral binding (62%, 68%), reverse transcription (75%, 98%), integration (82%, 98%), and protease (77%, 59%). The fractionated (5%; MCX, HLB and MAX) and *A. alternata* crude extracts showed low $CD4^+$ T cells (CD38 + HLA-DR+) T cell activation compared to treating with AZT and no activation in $CD8^+$ T cells. The crude extracts and the 5% HLB (Hydrophilic-Lipophilic Balance) showed low PD-1+CD4+ T cell exhaustion, and HLB and MAX showed low PD-1+CD8+ T cell exhaustion.

Conclusion

Therefore, fractionated crude extract from *A. alternata* fungal endophytes (MCX fraction) has strong inhibition potential during reverse transcription and integration. Furthermore, the results imply that the crude extracts, MAX and HLB fractions may have compounds that have immunomodulatory activities, which modulate the immune response by targeting pathways or molecules involved in T cell exhaustion, ultimately enhancing the function of either $CD4^+$ T cell and $CD8^+$ T cells. Endophytic fungi can be the major source of new anti-HIV agents with immunomodulatory effects. Liquid chromatography-mass/mass chromatography can further identify the bioactive compounds from these fractionated crude extracts from *A. alternata*.

CHAPTER 1: INTRODUCTION

1.1 Background

The Human Immunodeficiency Virus (HIV) continues to be a major global health problem despite intense efforts in international and local initiatives to address the pandemic (Govender *et al.*, 2021). Since the beginning of the epidemic, 1.3 million (1.0 –1.7 million) people have been infected with HIV, and about 630,000 (480,000- 880,000) people have died of HIV (UNAIDS, 2023). Globally, 39.0 million (33.1 – 45.7 million) people were living with HIV at the end of 2022 (UNAIDS, 2023). An estimated 37.5 (31.1 – 45.7) of adults aged 15-49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions (UNAIDS, 2023). HIV/AIDS is a virus that affects both men and women equally and even affects children under the age of 18. A total of 15 million children are estimated to be HIV-1 positive by UNAIDS in 2023, and women and children make up 53% of those living with the virus. The introduction of antiretroviral therapy has reduced the burden of HIV in people living with HIV/AIDS by transforming it into a clinically manageable chronic illness. Despite the success of antiretroviral drugs, they have fallen short in eradicating the virus from the viral reservoirs. HIV-1 viral reservoirs are defined as cell types or anatomical sites in association with which replication-competent forms of the virus persist with more stable kinetic properties than the main pool of actively replicating viruses (Blankson *et al*., 2002; Van Lint *et al*., 2013). In addition, the development of HIV drug resistance in the currently used drugs has hindered the successful story of ARVs. Moreover, antiretroviral drugs currently in use to treat an HIV-1 infection are chemically synthesised and lead to the development of drug resistance and cause severe toxicities such as mitochondrial toxicity, which may manifest as peripheral neuropathy, myopathy, lipoatrophy, or hepatic steatosis with lactic acidosis (Wellensiek *et al.*, 2013; Margolis *et al*., 2014). Therefore, there is an urgent need to develop novel anti-HIV drug candidates with improved resistance profiles or that target new sites to be able to eradicate the virus in the reservoirs and reduce the emergence of HIV-1 drug resistance mutations (Thomas *et al*., 2020).

On the other hand, bioactive compounds isolated from natural biological sources offer a vast and unexplored diversity of chemical structures unmatched by even the most extensive combinatorial databases (Strobel and Daisy, 2003; Linnakoski *et al.*, 2018). Natural products have served as traditional medicine for thousands of years and still provide the most affordable treatment for diseases in many developing countries (Amzat and Razum, 2018). Bioactive compounds are naturally derived metabolites and by-products from microorganisms, plants, or animals (Baker *et al*., 2000; Linnakoski *et al*., 2018). Over the past 25 years, bioactive compounds from many traditional medicinal plants

have been screened for their antiviral activity by various research groups in Asia, the Far East, Europe, and America (Jassim and Naji, 2003; Linnakoski *et al*., 2018). Medicinal plants are considered one of the major reservoirs of endophytes with novel bioactive metabolites (Bacon and White, 2000). The importance of novel drug discoveries has been bioactive molecules of fungal origin. Fungi growing in unique environments, such as endophytic and marine fungi, are being constantly explored for their antibacterial, antiviral, and antifungal potential (Linnakoski *et al*., 2018).

Endophytic fungi are a microbial community that resides inside all plants without showing symptoms, promising to produce various bioactive molecules and novel metabolites that have applications in medicine, agriculture, and industrial setups (Meena *et al*., 2019). These fungi produce biologically active secondary metabolites, which are natural products beneficial to the host (Wellensiek *et al*., 2013). They synthesise various biologically active compounds with diverse biological activities, such as insecticidal, antioxidant, cytotoxic, antibacterial, antiviral, antifungal and antimalarial compounds (Meena *et al*., 2019). Some secondary metabolites produced by endophytic fungi are phenols, alkaloids, polyketides, quinones, steroids, enzymes, and peptides, which possess high therapeutic value.

Previous studies showed that endophytic fungi from desert plants serve as a promising source in identifying potent inhibitors in the replication of HIV-1 (Wellensiek *et al.*, 2013; Bashyal *et al.*, 2014). *Alternaria tenuissima* isolated from the Sonoran desert plant Queise emoryi was said to produce an antiviral compound called Altertoxins, which was effective against the HIV-1_{LAV} strain virus (Bashyal *et al*., 2014). Furthermore, Melappa *et al*. (2015) also revealed that partially purified coumarins isolated from *Alternaria* sp. showed high HIV-1 inhibition on three viral enzymes: integrase enzyme (98%), reverse transcriptase enzyme (82.81%) and protease activity (78%). Recently, Nzimande *et al.* (2022) isolated the endophytic fungi from *Hypoxis* spp and further isolated the secondary metabolites. This study showed that *A. alternata's* secondary metabolites have anti-HIV-1 activity and are not cytotoxic to TZM-bl cells (Nzimande *et al.*, 2022). These studies demonstrate that metabolites from endophytic fungi from plants can serve as a viable source for identifying potent inhibitors of HIV-1 replication. Some natural products from endophytic fungi have been found to have an immunomodulatory effect (Ali Reza *et al*., 2021).

Immunomodulators influence the immune system's role and efficiency through various exogenous and endogenous factors that result in either immunosuppression or immunostimulant (Ali Reza *et al*., 2021). Among different immunomodulators, plant-based secondary metabolites have a high potential

for immune defence and cellular immune response (Ali Reza *et al*., 2021). The immunomodulatory effects of plant extracts and their bioactive metabolites have been suggested due to their diverse mechanisms of modulation of the complex immune system and their multifarious molecular targets (Amirghofran *et al.*, 2000; Ali Reza *et al*., 2021). A previous study by Abood *et al*. (2014) indicates that secondary metabolites from *Tinospora crispa* have immunomodulatory effects through the stimulation of INF-γ, IL-6, and IL-8 expressions. Where the LC-MS phytochemical analysis showed that the *T. crispa* fraction has cordioside, quercetin, eicosenoic acid (paullinic acid), and boldine, which may be responsible for the immunostimulatory effect of *T. crispa* (Abood *et al*., 2014), therefore, this study aims to continue the previous study by screening the anti-HIV activity of the fractionated fractions and crude extracts from *A. alternata* (isolated from *Hypoxis* plant), and further evaluate the immunomodulatory properties of the fractionated crude extracts and establish the mechanism of action.

1.2 Problem Statement

Human Immunodeficiency Virus remains a major worldwide health problem, accounting for more than 60% of deaths. The development of HIV-1 drug resistance and its high transmission pose a problem in managing and treatment of HIV. Even though current HIV-1 therapy has reduced the death of people infected with HIV, there are current challenges, such as the failure of the current anti-HIV regimen to eradicate HIV in reservoirs and to escape mutant HIV strains for successful treatment. Urgent intervention requires the development of new drugs that will escape resistant HIV strains and even eradicate HIV in reservoirs. Current treatments include a combination of antiretrovirals commonly called highly active retroviral therapy (HAART) (Günthard *et al*., 2014). Antiretroviral drugs are unable to eradicate HIV in the latently $CD4^+$ T infected cells (Kim e*t al*., 2022). Even if durable viral suppression is achieved, many individuals fail to restore optimal immune function after several years of ART (Deeks *et al*., 2015). Moreover, the current treatment presents undesirable side effects, and ART has potential short-term and long-term toxicities and must be administered for life.

More HIV research is required to develop new HIV-1 drugs to cure HIV that are less toxic with few side effects to target the HIV reservoirs. The microbial species produce diverse compounds with varied properties, including anti-HIV activities (Pham *et al*., 2019). These compounds have been the backbone of molecular medicine until the adoption of synthetic chemistry. Now, synthetic derivatives cannot meet the growing need for new, effective drugs to escape resistance challenges. Advances in technologies have allowed for revisiting these structurally diverse microbial structures. The focus is

on niches and species not explored in the previous phenotypic screens. Several studies have demonstrated the potential of endophytic fungi to produce novel secondary metabolites with the potential to be developed as a treatment for various diseases such as cancer (Seca and Pinto, 2018), bacterial (Uche-Okereafor *et al*., 2019), viral and fungal infections (Wellensiek *et al*., 2013). Some examples of the bioactive compound isolated from endophytic fungi with anti-HIV properties include coumarins (Melappa et al., 2015), alterotoxin (Bashyal *et al*., 2014), and curcumin (Thimmulappa *et al*., 2021).

1.3 Aim

This study aims to investigate the fractionated crude extracts from *A. alternata* (PO_4PR_2) as potential anti-HIV and/ or immunomodulatory agents against HIV-1.

1.4 Specific Objectives

- To identify and evaluate the cytotoxicity effects and anti-HIV properties of the fractionated crude extracts from *A. alternata* isolated from the *Hypoxis* plant.
- To establish the mode of action of the fractionated crude extract(s) by first conducting time of addition assays to determine its inhibitory mechanism in the HIV-1 life cycle.
- To evaluate the effect of the fractionated crude extracts from *A. alternata* on $CD4^+$ and CD8+ T cell activation and exhaustion.

1.5 Research question

What is the mechanism by which *A. alternata* fractionated crude extracts from the *Hypoxis* plant ameliorate anti-HIV activity?

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CHAPTER 2: LITERATURE REVIEW

2.1 Human Immunodeficiency Virus (HIV)

The human immunodeficiency virus (HIV) was first discovered by French scientists in 1981 (Barré-Sinoussi *et al*., 1983; German Advisory Committee Blood, 2016). Epidemiologic and phylogenetic analyses currently available suggest that HIV was introduced into the human population around 1920 to 1940 (Huet *et al*., 1990; Castro-Nallar *et al*., 2012). HIV is grouped in the genus Lentivirus within the family of *Retroviridae*, subfamily *Orthoretrovirinae* (Chackerian *et al*., 1997; McCarthy *et al*., 2015). Based on genetic characteristics and differences in the viral antigens, HIV is classified into types 1 and 2 (HIV-1, HIV-2) (Fukasawa *et al*., 1988; Geretti, 2006). HIV-type 1 is further grouped into M, O, and N. Group M viruses radiated from Central Africa, began their expansion in humans approximately 70 years ago, and are responsible for the pandemic (Geretti, 2006; Métifiot *et al*., 2013). Phylogenetic analyses identify nine subtypes within group M, including A–D, F–H, J, and K, which are genetically equidistant, diverging by 20–30% in the *env* gene and 15–22% in the *gag* gene (Geretti, 2006). Subtypes A and F include the sub-subtypes A1, A2, and A3 (Meloni *et al.*, 2004; Geretti, 2006), F1 and F2. Subtype D may be more appropriately classified as sub-subtype B2 (Geretti, 2006). At least 21 inter-subtype circulating recombinant forms (CRFs) have been identified. Additional unique recombinant forms have been recognised, and several have been characterised by whole genome sequencing, the gold standard for subtype assignment (Geretti, 2006). HIV-1 might have evolved from non-human primate immunodeficiency viruses from Central African chimpanzees (SIVcpz) and HIV-2 from West African sooty mangabeys (SIVsm) (Gao *et al.,* 1999).

2.1.1 Epidemiology

The HIV infection pandemic is generalised across the adult population in many regions of sub-Saharan Africa, with much of the burden of the disease placed on women (Marsh *et al*., 2019). Since the beginning of the epidemic, 1.3 million (1.0- 1.7 million) people have been infected with HIV, and about 630,000 (480,000- 880,000) people have died of HIV (UNAIDS, 2023). Globally, 39.0 million (33.1 – 45.7 million) people were living with HIV at the end of 2022 (Figure 2.1) (UNAIDS, 2023). An estimated 37.5 (31.1 – 45.7) of adults aged 15-49 years worldwide are living with HIV, although the burden of the epidemic continues to vary considerably between countries and regions (UNAIDS, 2023). The World Health Organisation (WHO) African region remains most severely affected, with nearly 1 in every 25 adults (3.4%) living with HIV and accounting for more than two-thirds of the people living with HIV worldwide (UNAIDS, 2023). The African region is most heavily affected by

HIV, with 25.6 million (21.6 million–30.0 million) of all people living with HIV in the world (UNAIDS, 2023). With an estimated 660 000 (480 000 – 920 000) infections and 380 000 (300 000-540 000) deaths (UNAIDS, 2023).

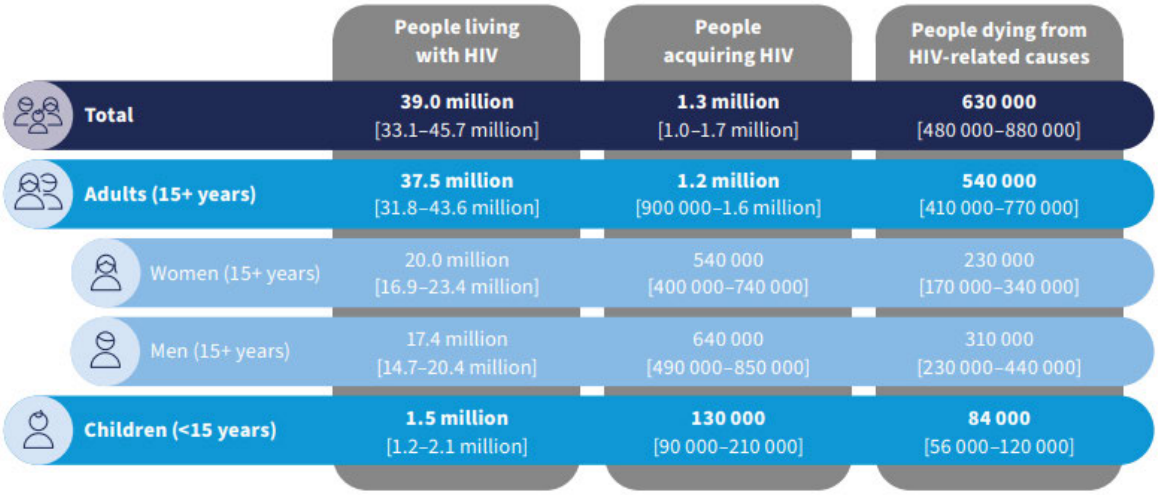

Figure 2. 1: The number of new HIV infections in 2023 (for both sexes and all ages) (UNAIDS, 2023).

2.1.2 HIV Genome

The HIV-1 genome comprises two identical single-stranded RNA molecules in the virus particle's core. The genome of the HIV provirus (Figure 2.2), also known as proviral DNA, is made by the reverse transcription of the viral RNA genome into DNA, degradation of the RNA, and integration of the double-stranded HIV DNA into the human genome (Salminen *et al.*, 1996; Lole *et al*., 1999; Acosta *et al*., 2014). The HIV genome contains nine genes that encode fifteen viral proteins. The DNA genome is flanked at both ends by LTR (long terminal repeat) sequences (Figure 2.2). The 5′ LTR region codes for the promotor for transcription of the viral genes. In the direction five ′ to 3′ the reading frame of the *gag* gene follows, encoding the proteins of the outer core membrane (MA, p17), the capsid protein (CA, p24), the nucleocapsid (NC, p7), and a smaller, nucleic acid-stabilizing protein (Grandbastien, 2008).

Three major genes, *gag*, *pol*, and *env*, encode for structural proteins (Matrix, Capsid, Nucleocapsid, p6), enzymes (Protease, Reverse transcriptase (RT), Integrase) and envelope proteins (gp120, gp41), respectively (Li *et al.*, 2015). The remaining genes code for regulatory (Tat, Rev) and accessory proteins (Vif, Vpr, Vpu/Vpx, Nef) (Frankel and Young, 1998; Acosta *et al*., 2014). These viral proteins can

exhibit multiple functions and interact with various human proteins during the viral life cycle (Engelman and Cherepanov, 2008; Acosta *et al*., 2014).

The trans-activator of transcription (Tat) functions is to enhance RNA polymerase II processivity of transcription from the HIV LTR (Laspia *et al*., 1989; Bohan *et al*., 1992; Casey *et al*., 2010) and viral envelope (rev) expedites RNA export from the cell nucleus and thereby decreases the extent to which viral RNA is spliced (Fischer *et al*., 1994; Casey *et al*., 2010). Negative factor (Nef), another protein expressed early after infection, down-modulates CD4 to control superinfection (Anderson e*t al*., 1993; Benson *et al*., 1993; Inoue *et al*., 1993; Mariani and Skowronski, 1993; Casey *et al*., 2010). Other functions have, however, also been ascribed to this protein and contribute to HIV biology (Roeth and Collins, 2006; Thoulouze *et al*., 2006; Casey *et al*., 2010). Viral protein U (vpu) similarly downmodulates CD4 expression at the cell surface (Willey *et al*., 1992) and significantly counteracts the cellular protein tethering, which retains newly produced virions at the cell surface (Neil *et al*., 2008; Van Damme *et al.*, 2008). Virion infectivity factor (Vif) regulates viral infectivity by preventing virion incorporation of APOBEC3G and other members of the family of cytidine deaminases (Conticello *et al*., 2003; Marin *et al*., 2003; Zheng *et al*., 2004; Andrew and Strebel, 2014).

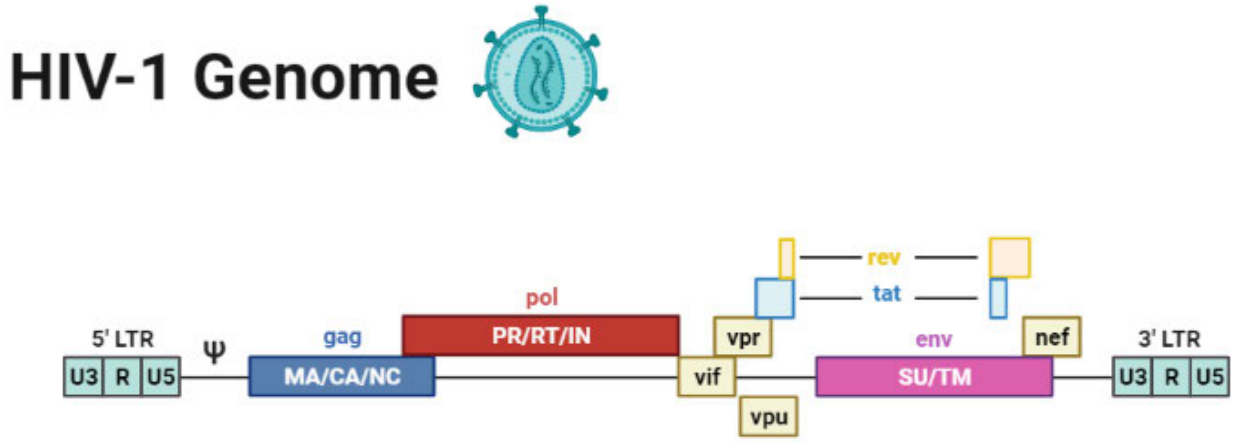

Figure 2. 2: Structure and organisation of the HIV-1 genome, shown are the reading frames of the genes coding for structure and regulatory proteins (Prepared by author,) (BioRender, Science suite Inc. Toronto, Canada) adapted from (Donovan and Weisstein, 2003).

2.2 Life cycle of HIV

The HIV-1 life cycle is complex and can roughly be divided into an early and a late phase of replication. The early phase begins with the virion attachment at the cell surface and ends with the integration of the proviral DNA into the host genome (Figure 2.3) (Engelman and Cherepanov, 2008). The late replication phase starts with the initiation of pro-viral transcription and ends with the release of fully infectious progeny virions. In highly activated $CD4^+$ T cells, the HIV life cycle lasts one to two days. It is associated with the programmed death of both virally infected cells and uninfected bystander cells. The viral life cycle shows some of the challenges related to HIV infection (Kirchhoff, 2013).

The HIV enters its target $CD4^+$ T cell via CC-chemokine receptor 5 (CCR5) or CXC-chemokine receptor 4 (CXCR4) through interaction with envelope (Env) glycoprotein (attachment and fission) (Ryu *et al*., 1990; Wang *et al*., 1990; Wu *et al*., 1997; Deeks *et al*., 2015). After fusion and uncoating, the viral RNA is reverse-transcribed into DNA by reverse transcriptase (Temin and Mizutani, 1970) during reverse transcription (Deeks *et al*., 2015). The pre-integration complex is then imported into the nucleus, and the viral DNA is then integrated into the host genome by the enzyme called integrase (Elliott and Kutluay, 2020) during integration. Mediated by host enzymes, HIV DNA is transcribed to viral mRNAs (transcription). These mRNAs are then exported to the cytoplasm, where translation occurs (translation) to make viral proteins and eventually mature virions. Then, HIV protease is responsible for processing Gag and Gag-Pol polyproteins during virion maturation (Gulnik *et al*., 2000).

Each step, HIV entry, reverse transcription, integration, and protein maturation in the HIV life cycle is a potential target for antiretroviral drugs (Engelman and Cherepanov, 2012b; Deeks et al., 2015). Integrase strand transfer inhibitors (INSTI), non-nucleoside reverse transcriptase inhibitors (NNRTI), nucleoside reverse transcriptase inhibitors (NRTI), and Protease inhibitors (PI) are classes of ART that are licensed by the FDA (Flexner, 2007). Nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) target the reverse transcription step that converts the viral genomic RNA into linear double-stranded DNA. In contrast, protease inhibitors (PIs) inhibit protease enzyme activity, critical for the maturation of viral particles that bud out from infected cells (Engelman and Cherepanov, 2012b). Two different inhibitors can block the entry of the virus into new target cells by thwarting either the interaction between the viral envelope glycoprotein gp120 and the CCR5 co-receptor (maraviroc) or the formation of the gp41 six-helix bundle that drives the fusion between the viral and cellular membranes (Fuzeon) (Engelman and Cherepanov, 2012b). The sole integrase strand transfer inhibitor (INSTI), raltegravir, blocks integrase's strand

transfer activity that is required to insert viral DNA into a host cell chromosome, an essential step in the viral lifecycle that is catalysed by the virally encoded IN protein within a nucleoprotein assembly called an intasome (Jóźwik *et al*., 2020). Highly active antiretroviral therapy, or HAART, routinely prescribes an NRTI, NNRTI, and PI as a single pill or in various combinations (Engelman and Cherepanov, 2012b). The use of a combinatorial strategy in drug treatment markedly reduces the likelihood of the emergence and proliferation of drug-resistant HIV-1 strains, a common occurrence when employing single-drug therapy (Engelman and Cherepanov, 2012b).

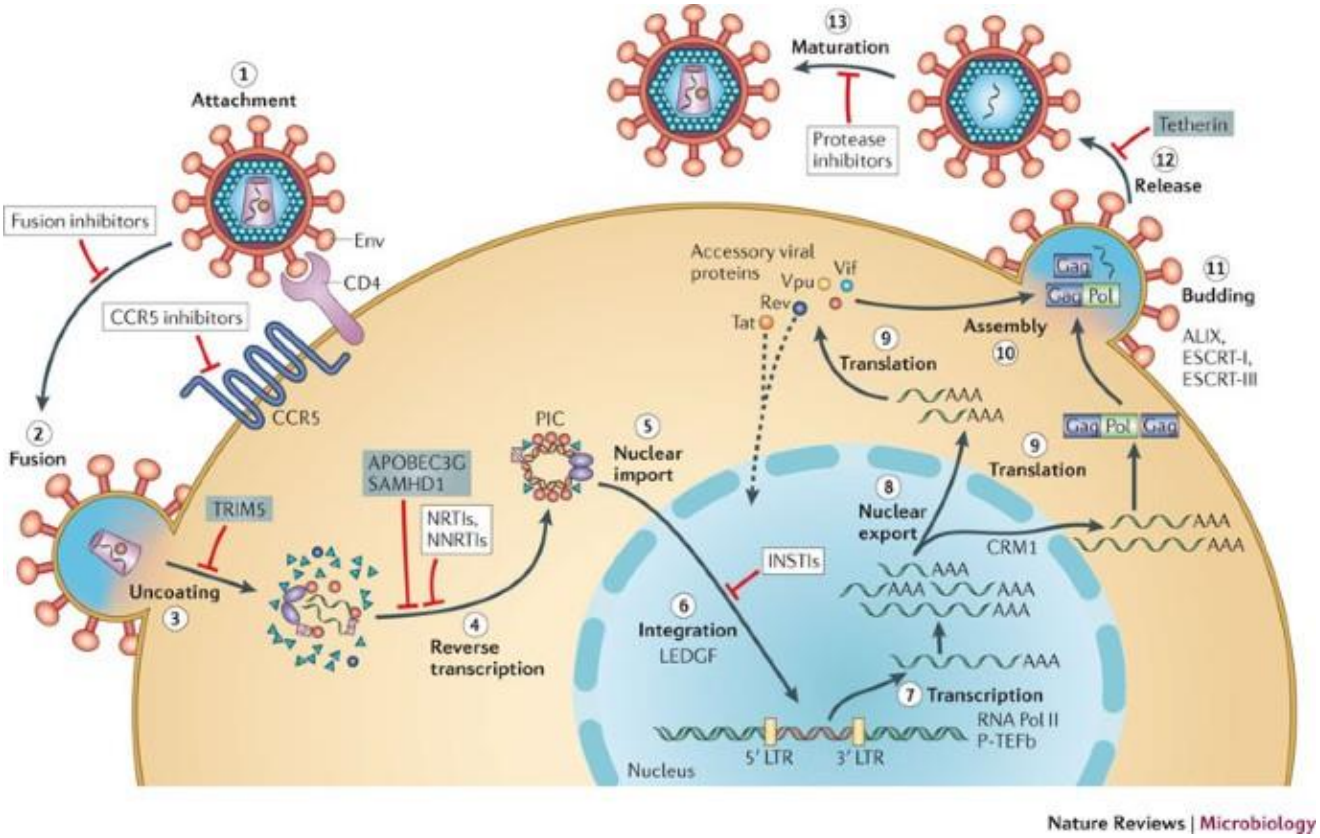

Figure 2. 3: The infographic illustrating the HIV replication cycle. The diagram shows the replication cycle steps illustrated by arrows starting with viral attachment (1), fusion (2), uncoating (3), RNA reverse transcription (4), nuclear import (5), DNA integration (6), mRNA transcription (7), nuclear export (8), translation (9), and lastly, cleavage and assembly of viral proteins (10), budding (11), release (12) and viral maturation (13) (Engelman and Cherepanov, 2012a).

2.3 Current HIV antiretroviral treatment

The introduction of anti-retroviral agents and highly active antiretroviral therapy (HAART) in 1996 significantly reduced the morbidity and mortality of HIV/AIDS (Salehi *et al*., 2018; Nastri *et al*., 2020). Antiretroviral drugs suppress HIV-1 replication to undetectable levels, resulting in HIV-1 being a manageable chronic infection (Arts and Hazuda, 2012). Nucleoside reverse transcriptase

inhibitor (NRTI) zidovudine was first approved by the Food and Drug Administration (FDA) in 1987 (Fischl *et al*., 1987; Kemnic and Gulick, 2022). Zidovudine functions as a viral DNA chain terminator by being incorporated into newly made viral DNA instead of thymidine (Sperling, 1998; Edwards *et al*., 2022). This inhibits the ability of HIV-1 reverse transcriptase to make viral DNA from the RNA template, which interferes with the HIV-1 life cycle (Edwards *et al*., 2022). In the early 1990s, the first HIV-specific antiviral drugs were given as monotherapy and dual therapy (such as dolutegravir plus rilpivirine and dolutegravir plus lamivudine) (Merigan, 1991; Vella *et al*., 2012; Baril *et al*., 2016; Soriano *et al*., 2017). Following that, HIV-1 treatment was revolutionised in the mid-1990s by the development of inhibitors of the reverse transcriptase and protease, two of three essential enzymes of HIV-1, and the introduction of combination antiretroviral therapy (cART) (Autran *et al*., 1997; Komanduri *et al*., 1998; Lederman *et al*., 1998; Maeda *et al*., 2019). The best strategy for combination drug therapy was to combine agents that do not share *In vitro* cross-resistance and have different mechanisms of action and dose-limiting toxicities (Merigan, 1991). Furthermore, these new drug regimens that combined these agents strongly suppressed viral replication and reduced plasma HIV-1 viral load, resulting in significant reconstitution of the immune system and durability of therapy to enhance the treatment's overall efficacy and durability (Maeda *et al*., 2019).

These drugs include nucleoside/nucleotide reverse transcriptase inhibitors (NRTIs) (Fischl *et al*., 1987), NRTI fixed-dose combinations, integrase strand transfer inhibitors (INSTI) (Métifiot *et al*., 2013; Zhao *et al.*, 2022), non-nucleoside reverse transcriptase inhibitors (NNRTIs) (De Clercq, 1998), protease inhibitors (PIs) (Oroszlan and Luftig, 1990; Konvalinka *et al*., 2015), Fusion entry inhibitor (Williams, 2003), and entry inhibitors (Kuritzkes *et al*., 2008; Günthard *et al*., 2014; Namasivayam *et al*., 2019). Highly Active Antiretroviral Therapy (HAART) targets different stages in the HIV-1 lifecycle, which include a viral attachment, reverse transcription, integration, transcription, protease cleavage, and maturation (Brechtl et al., 2001; Reeves and Piefer, 2005a).

Antiretroviral therapy is recommended for all patients who tested positive for HIV by the Department of Health and Human Services (DHHS) and the World Health Organization (WHO) (Kim *et al*., 2022). They have introduced the policy of “test and treat.” Although effective antiretroviral therapy (ART) can reduce HIV replication, viremia rebounds within weeks of ART interruption (Davey Jr *et al*., 1999; Li *et al*., 2022). This viral rebound might be fuelled by reservoirs that are established early in HIV infection and influenced by multiple virological and immunological factors (Li *et al*., 2022). HIV-1 viral reservoirs are defined as cell types or anatomical sites in association with which replication-competent forms of the virus persist with more stable kinetic properties than the main pool

of actively replicating viruses (Blankson *et al*., 2002; Van Lint *et al*., 2013). Although there are multiple reservoirs, the persistence of replication-competent HIV in resting memory $CD4^+$ T cells represents a significant obstacle to HIV eradication (Wei and Shao, 2021; Li *et al*., 2022). The HIV reservoirs can also form in macrophages, dendritic cells (DCs), and astrocytes (Sengupta and Siliciano, 2018; Zhao and Deng, 2020; Li *et al*., 2022). The elimination of these latently infected cells is critical to curing HIV infection (Li *et al*., 2022). The challenge with antiretroviral drugs is the inability to eradicate the virus from the viral reservoirs. Hence, there is an urgent need to find drugs that can activate HIV-1 latency and possess antiviral activity.

A typical initial HIV regimen includes three HIV medications from a minimum of two drug classes (Table 2.1). Although this treatment is not curative, it can provide longer lives for patients and reduce HIV transmission.

Table 2. 1: HIV drug classes approved by the FDA and their mechanism of action (Lipsky, 1996; Piscitelli and Gallicano, 2001; Cunha *et al*., 2021).

Drug class	Mechanism of Action	Approved drug
Nucleoside reverse transcriptase inhibitors.	Inhibit reverse transcriptase via chain termination.	Abacavir (ABC) Didanosine (DDI) Emtricitabine (FTC) Lamivudine (3TC) Stavudine (d4T) Tenofovir (EFV) Zidovudine (ZDV/ AZT)
Non-nucleoside reverse transcriptase inhibitors	Inhibit reverse transcriptase via direct binding and inactivation	Delavirdine (DLV) Efavirenz (EFV) Etravirine (ETR) Nevirapine (NVP) Rilpivirine (RPV)
Protease inhibitors	Inhibit HIV protease, an enzyme necessary for catalytic protein cleavage for viral replication.	Atazanavir (ATV) Darunavir (DRV) Fosamprenavir (FPV) Indinavir (IDV)

		Lopinavir (LPV) Nelfinavir (NFV) Ritonavir (RTV) Saquinavir (SQV) Tipranavir (TPV)
Fusion inhibitors	Block entrance of HIV into CD4 cells	Enfuvirtide (ENF)
Chemokine receptor antagonists (CCR5)	Block CCR5 receptors on CD4 cell surfaces, preventing HIV entrance	Maraviroc (MVC)
Integrase inhibitors	Inhibit integrase is an enzyme necessary to integrate viral DNA into host cells.	Dolutegravir (DTG) Elvitegravir (EVG) Raltegravir (RAL)

The ART can prevent new cells from becoming infected, but these drugs cannot eliminate infection once the viral DNA is successfully integrated into its target cell (Buzon *et al*., 2010). Moreover, there are several other drawbacks to the currently used ARV therapy, which include drug resistance, long-term drug therapy toxicity, drug-drug interactions, and lack of access to tissues and reservoirs (Desai *et al*., 2012).

Drug resistance is one of the other significant problems with anti-HIV drug therapy. HIV replication is rapid and error-prone, generating at least one mutation per genome (Alizon and Fraser, 2013; Andrews and Rowland-Jones, 2017). These genetic mutations enable the virus to develop resistance to anti-HIV drug therapy, mainly when monotherapy is employed (Ramana *et al*., 2014). Furthermore, the nature of HIV infection, including viral persistence in reservoirs, necessitates long-term, uninterrupted multi-drug ARV therapy. Treatment compliance is critical regardless of whether a patient is treatment-naïve or treatment-experienced since poor patient compliance is often a factor in treatment failure and viral rebound. The lack of patient adherence to complicated drug administration regimens is further exacerbated by the cumulative costs of combined ARV therapy (Ramana *et al*., 2014).

Furthermore, most of the anti-retroviral drugs cannot cross the blood–brain barrier (BBB); as a result of which, they are ineffective in entering microglial cells such as astrocytes that hoard HIV particles (Schweighardt and Atwood, 2001). A significant factor is the high protein binding of most anti-HIV drugs, which prevents their diffusion across the BBB. Also, it has been observed that the anti-

retroviral drugs are unable to reach the lymphatic system that harbours HIV. Thus, conventional therapy is ineffective in annihilating viral reservoirs as less than 2% of the lymphatic cells are in the systemic circulation (Gunaseelan *et a*l., 2010). The cells of the lymphatic system, like macrophages and dendritic cells, are involved in the transmission of the virus to CD4+ Th cells (helper T lymphocytes) (Gunaseelan *et al*., 2010), and failure to target such reservoirs of HIV increases the risk of a viral relapse post-treatment (Ramana *et al*., 2014).

Therefore, there is a global need for new antiviral compounds to solve drug resistance problems. Optimisation of these existing drugs is essential to work but may only partially alleviate the difficulties in using them for treatment. On the other hand, bioactive compounds isolated from natural biological sources offer a vast and unexplored diversity of chemical structures most unmatched by even the most extensive combinatorial databases (Strobel and Daisy, 2003; Linnakoski *et al*., 2018). Natural products have served as traditional medicine for thousands of years and still provide the most affordable treatment for diseases in many developing countries (Amzat and Razum, 2018). Several well-known natural products including polyphenols (e.g., curcumin, resveratrol), cardiotonic steroids (e.g., digoxin and bufalin), terpenoids (e.g., paclitaxel, artemisinin, and triptolide), polysaccharides (e.g., lentinan), saponins, capsaicin, have potential immunomodulatory effects (Bahrami *et al*., 2019).

Bioactive compounds are naturally derived metabolites and by-products from microorganisms, plants, or animals (Baker *et al.*, 2000; Linnakoski *et al*., 2018). Over the past 25 years, bioactive compounds from many traditional medicinal plants have been screened for their antiviral activity by various research groups in Asia, the Far East, Europe, and America (Jassim and Naji, 2003; Linnakoski *et al*., 2018). Medicinal plants are considered one of the major reservoirs of endophytes with novel bioactive metabolites (Bacon and White, 2000). The importance of novel drug discoveries has been bioactive molecules of fungal origin. Especially fungi growing in unique environments, such as endophytic and marine fungi, are being constantly explored for their antibacterial and antifungal potential (Linnakoski *et al*., 2018). Endophytes are relatively not well-studied microorganisms that has regained importance due to their different biological activities and bioactive compounds with a high level of structural diversity (Strobel *et al*., 2004; Selim *et al*., 2018). Endophytic fungi represent a diverse group of microorganisms abundant in biodiversity, typically residing asymptomatically within plant tissues or intercellular spaces (Wen *et al*., 2022). These fungi contribute to host plant growth by directly producing secondary metabolites, bolstering the plant's resilience against biotic and abiotic stresses (Wen *et al*., 2022). They are a rich source of novel natural metabolites for exploitation in medicine, industry, and agriculture (Selim *et al*., 2018). Medicinal plants are regarded as one of the major

reservoirs of endophytes with novel bioactive metabolites (Bacon and White, 2000; Selim *et al*., 2014; Selim *et al*., 2018). In this current study, we exploit endophytic fungi to evaluate the anti-HIV of the fractionated crude extract from *A. alternata* isolated from the *Hypoxis* sp. plant and establish the mode of action (mechanism).

2.4 *Hypoxis* species

The *Hypoxis* species commonly known as the African potato, belongs to the Hypoxidaceae family. Other common names include star lily, magic muthi, or yellow stars (Gail *et al*., 2015; Matyanga *et al*., 2020). In KwaZulu-Natal it is known as 'inkonfe' or 'ilabatheka' in isiZulu (Drewes *et al*., 2008a). Its use in Africa is widespread among medicinal plants of intense commercial and scientific interest (Van Wyk, 2015; Gail *et al*., 2015; Matyanga *et al*., 2020). The plant grows in the wild and is prevalent primarily in Southern Africa (mainly South Africa, Lesotho, Mozambique, and Zimbabwe (Matyanga *et al*., 2020). It is also found further into East Africa. The plant is characterised by strap-like leaves and bright yellow, star-shaped flowers shown in Figure 2.4 (Van Wyk, 2011).

The tuberous rootstock (corm) is traditionally used to treat various ailments (Matyanga *et al*., 2020). Traditionally, the extracts of the corm are used to make decoctions, which are taken as tonics against wasting diseases, common cold, flu, arthritis (Mills *et al*., 2005), barrenness, bad dreams, intestinal parasites, cardiac diseases (Matyanga *et al*., 2020), urinary infection (Watt and Breyer-Brandwijk, 1962) tuberculosis, testicular tumours, other cancers, and HIV/ AIDS (Drewes *et al*., 2008a). The main chemical constituent of the plant includes hypoxoside, a nonlignan glycoside. Sterols (β-sitosterol, stigmasterol) and their glycosides and stanols, namely stigmastanol, are also present in the plant (Seebaluck-Sandoram and Mahomoodally, 2017).

The South African traditional healers community previously uses *Hypoxis* sp. as an immunostimulant for patients with HIV/AIDS (Mills *et al*., 2005). Albrecht *et al*. (1995), who thoroughly researched African Potatoes, administered a methanolic extract of *Hypoxis hemerocallis* to patients with HIV over two years in the mid-1990s. He reported that the $CD4^+$ T lymphocyte counts in these patients remained stable. At the same time, the serum p24 HIV antigen decreased, and there was a decrease in the expression of the HLA-DR $CD8^+$ lymphocyte activation marker (Albrecht *et al*., 1995). The HLA-DR CD8+ is used to identify T lymphocytes, and elevated levels are observed in HIV infection (Viallard *et al*., 2006; Matyanga *et al*., 2020).

On the other hand, *Hypoxis hemerocallidea* showed interference with the efflux of nevirapine across intestinal epithelial cells and potentially increased the bioavailability of this antiretroviral drug when taken concomitantly (Brown e*t al*., 2008; Ncube *et al*., 2013). However, this combination may lead to increased side effects of the retroviral drug and, consequently also, a decrease in patient compliance (Brown *et al*., 2008). Previous studies reported that many medicinal plants were becoming less common as a result of habitat loss and overexploitations(Williams *et al*., 2013), climate change (Lawal *et al*., 2019), and invasive species (Groner *et al*., 2022). Approximately 15,000 species of medicinal plants may be in danger of going extinct, which would have a negative impact on the health and wellbeing of millions of people, according to earlier studies (Ross, 2005; Chen *et al*., 2016). Therefore, it is crucial to highlight the connections between endophytic microorganisms and their host plants. This is because endophytes can generate a wide range of compounds, including those with significant biological activities derived from the host plants due to horizontal gene transfer that may have occurred during the co-evolution between the endophytic fungi and the host plant (Gómez and Luiz, 2018; DeMers and May, 2021; Lacerda Í *et al*., 2022). Thus, to safeguard the medicinal plants and continue to exploit their bioactive compounds use within, isolation of endophytic fungi is vital because they can also exploit the same bioactive compounds at a higher concentration within a short period.

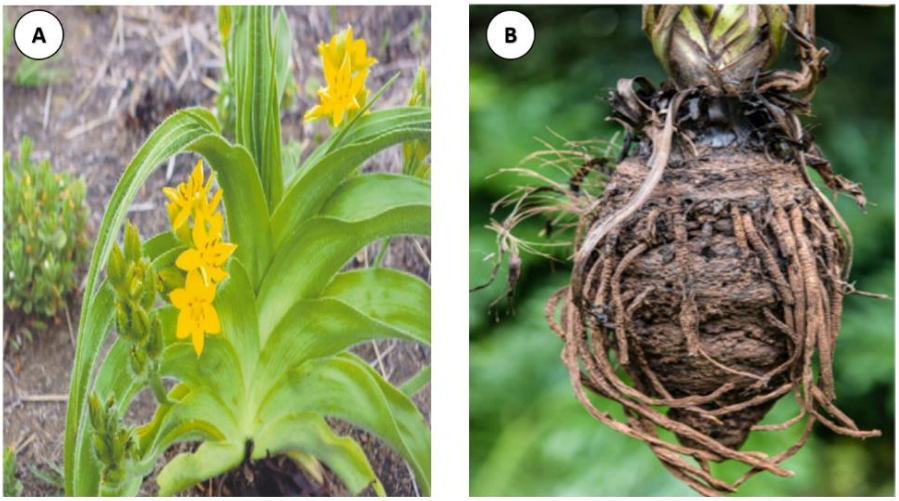

Figure 2. 4: An image of the *Hypoxis* plant displaying bright yellow, star-shaped flowers (A) (Drewes et al., 2008b) and the large, oblong corm with protruding roots (B) (Bassey *et al*., 2023).

2.5 Endophytic Fungi

Endophytic fungi are a group of host-associated fungal communities that colonise the intercellular or intracellular spaces of host tissues, providing beneficial effects to their hosts while gaining an advantage of a secure habitat (Tan and Zou, 2001; Alam *et al*., 2021). Endophytic fungi colonise the inside of the host tissue and help promote the production of plant hormones and the accumulation of secondary metabolites (Shwab and Keller, 2008; Rai *et al*., 2021). The plant helps fungal endophytes in a symbiotic relationship by providing nutrients, shelter, and seed dissemination. In contrast, fungal endophytes transform the bioactive compounds synthesised by the host plant into multifunctional products (Schouten, 2019). Fungal endophytes are also known to influence the biosynthesis of enzymes, phytohormones, and bioactive compounds in plants (Rozpądek *et al*., 2015; Vergara *et al*., 2017; Satheesan and Sabu, 2020). The crosstalk between plant–endophyte and endophyte–endophyte has been reported to trigger the biosynthesis of bioactive compounds (Kusari *et al.*, 2012; Rai *et al*., 2021). However, our understanding of host (plant)–endophyte (fungal) relationships is still inadequate regarding biochemistry and physiology. The intricacies of the fungal endophytes and host plants are thought to vary from microbe to microbe and host to host (Verma *et al*., 2009; Rai *et al*., 2021). Therefore, understanding the relationship between the host plant and fungal endophytes may aid in the complete manipulation of drugs with enhanced quality through the application of modern biological tools and the manipulation of medicinal plant growth conditions (Firáková *et al*., 2007; Rai *et al*., 2021).

2.5.1 Plant-endophytes relationship

Endophytic fungi can form mutualistic relationships with plants**.** Studies have shown that almost all plants contain endophytic fungi, including colonised plants in the Arctic and Antarctic regions, deserts, oceans, and tropical rainforests (Krings *et al*., 2007; Chadha *et al*., 2015; Busby *et al*., 2016; Baron and Rigobelo, 2022; Jin *et al.*, 2021). They have been isolated and cultured from the roots and above-ground parts of various plants, including algae, mosses, ferns, gymnosperms, and angiosperms (Wang *et al*., 2022). Endophytic fungi can produce various bioactive substances and have compounds identical to or like pharmacological activities identified from plants (Jin *et al*., 2018; Wen *et al*., 2022). Endophytic fungi can serve as an alternative source for valuable active plant compounds in searching for bioactive molecules as pro-drug compounds or an alternative source for useful active plant compounds in searching for bioactive molecules as pro-drug compounds or developing medicines. Endophytic fungi can be harnessed to produce bioactive compounds for human pharmaceutical use when the bioactive secondary metabolites are not commercially available, derived from slow-growing or rare and endangered plants, and challenging to synthesise due to heavy molecular weight or structural complexity (Zheng *et al*., 2021). Endophytic fungi secondary metabolites have drawn extensive attention among medicinal plants, mangroves, and marine microorganisms (Rustamova *et al*., 2020; Zheng *et al*., 2021).

The secondary metabolites produced by some endophytic fungi are phenols, alkaloids, polyketides, quinones, steroids, enzymes, and peptides, which possess higher therapeutic value (Akone *et al*., 2016). The secondary metabolites synthesise a variety of biologically active compounds with diverse biological activities, such as insecticidal, antioxidant, cytotoxic, antibacterial, antiviral, antifungal, and antimalarial compounds (Akone *et al*., 2016; Meena *et al*., 2019; Kouipou Toghueo and Boyom, 2019). This chemical diversity also protects host plants from pathogens by inhibiting plant pathogen growth and boosting the host immune system to amplify plant defence mechanisms (Fadiji and Babalola, 2020). The endophytic fungi and their secondary metabolites have a beneficial role to play in the field of medicine and agriculture.

Previous studies showed that endophytic fungi from desert plants are a promising source in identifying potent inhibitors in the replication of HIV-1 (Wellensiek *et al*., 2013; Bashyal *et al*., 2014). *Alternaria tenuissima* isolated from the Sonoran desert plant *Queise emoryi* was reported to have an antiviral compound, Altertoxins, effective against the HIV-1_{LAV} strain virus (Bashyal *et al*., 2014). Altertoxins V inhibited HIV-1_{LAV} with an IC_{50} of 0.50 μM, and I, II, and III interfered with an IC_{50} of 2.20, 0.30,

and 1.50 µM. Using a combination of the IC_{50} and cytotoxicity data, the therapeutic indexes for altertoxins I, II, III, and V, respectively (Bashyal *et al*., 2014). While these indexes are low, they provide a narrow window for further development. They suggest that epoxy perylene structure may be a promising scaffold for further manipulation to afford potent and non-toxic anti-HIV therapeutics (Bashyal *et al*., 2014).

Furthermore, Melappa *et al*. (2015) also revealed that partially purified coumarins isolated from *Alternaria* sp. showed high HIV-1 inhibition on three viral enzymes: integrase enzyme (98%), reverse transcriptase enzyme (82.81%) and protease activity (78%). The safety and pharmacokinetic of polycyclic-coumarin(+)-Calanolide A,(+)-[10*R*,11*S*,12*S*]-10,11-*trans*-dihydro-12-hydroxy-6,6,10,11-tetramethyl-4-propyl-2*H*,6*H*-benzo[1,2-*b*:3,4-*b'*:5,6-*b*"]tripyran-2-one, which is a novel nonnucleoside reverse transcriptase inhibitor (NNRTI) with potent activity against HIV-1 was examined in four successive single-dose cohorts (200, 400, 600, and 800 mg) in healthy, HIV-negative volunteers (Kashman et al., 1992; Xu et al., 2000; Creagh et al., 2001). Where the results showed that this study demonstrated the safety and favourable pharmacokinetic profile of single doses of (+)-calanolide A in healthy, HIV-negative individuals (Creagh *et al*., 2001). Furthermore, curcumin has been an effective antiviral agent against many enveloped viruses, including respiratory viruses such as influenza A and Respiratory Syncytial Virus (Praditya *et al*., 2019). Curcumin and its analogues were also effective in inhibiting HIV replication by targeting HIV protease (Sui *et al*., 1993), HIV integrase (Mazumder *et al*., 1995; Vajragupta *et al*., 2005), and HIV-1 Tat protein (Ali and Banerjea, 2016; Thimmulappa *et al*., 2021). Similarly, curcumin has been an effective antiviral agent against many enveloped viruses, including respiratory viruses such as influenza A and Respiratory Syncytial Virus (Praditya *et al*., 2019)

A recent study by Nzimande *et al.* (2022) showed that a crude extract from *A. alternata* (strain ID PO_4PR_1, PO_4PR_2 and PO_2PL_1) has 100% HIV-1 inhibition in TZM-bl cell line with an IC_{50} value ranging from 0.017 to 1.170 µg/mL. These three crude extract strains also inhibited HIV-1 replication on PBMCs and $CD4^+$ T cells at a concentration ranging from 0.3 to 50.2 ng/mL (Nzimande *et al.*, 2022). Moreover, many other fungal endophytes were reported as potent viral inhibitors. Previously, it was reported that endophytic *Streptomyces* sp. strains isolated from the mangrove plant *Bruguiera gymnorrhiza* produced xiamycin, which exhibits selective anti-HIV activity (Geometric mean IC_5 than µM) by blocking R5. Still, it did not affect X4 tropic HIV-1 infection (Ding *et al*., 2010; Selim *et al*., 2018). Additionally, the endophytic *Emericella* sp. (HK-ZJ) isolated from another mangrove plant, *Aegiceras corniculatum*, produces several bioactive isoindoline compounds (pyrimidine A and

B, emeriphenolicins A and D, spermidine A and B, Austin, austinol, dehydroaustin, and acetoxydehydroaustin), for which two of them showed moderate activity against influenza A virus (H_1N_1) (Zhang *et al*., 2011; Selim *et al*., 2018). A recent study shows that endophytic fungi, *Penicillium chrysogenum* $P0_3MB_2$, showed anti-HIV activity with an IC_{50} of 0.6024 µg/mL compared to untreated fungal crude extract (IC_{50} =5.053 µg/mL) when treated with sodium butyrate (Makhwitine *et al.,* 2023). The main objective of this study is to evaluate the anti-HIV properties of the fractionated crude extracts of *A. alternata* that were isolated from the *Hypoxis* plant. Specifically, the mode of action in HIV inhibition of the HIV-1 life cycle is assessed.

2.5.2 Bioactive compounds from fungi with antiviral activity.

There are several ways bioactive compounds isolated from fungi can interfere with viral infections (Table 2.2). Viruses can be directly attacked by outside cells to destroy the viral particles before their attachment to cellular receptors. Such agent compounds could irreversibly modify viral particles on different surfaces or, if non-toxic, also in the human body (Linnakoski *et al.*, 2018). The antiviral activity of an aqueous extract of *Agaricus subrufescens*, a basidiomycete, was assessed against herpes simplex type 1 (HSV-1) and bovine herpes type 1 (BoHV-1) in Hep-2 cell lines (Bruggemann *et al*., 2006). The viral replication inhibition was evaluated by plaque assay and immunofluorescence test. The aqueous extract of *Agaricus subrufescens* demonstrated virucide action for both viruses, being more effective against HSV-1 and inhibiting its infectivity in 78.4 and 73.9% at 50 and 100 µg/mL concentrations, respectively. Moreover, a reduction of 47% in the number of fluorescent cells was observed for both concentrations (Bruggemann *et al*., 2006).

De Sousa Cardozo *et al*. (2014) also demonstrated that chemically emerocal derivative (FR-S); (1 → 6) –(1 → 3)- β-D -glucan isolated from *Agaricus brasiliensis* showed antiviral activity when studied using viral plaque assay. FR-S displayed promising anti-HSV-1 and anti-HSV-2 activities in simultaneous and postinfection treatments, resulting in higher selectivity indices (CC_{50} /IC_{50}) than 393. FR-S had no virucidal effect but significantly suppressed HSV-1 (EC_{50} = 0.32 µg/mL) and HSV-2 (EC_{50} = 0.10 µg/ml) adsorption (de Sousa Cardozo *et al*., 2014). FR-S was less effective in the adsorption inhibition of mutant virus strains devoid of gC (HSV-1 gC – 39 and HSV-2 gCneg1), indicating a possible interaction with this glycoprotein. The reduction of viral adsorption upon cell pretreatment with FR-S also suggests its interaction with cellular components (de Sousa Cardozo *et al.*, 2014).

Another study by Wang *et al*. (2007) showed that the extract of *Russula paludosa* demonstrated inhibitory activity on HIV-RT with 97.6%. Then *Russula paludosa* was purified using anion exchange

chromatography on DEAE-cellulose and gel filtration on Superdex 75, resulting in SU2 fraction, which exhibited a potent inhibitory activity on HIV-1 RT. At the concentrations of 1 mg/ml, 0.2 mg/ml, and 0.04 mg/mL, the inhibition ratios were 99.2%, 89.3%, and 41.8%, respectively, giving an IC_{50} of 11 mM (Wang *et al*., 2007). Sato *et al*. (2009) isolated lanostane-type triterpenoids from *Ganoderma sinense*; among the isolated triterpenoids, ganoderic acid GS-2, 20-hydroxylucidenic acid N, 20(21)-dehydrolucidenic acid N and ganoderiol F inhibited HIV-1 protease with IC_{50} values of 20—40 mM.

The two *Ganoderma lucidum* triterpenoids, lanosta-7,9(11),24-trien-3-one,15;26-dihydroxy (GLTA) and ganoderic acid Y (GLTB), showed antiviral activity against Enterovirus 71 (EV71) infection (Zhang *et al*., 2014b). The mechanisms by which the two triterpenoids affect EV71 infection were elucidated by three action modes using Ribavirin, a common antiviral drug, as a positive control. The results suggested that GLTA and GLTB prevent EV71 infection by interacting with the viral particle to block the adsorption of the virus to the cells (Zhang *et al*., 2014b). In addition, the interactions between EV71 virion and the compounds were predicated by computer molecular docking, which illustrated that GLTA and GLTB may bind to the viral capsid protein at a hydrophobic pocket (F site) and thus may block the uncoating of EV71 (Zhang *et al*., 2014b). Sacramento *et al*. (2015), investigated the anti-influenza activity of a fungi-derived natural product, aureonitol, which inhibited influenza A and B virus replication. This compound was effective against influenza A(H3N2), with an EC_{50} of 100 nM. Aureonitol cytotoxicity was also very low, with a CC_{50} value of 1426 μM. Aureonitol inhibited influenza hemagglutination and significantly impaired virus absorption (Sacramento *et al*., 2015).

Table 2. 2: Fungi order, phylum, bioactive agents from secondary metabolites, targeted virus, and the mechanism of action.

Fungal order (source)	**Common name**	**Antiviral screening assay**	**Phylum**	**Bioactive agent**	**Target virus**	**Mechanism**	**Cell line**	**Reference**
Agaricus subrufescens	Almond mushroom	Plaque assay and immunofluorescence test	Basidiomycetes	-Beta-glucan-protein -Polysaccharides	-HSV-1, BoHV-1 -HSV-1(KOS), HSV -PV	-Directly on virus particle -Adsorption -Replication	Hep-2	(Bruggemann *et al*., 2006)
Russula paludosa	Tall Bog Russula	Assay of HIV-1 integrase inhibitory ac	Basidiomycetes	4.5 kDa protein (peptide)	HIV protease	Virus protein as a target	E. coli BL21(DE3)	(Wang *et al*., 2007)
Grifola frondose	Hen-of-the-woods	Plaque reduction assay	Basidiomycetes	GFAHP	HSV	Directly on virus particle	Vero cells	(Gu *et al*., 2007)
Ganoderma sinense	Zizhi/ purple Ganoderma	HIV-1 Protease Inhibitory Assay	Basidiomycetes	Triterpenoids	HIV protease	Virus protein as a target	-	(Sato *et al*., 2009)
Lentinula edodes	Shiitake	viral plaque assay	Basidiomycetes	-	HSV-1	Adsorption	Vero cells	(de Sousa Cardozo *et al*., 2014)
Ganoderma lucidum	Reish/ Lingzhi	MTT and molecular docking	Basidiomycetes	-Ganoderic acid -Triterpenoids	-HIV protease - EV71	Virus protein as a target	Huma RD cells	(Zhang *et al*., 2014b)
Trametes versicolor	Turkey tail	Cell-free assays using the NA-Star kit	Basidiomycetes	Polysaccharopeptide -	-HIV -Influenza and HSV	-Adsorption -Replication	HEK293	(Sacramento *et al*., 2015)

2.5.3 *Alternaria alternata.*

Endophytic fungi *A. alternata* is naturally found in the soil and water and can be isolated from medicinal plants. It can be isolated from *Vitex rotondifolia* (Lee *et al*., 2019), *Ziziphus spina-christi* (Elghaffar *et al*., 2022)*, Hypoxis* spp (Nzimande *et al*., 2022), and other sources such as *Azadirachta indica* (Chatterjee *et al*., 2019). *Alternaria alternata* essential has two critical features: the production of melanin and the biosynthesis of many host-specific toxins (HSTs) or non-HSTs. These secondary metabolites play a role in the pathogenicity of hosts (Hadi, 2019). The genus *Alternaria* contains many species that grow in different environments and from other parts of the world (Barkai-Golan and Paster, 2011). It belongs to the class of Hyphomycetes fungi from the sub-division Deutromycotina of the Eumycota division (Girbardt, 1976; Hadi, 2019). This genus is saprophytic fungi that play an essential role in the decomposition processes of organic matter in nature due to its derivatives of cellulose enzymes and other analytic enzymes. However, many of them are opportunistic pathogens (Hadi, 2019).

Previous chemical investigations of *A. alternata* JS0515 identified phenolics, quinones, steroids, terpenoids, and nitrogen-containing metabolites, some of which exhibited phytotoxic, cytotoxic, antifungal, antiviral, and antimicrobial activities (Abbas and Riley, 1996; Lou *et al*., 2013; Shaaban *et al*., 2012; Lee *et al*., 2019). Studies have displayed the antiviral activity of the above chemical compounds. The phenolics can inhibit the replication of distinct types of viruses, such as coronavirus, influenza, and hepatitis, by interfering with their key enzymes and proteins (Ikeda *et al*., 2020; Attia *et al*., 2021; Arimboor, 2021). Quinones have also been reported to have antiviral activity against influenza (Uddin *et al*., 2016) and SARS-CoV (Caruso *et al*., 2020; Cui and Jia, 2021; Chan-Zapata *et al*., 2023). Steroids and terpenoids were also reported to have antiviral (Lacerda Í *et al*., 2022) and nitrogen-containing metabolites (Tran and Henary, 2022). Therefore, endophytic fungi are the major source of bioactive compounds which may possess antiviral activity. Hence, it is a good source for the development of new HIV-1 antiretroviral. These bioactive compounds may have immunomodulatory effects.

2.5.4 Immune response to HIV infection

T-cell activation and exhaustion are two important aspects of the immune response to HIV infections. T cell activation refers to the process by which T cells recognize and respond to viral antigens, leading to proliferation, differentiation, and effector functions (Fenwick *et al*., 2019). On the other hand, T cell exhaustion is a state of impaired T cell function that results from chronic exposure to high levels of antigen and inflammation (Nakanjako *et al*., 2011). Exhausted T cells express high inhibitory receptors, such as PD-1, that dampen their responsiveness to stimulation (Nakanjako *et al*., 2011). T cell exhaustion is associated with poor viral control, disease progression, and suboptimal CD4+ T cell recovery in HIV-infected individuals (Zheng *et al*., 2022). The mechanisms of T cell activation and exhaustion in HIV infections are complex and involve multiple factors, such as viral load, viral diversity, antigen presentation, co-stimulation, cytokine signalling, metabolic dysfunction, and epigenetic regulation (Nakanjako *et al*., 2011; Fenwick *et al.,* 2019; Kervevan and Chakrabarti, 2021). The $CD8^+$ T cells represent a subset of T suppressor/cytotoxic cells, and the proportion of $CD4^+/CD8^+$ T cells is decreased, indicating the inhibition of T cell immunity (Olguín *et al*., 2018; Lu *et al*., 2020). It was reported that $CD38^+$ T cells were identified as surface markers of some tumour-infiltrating lymphocytes (TILs) and expression of these is associated with chromatin state (Philip *et al*., 2017; Lu *et al*., 2020).

Loss of $CD8^+$ T cell function is associated with impaired immune responses. The CD38 and HLA-DR molecules are transmembrane glycoproteins on immature T and B lymphocytes and re-expressed during cellular immune response (Lu *et al*., 2020). The CD38 and HLA-DR on $CD8^+$ T cells are markers of immune activation. Some of these factors are modulated by the virus itself, such as the downregulation of HLA molecules by Nef and Vpu, which reduces the recognition and elimination of infected cells by $CD8^+$ T cells (Fenwick *et al*., 2019). Other factors are influenced by the host immune system, such as the production of IL-10 and TGF-β, which induce and maintain T-cell exhaustion (Nakanjako *et al*., 2011). Moreover, some factors are interrelated and mutually reinforcing, such as the metabolic dysfunction and epigenetic changes that occur in exhausted T cells and impair their function and survival (Fenwick *et al*., 2019; Kervevan and Chakrabarti, 2021). Understanding the role of T cell activation and exhaustion in HIV infections is important for developing effective strategies to enhance the immune response and achieve a functional cure (Nakanjako *et al*., 2011). Several approaches have been explored or proposed, such as therapeutic vaccination, immune checkpoint blockade,

cytokine therapy, metabolic reprogramming, and epigenetic modulation (Nakanjako *et al*., 2011; Fenwick *et al*., 2019; Kervevan and Chakrabarti, 2021). However, the optimal combination and timing of these interventions remain to be determined, and the potential risks and benefits need to be carefully evaluated. Therefore, further research is needed to elucidate the molecular and cellular mechanisms of T cell activation and exhaustion in HIV infections and to identify novel targets and biomarkers for immunotherapy.

2.5.5 Immunomodulation

In healthy organisms, the immune system maintains homeostasis within the body (Jantan *et al*., 2015). The function and efficiency of the immune system are influenced by various exogenous and endogenous factors, resulting in either immunosuppression or immunostimulant (Wen *et al*., 2012; Jantan *et al*., 2015). Several agents possessing an activity to normalise or modulate pathophysiological processes are called immunomodulators (Puri *et al*., 1994; Jantan *et al*., 2015). Immunomodulation refers to any improvement in the immune system's response, including stimulation, expression, amplification, or suppression of any part or stage of the immune response (Maggini *et al*., 2018; Di Sotto *et al*., 2020; Ali Reza *et al*., 2021). Immunomodulators, therefore, influence the immune system's role and efficiency through various exogenous and endogenous factors that result in either immunosuppression or immunostimulant (Ali Reza *et al*., 2021). Numerous proteins, amino acids, and natural substances, such as steroids, dimethylglycine, and interferon-γ (IFN-γ), have demonstrated a notable capacity to regulate immune responses (Akira *et al*., 2006; Ali Reza *et al*., 2021). They have anti-inflammatory qualities as well as humoral and cellular immunomodulatory actions.

Immunostimulants, including glucan, chitin, lactoferrin, levamisole, as well as other plant polysaccharide extracts or compounds, have been used to modulate the immune response to promote the role of phagocytic cells, enhance their bactericidal functions (Moser and Leo, 2010; Ali Reza *et al*., 2021). Immunomodulators are synthetic or natural products that enhance or inhibit the host defence response, which can be used as a protective measure or in combination with other treatment approaches and activate biological killer cells, complement, lysozyme activity, and antibody responses, thereby enhancing protection from infectious diseases (Mohamed *et al*., 2017; Ali Reza *et al*., 2021). Among different

immunomodulators, plant-based secondary metabolites have a high potential for immune defence and cellular immune responsiveness (Mukherjee *et al.*, 2012). These natural immunomodulators can be developed into safer alternatives to the clinically used immunosuppressants and immunostimulants cytotoxic drugs, which possess severe side effects (Di Sotto *et al*., 2020).

2.5.5.1 Medicinal plants with Immunomodulatory effects

Many plants of different species have been reported to possess strong immunomodulating properties (Shukla *et al*., 2014; Ali Reza *et al*., 2021). The immunomodulatory effects of plant extracts and their bioactive metabolites have been suggested due to their diverse mechanisms of modulation of the complex immune system and their multifarious molecular targets (Amirghofran *et al*., 2000; Ali Reza *et al*., 2021). Several plant species and plant-based products have been reported to possess potent immunomodulating properties, which include *Echinacea purpurea L, Dioscorea batatas decline, Allium sativum, Berberis vulgaris, Clerorodendrium splendens, Tinospora cordifolia, Curcuma domestica, and Hypoxis emerocallis* (Ali Reza *et al*., 2021).

Echinacea purpurea L. is one of the most medicinally recognised plants; it shows effective antioxidant, anti-inflammatory, hypoglycemic, and antiproliferative activities (Dobrange *et al*., 2019). Root and above-ground parts extracts reportedly signal and stimulate immune responses by influencing macrophages, dendritic cells, monocytes, and NK cells and show antiviral activity (Dobrange *et al*., 2019). The chemical constituents responsible for the antiviral activity and activating the body's immune system include glycoproteins, soluble polysaccharides, phenolic compounds, caffeic acid derivatives, and alkyl amides (Aarland *et al*., 2017; Dobrange *et al.*, 2019).

Dioscora batatas Decne is extensively used in traditional and modern medicines, and the tuber of the plant contains several active components, including dioscorin, diosgenin, mucopolysaccharides, batatasins, and glycoproteins, exhibiting immunomodulation activities upon oral administration (Alhazmi *et al*., 2021). Tuber protein and dioscorin displayed mucosal and systemic immunomodulation activity through TLR4-induced macrophage activation by stimulating signalling molecules such as NF-kB, JNK, p38, and ERK, as well as by expressing cytokines such as TNF-a and IL-6 (Huong and Jeon, 2011; Wen *et al*., 2012). The ethanolic extract of bark inhibited iNOS and COX-2 expression in

RAW 264.7 cells via NF-kB and ERK1/2, conferring anti-inflammatory activity (Jin *et al*., 2010; Lim *et al.,* 2019). Tuber extract also improved immunomodulation by remarkably enhancing the granulocyte-macrophage colony-stimulating factor (GM-CSF) promoter activity in inflamed and normal skin (Su *et al*., 2008; Wen *et al*., 2011).

Allium sativum, commonly known as garlic, is an onion species. The biological activity of garlic shows free radical scavenger, cardiovascular diseases, immune stimulant, anti-cancer, anti-inflammatory, anti-infectious diseases, antioxidant, anti-allergic, and anti-bacterial properties (Arreola *et al*., 2015; Moutia *et al*., 2018). The bioactive chemical constituents of garlic mainly include S-allyl-L-cysteine sulfoxide (alliin), gamma-glutamyl cysteine, Diallyl Disulphide (DADS), Dithiines, (E-Z) ajoene and diallyl thiosulphate (allicin) (Harris *et al*., 2001; Swaroop *et al*., 2022).

Different compounds and formulations show immunomodulatory effects, including cytokine secretion, regulation, stimulation of phagocytosis, macrophage activation, and immunoglobulin production (Arreola *et al*., 2015; Swaroop *et al*., 2022). Alliin is a bioactive chemical constituent extracted from *Allium sativum* that shows enhancement of IL-6 and MCP-1 (pro-inflammatory cytokines). Garlic extract shows cytokine modulation at low concentrations, including an increase of IL-10 and a decrease of IL-12, IL-1alpha, IFN-gamma, TNF-alpha, IL-6, and IL-8 (Quintero-Fabián *et al*., 2013). Other active constituents of garlic allitridin, S-allyl-L-cysteine, caffeic acid, uracil, and diallyl sulphide inhibit transcription factors NF-KB, IL-6, MCP-1, TNF-alpha, IL-1beta, IL-12 (Kim *et al*., 2013). Allicin is administered to mice infected by *Plasmodium yoelii* show reduced parasitaemia due to stimulation of proinflammatory cytokines like IFN-gamma, macrophage activation, CD4+ T-cells, CD40, maturation of dendritic cells (Feng *et al*., 2012; Swaroop *et al*., 2022).

Berberis vulgaris contains benzodioxol quinolizine alkaloid (berberine), which is shown to significantly induce interleukin (IL)-12 production in a dose-dependent manner to counter the effects of HCV infection (Alhazmi *et al*., 2021). IL-12 consequently increased the IFN-g production and decreased the IL-4 level in antigen-primed CD4+ T cells. Berberine also stimulates T helper lymphocytes subset 1 (Th1) cytokine synthesis and reduces Th2 (Ghareeb *et al*., 2016; Kalmarzi *et al*., 2019). The plant's fruit has medicinal importance due to its antioxidant, anticancer, anti-inflammatory, antidiabetic, antibacterial, and hepatoprotective properties (Rahimi-Madiseh *et al*., 2017). The polysaccharide obtained from the leaves of

Clerodendrum splendens also exhibited potent immunomodulation activity (Alhazmi *et al*., 2021). Its high-molecular-weight subfraction induced nitrous oxide and cytokine, TNF, granulocyte macrophage-colony stimulating factor, peripheral blood mononuclear cells, and monocytes with other wide-ranging agonist activities that considerably reduced disease severity (Kouakou *et al*., 2013; Wang *et al*., 2018).

Extracts of *Tinospora cordifolia* show a broad essential anti-pyretic wide spectrum of immunotherapeutic properties ranging from essential tonic, anti-inflammatory, anti-arthritic, anti-malarial, aphrodisiac, anti-allergic, anti-diabetic, anti-hepatotoxic, wound healing effect. Antipyretic properties have relatively low toxicity (Saha and Ghosh, 2012). *Tinospora. cordifolia* is a herbal medicine; annually, its preparation utilises 10,000 tonnes of crude herbal extract (Singh *et al*., 2004a). The active phytochemical constituents of *T. cordifolia* include clerodane Furano diterpene glycoside, cordioside, syringin, Cordifolioside A, Cordifolioside B, cordial (Khan *et al*., 2007; Saha and Ghosh, 2012; Nair *et al*., 2007)**.** Aqueous and alcoholic extracts of *T. cordifolia* have been tested and reported successfully as immunomodulatory activity (Rege *et al*., 1999). *Curcuma domestica* (turmeric) is a traditional Indian medicine popular as a dietary spice. Its polyphenolic compound, curcumin, is the main compound isolated from the rhizome (Panaro *et al*., 2020). Curcumin has shown remarkable efficacy in treating cerebral malaria through immunomodulation mechanisms (Panaro *et al*., 2020). It inhibits NF-kB activation and reduces proinflammatory cytokine production by expressing cyto-adhesion molecules on endothelial cells (Mimche *et al*., 2011; Yadav *et al*., 2015).

Curcumin abrogate the production of inflammatory cytokines and chemokines through genital epithelial cells *In vitro* (Praditya *et al*., 2019; Vitali *et al*., 2020). The polyphenolic component also competently blocked Kaposi's sarcoma-associated herpes virus replication and inhibited the pathogenic processes of angiogenesis and cell invasion (Li *et al*., 2019a). Several other medicinal plants have shown good to moderate immunomodulation and antiviral activities in various *In vitro* and *In vivo* models (Alhazmi *et al*., 2021). For instance, oleanolic acid in rosemary (*Salvia rosmarinus*) has been proven to have a good effect against herpes, HIV, influenzas, and hepatitis A viruses (Jiang *et al*., 2005). In many laboratory experiments, Sambucus, licorice, ginger, ginseng, and dandelion showed dominant antiviral activities (Zeedan and Abdalhamed, 2021). Glycyrrhizin and lycorine from *G. glabra* and lycoris radiate showed good activity against SARSCoV (Mukherjee, 2019).

2.5.5.2 Endophytic fungi with Immunomodulatory effects

The bioactive compounds derived from endophytic fungi within plants emerge as intriguing agents that influence the delicate balance of immune responses (Kaur *et al*., 2015). Immunomodulators have become valuable agents in relieving the pathology associated with viral infections in the future (Suwannarach *et al*., 2020). *Pestalotiopsis leucothe's*, an endophytic fungus from *Tripterygium wilfordii*, showed considerable inhibition of cytokines production (Interleukins, interferons, and tumour necrosis factor) and IgG and IgM by PBMC (Kumar *et al*., 2005). It also showed the production of interleukin-2 and Sil-2R, suggesting that *P. leucothes* is a T-cell-specific immunosuppressant with B-cell immunosuppressive effects (Kumar *et al*., 2005). The study by Abood *et al*. (2014) indicates that secondary metabolites from *T. crispa* have immunomodulatory effects through the stimulation of IFN-γ, IL-6, and IL-8 expressions where the LC-MS phytochemical analysis showed that the *T. crispa* fraction has cardioid, quercetin, eicosenoic acid (pollinic acid), and boldine, which may be responsible for the immunostimulatory effect of *T. crispa* (Abood et al., 2014).

The endophytic fungus *Fusarium subglutinans*, isolated from *T. wilfordii*, produces the immunosuppressive but noncytotoxic diterpene pyrones subglutinol A and B. Subglutinol (Lee *et al*., 1995). Another study by Shanavas, (2014) showed that WEF-7 *Fusarium solani* isolated from *Withania somnifera* showed immunomodulatory potential by increasing the phagocytic activity (Shanavas, 2014). Another study by Ujam *et al*. (2019) showed that the endophytic fungi (code name ACL4) extract isolated from fresh leaves of *Ageratum condyloid*, showed immune-stimulatory activity in a dose-related manner with a significant increase in the total white blood cells and neutrophil levels at 100 and 200 mg/kg. The extract also exhibited immune-protective properties by inhibiting the immune-suppressive activity of cyclophosphamide (Ujam *et al*., 2019). Morales *et al*. (2018) also demonstrated that *A. alternata* allergoid could be an effective immunotherapy treatment (in mice, guinea pigs and zebra fish models), leading to cytokine stimulation and inducing synthesis of IgG antibodies able to block IgE binding to the allergen (Morales *et al*., 2018). Olwenyi *et al*. (2021) study also showed that the final concentration of 0.500 µg/mL of *Azadirachta indica,* a significant downregulation of CD4 + CD38 + HLA-DR+ expression was observed in HIV negative ($p < 0.0001$) and both HIV infected groups ($P = 0.0313$). This plant extract also significantly lowered SEB induced % CD4+ T cell HLADR, PD-1 and Tim-3 levels. PD-1 and CD69 markers were only significantly downmodulated in only the HIV negative (($p = 0.0001$ and $p = 0.0078$ respectively) and viral load< 1000 copies per mL ($p = 0.0078$) groups

(Olwenyi et al., 2021). These findings show that endophytic fungi bioactive compounds can modulate the immune system by enhancing or suppressing its responses, depending on the context and the target cells.

2.6 METHODOLOGY REVIEW

2.6.1 Antiviral assays

2.6.1.1 Assays and methods for screening and evaluation viral entry inhibitors

Different assays are used to evaluate HIV-1 inhibitors, including cell-to-cell fusion, cell-virus fusion with pseudo-typed viral particles, and *In vitro* biochemical assays developed to screen viral entry inhibitors (Rumlová and Ruml, 2018).

Cell-to-cell and cell-virus fusion assay

A cell-to-cell and cell-virus fusion assay is a method to measure the ability of cells or viruses to fuse. Fusion is a process that involves the merging of two or more membranes to form a single membrane (Zhao *et al*., 2021; Chan, 2022). The Cell-cell fusion assays involve two types of cells, namely, the effector cells that stably (Bradley *et al*., 2004) or inducible (Ji *et al*., 2006; Herschhorn *et al*., 2011) express HIV-Env glycoprotein, and target cells that express CD4 and either CXCR4 or CCR5 (Rumlová and Ruml, 2018). Co-cultivation of these cells leads to HIV-1 Env-mediated cell membrane fusion, forming multinucleated syncytia (Edinger and Doms, 1999; Rumlová and Ruml, 2018). This approach uses effector cells that express both HIV-1 Env and the Renilla luciferase (R-Luc) reporter protein using inducible tetracycline-controlled transactivator (tTA) and target cells that express the HIV-1 receptor (CD4) and co-receptor (CCR5) and contain the firefly luciferase (F-Luc) reporter gene under the control of a tTA-responsive promoter (Herschhorn *et al*., 2011). Upon fusion of the effector and target cells, tTA enters the target cells and activates the expression of the F-Luc reporter (Herschhorn *et al*., 2011). The inhibition of fusion of cellular membranes is determined as a decrease in F-Luc luminescence, and the inhibitor specificity is measured as the R-Luc activity (Herschhorn *et al*., 2011).

Virion-based fusion assays are another category of cell-based fusion assays. One such approach is based on producing chimeric HIV-1 virions carrying β-lactamase–Vpr chimeric protein (BlaM-Vpr) (Cavrois *et al*., 2014). Chimeric HIV released into the cell culture media is isolated by ultracentrifugation and used to infect target cells. Entry of the virions into the cytoplasm is detected by cleavage of a fluorescent substrate by β-lactamase (Cavrois *et al*., 2014; Rumlová and Ruml, 2018). The fluorescence shift corresponds to the fusion efficacy and is measurable by fluorescence microscopy, flow cytometry, or fluorometric plate reader (Cavrois *et al*., 2002; Cavrois *et al*., 2004; Cavrois *et al*., 2014).

2.6.1.2 Assays and methods for screening viral capsid disassembly/uncoating inhibitors

Two main techniques are used in screening viral capsid disassembly or uncoating inhibitors assays: the ultracentrifugation or utilization of HIV-1 specific cellular restriction factors (Rumlová and Ruml, 2018). The *"In vitro* core-stability assay" is based on ultracentrifugation of released HIV-1 virions through a detergent layer, where the viral membrane dissolves into a sucrose gradient, and the viral cores are concentrated (Shah and Aiken, 2011). The result of this assay is determined by the isolation of the cores by sedimentation of concentrated virions through a layer of detergent and into a linear sucrose gradient in the cold. To quantify uncoating, the isolated cores are incubated at 37°C for various timed intervals and pelleted by ultracentrifugation (Shah and Aiken, 2011). The extent of uncoating is analysed by quantifying the fraction of CA in the supernatant. Then, the "fate of capsid" assay uses ultracentrifugation through a sucrose cushion to separate the HIV-1 core from a whole cell lysate prepared shortly after infection (Stremlau *et al*., 2006).

Previously, a novel entry/uncoating assay (EURT), an alternative to BlaM-Vpr (d+), was reported where it quantifies the protein product of a virion-packaged mRNA reporter upon uncoating (Da Silva Santos *et al*., 2016). A method to monitor the uncoating/disassembly of the capsid of the influenza A virus, which enters the cell by endocytosis, is also based on ultracentrifugation (Zhirnov, 1990; Au - Stauffer *et al*., 2016). Purified virions are separated using velocity gradient centrifugation through a two-layer glycerol gradient (Au - Stauffer *et al*., 2016). The advantage of this assay is that it can provide quantitative data on the thermodynamic parameters of protein stability, such as melting temperature, enthalpy, entropy, and free energy. It may not reflect the proper stability of proteins *in vivo*, subject to

various factors such as post-translational modifications, molecular crowding, and chaperones (Da Silva Santos *et al.,* 2016; Au - Stauffer *et al*., 2016).

2.6.1.3. Assay and methods for screening and evaluating integrase inhibitors.

Retroviral integrase inhibitors are a new type-approved a new type of inhibitor imposed by the emergence of drug-resistant mutants. HIV integrase activities, integrase inhibitors, and drug resistance have been discussed in detail elsewhere (Liao *et al*., 2010; Andrake and Skalka, 2015; Hajimahdi and Zarghi, 2016; Thierry *et al*., 2017; Anstett *et al*., 2017; Podany *et al*., 2017). Methods to assess the two significant activities of integrase end processing of the reverse transcription product and its joining to target chromosomal DNA—have been reviewed in detail by several groups (Marchand *et al*., 2001; Merkel *et al*., 2009; Engelman and Cherepanov, 2014). Initial methods used radioactively labelled DNA oligonucleotides comprising the terminal *cis*-acting sequences of linear viral DNA required for integration. The joining of the processed strand to the other strand (self-integration) or supplemented target DNAs can be analysed by polyacrylamide gel electrophoresis (PAGE) (Katzman *et al*., 1989; Katz *et al*., 1990; Rumlová and Ruml, 2018).

A less time-consuming, non-radioactive method involves time-resolved fluorescence anisotropy measurement using a 21-metric oligonucleotide fluorescently labelled on the terminal GT dinucleotide (Guiot *et al.*, 2006; Mohanty *et al*., 2017). This assay monitors integrase binding to the substrate and the subsequent 3′-processing reaction, changing the anisotropy (Guiot *et al.*, 2006; Rumlová and Ruml, 2018). Alternatively, the yields of both the processing and joining reactions can be measured upon separating the radioactively labelled product from the rest of the DNA molecule using adsorption to PEI-cellulose (Müller *et al.*, 1993). A real-time HTS method measures fluorescence emission by removing the 3′-terminal dinucleotide, labelled with a quencher, by integrase (HE *et al*., 2007). Han et al. (2013) described a fluorescence method to screen molecules that inhibit integrase binding to viral DNA (Han *et al*., 2013). Techniques evaluating the integrase strand transfer reaction have been modified to a high-throughput format using magnetic beads (HE *et al*., 2008) or streptavidin-coated microplates. A method to assess strand transfer by time-resolved FRET

with a europium-streptavidin-labelled substrate has been optimized for 384- and 1536-well plate formats (Wang *et al*., 2005).

In this study, we used Luciferase TZM-bl cell-based assay to evaluate HIV-1 virus inhibition by the fungal crude extracts. Luciferase assay (measuring luciferase activity) is one example of a more rapid single-round infectivity assay that is widely used to assess the activity of compounds, including those from microbial natural products (e.g., fungal extracts) in HIV-1 strains (Nzimande *et al*., 2022; Nzimande *et al*., 2023). The TZM-bl assay measures antibody-mediated neutralization of HIV-1 as a function of reductions in HIV-1 Tat-regulated firefly luciferase (Luc) reporter gene expression after a single round of infection with Env-pseudotyped viruses (Montefiori, 2009). The Luciferase TZM-bl cell-based assay was derived from the TZM-bl neutralisation assay. The TZM-bl neutralisation assay measures the ability of antibodies to neutralise HIV-1 (Sarzotti-Kelsoe et al., 2014); it is also utilised for standardized assessments of vaccine-elicited neutralizing antibodies, for studies of monoclonal antibodies and the neutralizing antibody response in HIV-1 infected people (Sarzotti-Kelsoe et al., 2014). The luciferase assay uses the enzyme luciferase and a substrate (such as luciferin) to produce bioluminescence, which can be measured by a luminometer in relative luminescence units (RLU). The amount of light emitted is proportional to the activity of the promoter region of the target gene (Cheng et al., 2010; Thorne et al., 2010; Sarzotti-Kelsoe et al., 2014; Cherne et al., 2019).

The TZM-bl cell line is derived from a HeLa cell clone that was engineered to express CD4, CCR5, and CXCR4 (Platt *et al*., 1998) and to contain integrated reporter genes for firefly Luc and *E. coli* β-galactosidase under the control of an HIV-1 long terminal repeat (Wei *et al*., 2002), permitting sensitive and accurate measurements of infection. The cells are highly permissive to infection by most strains of HIV, SIV, and SHIV, including primary or molecularly cloned viral isolates and molecularly cloned Env-pseudotyped viruses (Sarzotti-Kelsoe et al., 2014). The viral replication activates the reporter gene, and the activity can be measured in a calorimetric, luminometric or fluorometric assay (McMahon *et al*., 2009; Nzimande *et al*., 2023). The half maximum inhibitory concentration (IC_{50}) values (reciprocal dilution needed to avoid virus-induced cytolysis by 50%) are used to express viral activity. Luciferase assay (measuring luciferase activity) is one example of a more rapid single-round infectivity assay that is widely used to assess the activity of compounds, including those from

microbial natural products (e.g., fungal extracts) in HIV-1 strains (Nzimande *et al*., 2022; Nzimande *et al*., 2023).

To determine the stage of the HIV replication cycle with which this anti-HIV compound interferes, we used the time of addition assay (TOA) coupled with p24 ELISA. Time of Addition assay was performed according to Daelemans et al. (2011) and Wang et al. (2010) with some modifications. The time of addition assay can be used to narrow down the mechanism/ or target of action of newly identified anti-HIV-1 agents in cell cultures by comparing it to time intervention with well-characterized inhibitors (Daelemans *et al*., 2011; Corona *et al*., 2020). This assay is based on the principle that viral replication occurs in sequential stages that can be timed (Daelemans *et al*., 2011; Nzimande *et al*., 2023). The compound target site can be predicted based on its relative position compared to the reference drug used as the training set on the time scale (Nzimande *et al*., 2023). Then, the HIV-1 viral load in the culture supernatant from TOA was measured using a Quicktiter Lentiviral Quantification kit (p24).

The p24 is a specific HIV structural protein that forms most virus cores. This protein is secreted in the blood serum of an infected person and is therefore also recognized as an early biomarker of viral infection (Gray *et al*., 2018; Passaes *et al*., 2021; Nzimande *et al*., 2023). A study by Fujioka *et al*. (1994) was undertaken to evaluate the anti-HIV activity of betulinic, platanic, and dihydrobetulinic acid components from *S. claviflorum* leaves, which exhibited anti-HIV activity by inhibiting the replication of the HIV-1 IIIB in H9 cells, as measured by detecting p24 antigen using enzyme-linked immunosorbent assay (ELISA), without significantly altering cell growth; this indicated that these compounds have a direct effect on viral replication and that the anti-HIV activity not caused by generalized cell cytotoxicity (Fujioka *et al*., 1994; Serna-Arbeláez *et al*., 2021).

2.6.2 Analytical processes (Extraction, isolation, and preparation of samples)

Extraction is the first step to separate the desired natural products from the raw materials. Extraction methods include solvent extraction, distillation, pressing, and sublimation according to the extraction principle (Zhang *et al*., 2018). The extraction of natural products progresses through different stages; firstly, the solvent penetrates the solid matrix, then the

solute dissolves in the solvents, and then the solute is diffused out of the solid matrix, and lastly, the extracted solutes are collected (Li *et al*., 2008; Du *et al*., 2011; Yi *et al*., 2012; Zhang *et al*., 2018). The conventional extraction methods, including maceration, percolation, and reflux extraction, usually use organic solvents and require a large volume of solvents and a long extraction time (Zhang *et al*., 2018). Some modern extraction methods include super-critical fluid extraction (SFC), ultrasound-assisted extraction (UAE), and microwave-assisted extraction (MAE) (Zhang *et al*., 2018).

2.6.2.1 Maceration

Maceration is an extraction technique that is used to extract thermolabile components. The limitation of the maceration is that it results in low extraction efficiency and longer extraction time (Zhang *et al*., 2018). Even though it has some limitations, Ćujić *et al*. (2016) achieved high yields of total phenols and total anthocyanins from chokeberry fruit, suggesting that it is an effective method for extracting phenolic compounds (Ćujić *et al*., 2016). Then, there is Percolation, which is more efficient than maceration because it is a continuous process where saturated solvents are replaced with fresh solvents. It was found that percolation yielded more fucoxanthin than the refluxing method when both were used to extract *Undaria pinnatifida* (Zhang *et al*., 2014a). Reflux extraction cannot extract thermolabile natural products (Zhang *et al*., 2018).

2.6.2.2 Supercritical fluid extraction

Supercritical fluid extraction (SFE) uses supercritical fluid (SF) as the solvent, such as carbon dioxide, water, ethane, methane, ethylene, propane, methanol, propylene, ethanol and acetone (Budisa and Schulze-Makuch, 2014; Uwineza and Waśkiewicz, 2020). Supercritical carbon dioxide (S-CO_2) is widely used in SFE because of its attractive merits, such as low critical temperature (31 °C) and capability to extract thermally labile compounds (Budisa and Schulze-Makuch, 2014; Conde-Hernández *et al.*, 2017). A study by Conde-Hernández *et al*. (2017) extracted the essential oil of rosemary (*Rosmarinus officinalis*) by S-CO_2 extraction, hydro distillation, and steam distillation. The study found that both yields of important oil and antioxidant activity of SFE extract were higher than those from other methods (Conde-Hernández *et al*., 2017).

2.6.2.3 Ultrasonic assisted extraction

Ultrasonic-assisted extraction (UAE), also called ultrasonic extraction or sonication, uses ultrasonic wave energy in the extraction (Zhang *et al*., 2018). Ultrasound in the solvent-producing cavitation accelerates the dissolution and diffusion of the solute as well as the heat transfer, which improves the extraction efficiency (Barba *et al*., 2016). It is applicable for extracting thermolabile and unstable compounds and is commonly used in natural product extraction (Barba *et al*., 2016; Chemat *et al*., 2017). Jovanović *et al*. (2017) achieved a higher yield of polyphenols from *Thymus serpyllum* L. by UAE at an optimized condition (50% ethanol as a solvent; 1:30 solid-to-solvent ratio; 0.3 mm particle size and 15 min time) than maceration and heat-assisted extraction methods (Jovanović *et al*., 2017).

2.6.2.4 Microwaves

Microwaves generate heat by interacting with polar compounds such as water and some organic components in the plant matrix following the ionic conduction and dipole rotation mechanisms (Zhang *et al*., 2018). There are two types of MAE methods: solvent-free extraction (usually for volatile compounds) and solvent extraction (usually for non-volatile compounds) (Cravotto, 2013; Vinatoru *et al*., 2017). Then, separation methods further separated and purified the natural products to obtain the active fraction. Natural products can be split based on various properties, including adsorption properties, partition coefficient, molecular size (membrane filtration and gel filtration chromatography), and ionic strength (Zhang *et al*., 2018). Other modern separation techniques include molecular distillation, preparative gas chromatography, supercritical fluid chromatography, molecular imprinted technology, simulated moving bed chromatography, and multi-dimensional chromatographic separation (Zhang *et al*., 2018).

2.6.2.5 Mixed mode solid phase extraction

Solid-phase extraction (SPE) is a solid-liquid extractive technique that separates dissolved or suspended compounds from other compounds mixed according to their physical and chemical properties. Analytical laboratories use solid-phase extraction to concentrate and purify samples for analysis (Hennion, 1999; Augusto e*t al*., 2013). The SPE uses the affinity of solutes dissolved or suspended in a liquid (the mobile phase) for a solid through which the sample is passed (known as the stationary phase) to separate a mixture into desired and undesired components. The result is that either the desired analytes of interest or undesired impurities in the sample are retained in the stationary phase (Augusto *et al*., 2013).

Suppose the portion retained on the stationary phase includes the desired analytes. In that case, they can then be removed from the stationary phase for collection in an additional step, in which the stationary phase is rinsed with an appropriate eluent (Buszewski and Szultka, 2012). SPE has become a technique of choice for sample clean-up and trace enrichment in pharmaceutical, clinical, food, and environmental analysis (Verette, 2000). Indeed, it offers better versatility and selectivity than other sample preparation techniques (Verette, 2000). Compared with liquid-liquid extraction, it is quicker, requires much less solvent, prevents the possible formation of emulsions, has smaller sample volumes, and is much more amenable to automation (Verette, 2000).

In this study, we used solid phase extraction (SPE) based on multi-dimensional chromatographic separation. Multi-dimensional chromatographic separation combines multiple columns with different stationary phases, improving separation efficiency (Zhang *et al*., 2018). Solid-phase extraction (SPE) is a solid-liquid extractive technique that separates dissolved or suspended compounds from other compounds mixed according to their physical and chemical properties. Analytical laboratories use solid-phase extraction to concentrate and purify samples for analysis (Hennion, 1999; Augusto *et al*., 2013). Different types of solid phase extraction fall into several categories: reversed phase, normal phase, ion exchange (anion/cation), and mixed-mode phase. The SPE efficiently reduces hazardous organic solvents, has no foaming and emulsion formation, has a high recovery percentage, is easy to operate, and can pair with analytical devices (Hamidi, 2023). It has various columns such as Bond Elut-SPE (Sep-Pak C18), hydrophobic/ hydrophilic (Oasis HLB), polymeric cation exchange resins (Bond Elut Plexa PCX or Oasis MCX), and Oasis MAX, Bond which all contain different sorbents (Bertol and Vaiano, 2016; Su *et al*., 2017). In the study, we only used HLB (Hydrophilic-Lipophilic Balance), MCX (Mixed-mode, strong Cation-eXchange), and MAX (Mixed mode, strong Anion-eXchange) cartridges obtained from Waters Corporation,

2.6.3 Immune Functional assays

In vivo and *In vitro* methods of assessing T cell number and functions are delayed-type hypersensitivity, ELISPOT, flow cytometry-based analysis of cytokine expression, and PCR-

based detection of T cell receptor gene usage or cytokine production (Intracellular cytokine staining) (Clay et al., 2001).

1.6.3.1 Delayed-type hypersensitivity (DTH)

Delayed-type hypersensitivity (DTH) reactions (Type IV hypersensitivity) are T-cell-driven inflammatory responses against various bacterial and eukaryotic pathogens. In contrast to Type I, II, and III hypersensitivity reactions mediated by antibodies, DTH reactions are typically antibody-independent (Pepys, 1973; Kobayashi *et al*., 2001). The response is comprised of both sensitization and elicitation (challenge) phases. During the sensitization phase, the host's antigen-presenting cells (APC) take up, process, and present antigenic peptides derived from pathogens to T-cells at the site of the infection (Thorn *et al*., 2015). Sensitized T-cells are formed because of the initial exposure. Upon secondary challenge with the same antigen, a vigorous response by the sensitized T-cells is elicited, accompanied by the release of several cytokines and tissue swelling (Kobayashi *et al.*, 2001; Thorn *et al*., 2015). Because DTH relies on antigen processing by APC and subsequent presentation to T-cells during the elicitation phase, at least 24–72 h are needed for the response to occur, as opposed to antibody-mediated hypersensitivity which may take only minutes to manifest (Liew, 1982; Actor and Ampel, 2009). The DTH responses are associated with immune protection against various infectious organisms, including *Mycobacterium tuberculosis* and *C. albicans* (North and Jung, 2004; Almeida, 2008).

One of the hallmarks of DTH is the production of interferon (IFN)-γ, a cytokine with multiple functions in the immune system including the induction of cytotoxic $CD8^+$ T-cells, promotion of NK cell function, stimulation of antigen presentation, and macrophage activation (Dvorak *et al*., 1974; Tartof *et al*., 1984; Fong and Mosmann, 1989; Morikawa *et al*., 1992; Higashi *et al*., 1995). Unlike IFNα and IFN-β that a variety of cells may produce, IFN-γ secretion is limited to T-helper (T_H)-1 cells, cytotoxic T cells, and NK cells, thus offering a specific biomarker of DTH responses (Iwakura *et al*., 2008).

A study by Smith and White, 2010 explored the sensitization of mice with *C. albicans* followed by footpad challenge with *C. albicans*-derived chitosan, and subsequent measurement of footpad swelling is an established assay to assess DTH responses in mice and to evaluate

potential immunosuppressive compounds (Smith and White Jr, 2010). This assay showed superior sensitivity to the footpad swelling assay.

1.6.3.2 Enzyme-Linked Immunospot assay

The enzyme-linked immunospot (ELISpot) assay is one of the most used immunoassays to measure antigen-specific T cells in mice and humans (Slota *et al*., 2011). Although the ELISpot assay was initially developed to detect antibody-secreting cells (Czerkinsky *et al*., 1983), it has been widely used to evaluate both $CD4^+$ and $CD8^+$ T cells responding to an antigenic or mitogenic stimulus (Taguchi *et al*., 1990; Miyahira *et al*., 1995; Slota *et al*., 2011). ELISpot is a quantitative assay that measures broad range of magnitude of responses and cytokines secretion. The ELISpot assays have various types of assays, such as IFN-gamma, , Granzyme B, TGF-β1, Fluorescent, and single-color ELISpot assay (Slota *et al*., 2011). Among the various types of ELISpot, the IFN-γ-based assay is the most common application of the technique (Slota *et al*., 2011). ELIspot assay is used to evaluate T cells, B cells and innate immune cells functions. It is now not only a research tool but also used in clinical trial assay. In vaccine clinical trials, ELISpot may be used as a biomarker assay to evaluate clinical benefit after therapeutic immune modulation.

The ELISpot assay has been used as a detection method in human tuberculosis because of its higher sensitivity and precision in assessing T-cell-specific responses (Sattah *et al*., 2012). In addition, the ELISpot assay has been used to explore the contribution of different phenotypes of T cells in the immune response to *M. bovis* (Blunt *et al*., 2015; Maggioli *et al*., 2015) and the potential to distinguish different stages of Btb (Veerasami *et al*., 2011; Parthasarathy *et al*., 2012; Steinbach *et al*., 2019).

1.6.3.3 Measurement of Cytokine mRNA Levels by Real-Time Quantitative RT-PCR

Quantitative RT-PCR is a highly accurate molecular method for measuring the levels of transcripts of a gene or genes of interest in sample RNA (Heid *et al.*, 1996; Clay *et al*., 2001). Kruse *et al.* (1997) applied the technique to analyse cytokine mRNA from cryopreserved standard donor blood samples (Kruse *et al*., 1997). A previous study by Kammula *et al.* (1999) used the technique in clinical trials of melanoma peptide-based vaccines to detect

antigen-specific T-cell responses by comparing pre- and post-vaccine samples from melanoma patients. Peripheral blood samples and tumour tissues obtained by fine needle aspiration were evaluated. Quantitative RT-PCR was then used to measure cytokine mRNA levels in the samples (Clay *et al*., 2001). Data were normalized to the expression of a control gene, such as CD8. The study showed that quantitative RT-PCR can detect antigen-specific T-cell responses in peripheral blood samples. Additionally, the localization of antigen-specific T cells to tumour sites was demonstrated by analysis of biopsy samples without any *In vitro* stimulation step (Clay *et al*., 2001).

1.6.3.4 Flow cytometry-based analysis of cytokine expression

Cytokines are polypeptide hormones regulating the growth and function of various cells (Suni *et al.*, 1998; Qiu *et al*., 2014). They execute various pleiotropic actions, including cell growth, differentiation, apoptosis, wound healing, signal transduction, and homeostasis (Baraldi-Junkins *et al*., 2000). Inappropriate activation of the cytokine network is associated with many diseases. Therefore, as cytokines form a complex network and many are regulated through the exact mechanisms, it is critical to profile cytokines expression (Qiu *et al*., 2014). Cytokines in supernatants are customarily measured by ELISA, limiting dilution analysis (LDA), immunohistochemical staining, and other methods (Thorpe *et al*., 1992). Since the cytokine production by individual cell subsets cannot be determined, these methods, though useful, provide only an incomplete picture (Qiu *et al*., 2014). Thus, a method to detect cytokines at the single-cell level would help study the contribution of different cells to cytokine production in heterogeneous cell populations (Qiu *et al*., 2014). However, flow cytometry (FCM), a method to detect cytokines at a single cell level, can be useful in studying the contribution of different cells to cytokine production in heterogeneous cell populations (Sander *et al*., 1991; Qiu *et al*., 2014). The capability of multi-parameter FCM permits the simultaneous detection of two or more cytokines in a single cell with the high throughput inherent to the instrument, giving cytokine staining a tremendous advantage over existing signal-cell methods (Foster et al., 2007).

2.6.4 Cytotoxicity and Cell viability assays

In vitro, cytotoxicity testing of drugs is essential at the early preclinical stage, which decides the fate of a drug candidate in the journey of clinical research. Cytotoxicity screening quantitatively measures the effect of any drug candidates on cell viability and cell growth

and enumerates the cells upon exposure to drug candidates (van Tonder *et al*., 2015; Kuete *et al*., 2017). It is usually determined before commencing the efficacy studies on antiretrovirals. Cell viability is the number of living cells in a specific population, and measuring the number of proliferating cells is a vital indicator for cell survival or death in response to drugs or chemical agents (Adan *et al*., 2016). A broad spectrum of cytotoxicity assays is currently used in toxicology and pharmacology (Gaikwad *et al*., 2023). Cell viability and cytotoxicity assays are based on various cell functions such as cell membrane permeability, enzyme activity, cell adherence, ATP production, co-enzyme production, and nucleotide uptake activity (Ishiyama *et al*., 1996; Özlem Sultan, 2017). Researchers use different types of assays to screen the outcome of developing therapeutics that target mainly cancer cells (Adan *et al*., 2016).

The basic principle of cytotoxicity experiments is based on the ability of the cell to reduce compounds, such as 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyl-2H-tetrazolium bromide (MTT), 2,3-bis (2-methoxy-4-nitro-5-sulfophenyl)- 5-[(phenylamino) carbonyl] -2H-tetrazolium hydroxide (XTT) in presence of candidate drugs (Gaikwad *et al*., 2023). Common experimental platforms have been established based on these reactions. In addition, the Neutral Red Uptake (NRU), Resazurin reduction (RES), and sulforhodamine B (SRB) assays have also been used for cell enumeration (van Tonder *et al*., 2015). The MTT assay is the popular and gold standard method (Lü *et al*., 2012; van Tonder *et al*., 2015). It is a colorimetric assay that measures the enzymatic activity of cellular oxidoreductase and dehydrogenase enzymes present in the endoplasmic reticulum and mitochondria of living cells (Ghasemi *et al*., 2021). These enzymes reduce the tetrazolium compound MTT into its water-insoluble, purple colour formazan crystals (Berridge *et al*., 2005; Ghasemi *et al*., 2021). Here, the formation of formazan crystals is influenced by the cell's metabolic rate and number of mitochondria (Kuete *et al*., 2017). These water-insoluble crystals are then dissolved in dimethyl sulfoxide (DMSO), and the absorbance of the coloured product is measured by a spectrophotometer (Patravale *et al*., 2012). Thus, absorbance is directly proportional to the cell viability, indirectly indicating the level of cellular toxicity. Given this, the dose-dependent toxicity of an investigational drug after exposure to the cells for a specified time interval is determined and compared with the appropriate drug control with a known toxicity profile (Patravale *et al*., 2012).

The results are expressed in terms of CC_{50} value, defining the concentration of the drug candidate at which at least 50% of cells are viable (Kumar *et al*., 2019). Thus, the CC_{50} value measures cellular toxicity and safety, further directing several viral infectivity assays. Likewise, the cytotoxicity screening is performed parallel to the infectivity assays to differentiate between the viral inhibition achieved by the candidate's activity or cell death due to the drug's toxicity or distressed atmospheric conditions (Weyermann *et al*., 2005). The Neutral Red Assay (3-amino-7-dimethylamino-2-methylphenazine hydrochloride) is a measure of viable cells' ability to incorporate and bind to supravital neutral red dye (Griffiths *et al*., 1994; Fotakis and Timbrell, 2006; Perez *et al*., 2017). The lysosomal uptake of neutral red depends on the cells' capacity to maintain pH gradients through ATP production, and it is independent of the enzymatic conversion of dye (Repetto *et al*., 2008). Though the method is sensitive, certain volatile and unstable compounds have presented interferences (van Tonder *et al*., 2015; Kuete *et al*., 2017).

On the other hand, the Resazurin assay (RES), also known as the Alamar blue assay, measures the metabolic activity of a living cell (Vieira-da-Silva and Castanho, 2023). The mitochondrial reductase enzyme occurs mainly in the mitochondria. It converts the nonfluorescent Resazurin to the strongly fluorescent Resorufin that can be monitored using a standard spectrophotometer (Conrad *et al*., 2004). Thus, the quantity of Resorufin is a measure of the metabolic activity of a cell. The assay is simple, rapid, and more sensitive than tetrazolium-based assays (Vieira-da-Silva and Castanho, 2023). The main advantage of Alamar blue is its non-toxic nature, which allows the return of exposed cells to the culture flask for performing any follow-up assays (Gong *et al*., 2020). The Sulforhodamine B (SRB) assay is also based on binding a fluorescent dye to the essential amino acids of cellular proteins. Here, the total protein mass related to the cell numbers can be evaluated colorimetrically (Nakatsu *et al.*, 2006; Orellana and Kasinski, 2016). The advantages include sole dependency on protein content, higher sensitivity and reproducibility, better linearity, and no compound interference (Orellana and Kasinski, 2016; Kuete *et al*., 2017).

Nevertheless, the assay sensitivity reduces with the nonadherent cells. Hence, it has been concluded that the MTT ((3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay possesses the most significant variation in linear range compared to the commonly used cell enumeration assays (Kuete *et al*., 2017). Overall, it indicates the vigilant selection of MTT assay for evaluating cellular viability and toxicity. The experimental data is analysed

like an MTT assay. It has been reported that the assay is less prone to artefacts than the other traditional cell viability assays and is sensitive to detect signals from a minimal number of cells(Gaikwad *et al.*, 2023). Another promising approach for monitoring the effect of drug candidates on cells is the determination of the level of Adenosine Triphosphate (ATP). As ATP is present in all metabolically active cells, it plays an essential role in the energy exchange and, hence, is recognized as a marker of the functional integrity of a cell (Suwara *et al*., 2023). This attribute makes ATP measurement essential while studying living processes. The basic principle of this method is a catalytic reaction between the enzyme Luciferase, obtained from firefly, *Photinus pyralis*, and substrate luciferin (Smale, 2010; Riss *et al*., 2016). The method has high specificity because only the viable cells can provide the ATPs required for the reaction.

The reaction proceeds with two steps response; first, the luciferin is activated to give luciferyl-adenylate and pyrophosphate, followed by the reaction between luciferyl-adenylate and molecular oxygen, yielding oxyluciferin (Oba *et al*., 2003; Gaikwad *et al*., 2023). When excited-oxyluciferin returns to the ground state, green to yellow luminescent light is emitted, and its intensity is then measured using a light-sensitive apparatus, Luminometer (Gosset *et al*., 2020). The readouts are expressed in Relative Luminescence Units (RLU) that are proportional to the concentration of ATP in a cell (Nakatsu *et al*., 2006; Gosset *et al*., 2020).. Moreover, to make the assay compatible with HTS platforms, it has been modified to generate a stable luminescent signal that can tolerate harsh cell lysis conditions and is resistant to luciferase inhibitors found in small molecule libraries. Appreciating these ATP determination assays' sensitivity, specificity, and simplicity, they have been widely utilized in preclinical studies (Gaikwad *et al*., 2023).

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CHAPTER 3

Anti-HIVs and Immunomodulatory properties of fractionated crude extracts isolated from *Alternaria alternata*

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3.1 Abstract

Background: Antiretroviral drugs have reduced the morbidity rate of people living with HIV-1 to a chronic, manageable disease. However, HIV drug resistance, viral reservoir development, and drug toxicity hindered the search for a functional HIV-1 cure. Therefore, there is an urgent need to develop novel anti-HIV drug candidates with improved resistance profiles and reduced drug toxicity or that target different sites of HIV-1 replication and eventually eradicate HIV. Here, we investigate the fractionated crude extract from the endophytic fungi *A. alternata* as a potential anti-HIV and/or immunomodulatory agent.

Materials and methods: *Alternaria alternata* PO_4PR_2 was fractionated using Solid Phase Extraction (SPE) providing different columns, which are MAX (Mixed mode, strong Anion-eXchange), MCX (Mixed-mode, strong Cation-eXchange) and HLB (Hydrophilic-Lipophilic Balance) columns. Cell viability and cytotoxicities of the fractionated crude extract *A. alternata* PO_4PR_2 determined by MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay in the TZM-bl cell lines, followed by the generation of the infectious virus by transfecting 293T cells with NL4.3 plasmid DNA. Then, the antiviral activity of the *A. alternata* PO_4PR_2 was evaluated by a luciferase-based antiviral assay using a TZM-bl cell line. A Time of Addition assay was performed to determine how and when the fractionated crude extract (*A. alternata* PO_4PR_2) inhibits the HIV life cycle. Jurkat cells were infected with pNL4.3, and fractionated *A. alternata* PO_4PR_2 and the known HIV-1 drugs were added at different time intervals (0, 1, 3, 8, 10, 14, 24, 30h) and measured the p24 titer using ELISA. Similarly, to determine the percent inhibition using the TZM-bl luciferase assay, the

TZM-bl cell line was infected with NL4.3, and *A. alternata* PO_4PR_2 was added at different time intervals (1, 3, 5, 6, 8, 10, 16, 20h). Maraviroc, Zidovudine, Raltegravir and amprenavir were used as positive control. To evaluate the immunomodulatory effect of the fractionated crude extracts from *A. alternata* on $CD4^+$ T cell, flow cytometry was performed by infecting the PBMC and staining with fluorochrome labelled monoclonal antibodies for activation (CD38 and HLA-DR) and exhaustion (PD-1).

Results and discussion: Cell cytotoxicity at 50% (CC_{50}) was determined to be 285 µg/mL for the crude extract, and cell viability was above 80% for all the fractions. The fraction eluted using MCX (basic compound & weak and strong cation exchangers) eluted with 0% methanol showed more anti-HIV inhibition than all the other columns with an IC_{50} of 0.03262 µg/mL and 86% inhibition compared to HLB (32-73% inhibition) with an IC_{50} ranging between 0.1474 to 5.146 µg/mL and MAX (40-62 % inhibition) with an IC_{50} ranging between 0.3313 to 5.240 µg/mL. Time of addition results show that crude extract and MCX fraction inhibit the virus during viral binding (62%, 68%), reverse transcription (75%, 98%), integration (82%, 98%) and protease (77%, 59%). The fractionated (5%; MCX, HLB, MAX) (*A. alternata* secondary metabolites) and *A. alternata* secondary metabolites (crude extracts) decrease the $CD4^+$ T cells (CD38 + HLA-DR+) T cell activation compared to treating with AZT and no activation in $CD8^+$ T cells. The crude extracts and the 5% HLB (Hydrophilic-Lipophilic Balance) showed decreased PD-1+CD4+ T cell exhaustion, and HLB and MAX showed a low PD-1+CD8+ T cell exhaustion.

Conclusion: Fractionated crude extract from *A. alternata* (MCX fraction) fungal endophytes has strong inhibition potential during reverse transcription and integration. *Alternaria alternata* crude extracts, and fractionated fractions from MAX and HLB may have compounds that has immunomodulatory with moderate anti-HIV-1 while MCX has less immunomodulation. Endophytic fungi can be the major source of new anti-HIV agents with immunomodulatory effects. Liquid chromatography-mass/mass chromatography can be further used to identify the bioactive compounds from these fractionated crude extracts from *A. alternata*.

3.2 Introduction

The Human Immunodeficiency Virus (HIV) continues to be a significant global health problem despite intense efforts in international and local initiatives to address the pandemic (Govender *et al.*, 2021). Nearly 39.0 million people are currently living with human immunodeficiency virus type I (HIV-1), with approximately 1.3 million people newly infected and 630,000 deaths per year (UNAIDS, 2023). Numerous strides have been made in understanding the molecular mechanisms of pathogenesis of human immunodeficiency virus type 1 (HIV-1) infection since the discovery of the virus in 1983 (Gallo *et al*., 1983; Ahmad *et al*., 1989; Levy, 2009; Levy, 2011). Several critical points in the HIV-1 lifecycle can be targeted for the development of anti-retroviral drugs (Reeves and Piefer, 2005b). Along these lines, many anti-retroviral drugs have been developed to target the viral enzymes reverse transcriptase, integrase and protease, capsids, latency- infected cells and the viral proteins facilitating entry into the host cell (Carr, 2003; De Clercq, 2010). Combined antiretroviral therapy (cART) includes tenofovir, disoproxil fumarate, lamivudine and dolutegravir (Weichseldorfer *et al*., 2021).

Antiretroviral drug development has reduced the HIV-1 burden in people living with HIV. However, they are unable to eradicate the virus from viral reservoirs (latently infected cells), and there is also drug toxicity, including mitochondrial toxicity, hypersensitivity, lipodystrophy, dyslipidaemia and type 2 diabetes, drug-drug interactions and viral rebound upon treatment interruption (Carr, 2003; Chawla *et al*., 2018). In addition, the development of HIV-1 drug resistance development has hindered the success of ART. Therefore, there is still a need to find an anti-retroviral compound that is less toxic and targets multiple sites of HIV-1 infection to eradicate the virus. On the other hand, bioactive compounds isolated from natural biological sources (such as medical plants) offer a vast and unexplored diversity of chemical structures unmatched by even the most extensive combinatorial databases (Strobel and Daisy, 2003; Linnakoski *et al*., 2018). Natural products have served as traditional medicine for thousands of years and still provide the most affordable treatment for diseases in many developing countries (Amzat and Razum, 2018).

Medicinal plants are considered one of the major reservoirs of endophytes with novel bioactive metabolites (Bacon and White, 2000; Selim *et al.*, 2014; Selim *et al*., 2018). Endophytic fungi are one such microbial community that resides inside all plants without

showing any symptoms, with the promise to produce diverse bioactive molecules and novel metabolites that have applications in medicine, agriculture, and industrial setups *(Meena et al*., 2019). These fungi produce biologically active secondary metabolites, which are natural products beneficial to the host (Singh et al., 2003; Singh et al., 2004a; Singh et al., 2004b; Wellensiek et al., 2013). They synthesise various biologically active compounds with diverse biological activities such as, antioxidant, immunosuppressive activities (Gunatilaka, 2006; Ravindran *et al.*, 2012; Yang *et al*., 2018; Hoque *et al*., 2023), cytotoxic (van de Loosdrecht *et al*., 1994; Hoque *et al*., 2023), antibacterial (Radić and Štrukelj, 2012), antiviral (Verma *et al*., 2009), antimicrobial (Malhadas *et al.*, 2017), antimalarial compounds (Ibrahim *et al*., 2018).

Previous studies have reported that an endophytic *Streptomyces* sp. strain isolated from the mangrove plant *Bruguiera gymnorrhiza* produced xiamycin, which exhibits selective anti-HIV activity. Xiamycin (Geometric mean IC_{50} >30 μM) blocks R5, but it did not affect X4 tropic HIV-1 infection (Ding *et al*., 2010; Selim *et al*., 2018). Similarly, curcumin isolated from *Curcuma longa* L has been an effective antiviral agent against many enveloped viruses, including respiratory viruses such as influenza A and Respiratory Syncytial Virus (Praditya *et al.*, 2019). Curcumin and its analogues were also effective in inhibiting HIV replication by targeting HIV protease (Sui *et al*., 1993), HIV integrase (Mazumder *et al*., 1995; Vajragupta et al., 2005), and HIV-1 Tat protein (Ali and Banerjea, 2016; Thimmulappa *et al*., 2021). Previous studies showed HIV-1 inhibition by Alterotoxin V from *Alternaria tenuissima* at an IC_{50} value of 0.9 μM (Bashyal *et al*., 2014). Bashyal *et al*. (2014) suggested that the epoxy perylene structural scaffold in altertoxins may be manipulated to produce potent anti-HIV therapeutics. Melappa *et al*. (2015) also revealed that partially purified coumarins isolated from *Alternaria* sp. showed high HIV-1 inhibition on three viral enzymes, integrase enzyme (98%), reverse transcriptase enzyme (82.81%) and protease activity (78%). These findings from this study emphasise that secondary metabolites from *A. alternata* are a promising source of anti-HIV-1 drug development.

The bioactive compounds from endophytic fungi may have anti-HIV, but that means they act directly on the virus, or they can act on post factors or the immune response, which means they have immunomodulatory effects (Ali Reza *et al.*, 2021). The bioactive compounds derived from endophytic fungi within plants emerge as intriguing agents that

influence the delicate balance of immune responses (Kaur *et al*., 2015). As we advance, immunomodulators have become agents in relieving the pathology associated with viral infections (Suwannarach *et al*., 2020). The study by Abood *et al*. (2014) indicates that secondary metabolites from *Tinospora. crispa* has immunomodulatory effects by stimulating INF-γ, IL-6, and IL-8 expressions. Where the LC-MS phytochemical analysis showed that the *T. crispa* fraction has cordioside, quercetin, eicosenoic acid (paullinic acid), and boldine, which may be responsible for the Immunostimulatory effect of *T. crispa* (Abood *et al*., 2014), another study by Ujam *et al*. (2019) also showed that the endophytic fungi (code name ACL4) extract isolated from fresh leaves of *Ageratum conyzoide* showed immune-stimulatory activity in a dose-related manner with a significant increase in the total white blood cells and neutrophil levels at 100 and 200 mg/kg. The extract also exhibited immune-protective properties by inhibiting the immune-suppressive activity of cyclophosphamide (Ujam *et al*., 2019). These findings show that endophytic fungi bioactive compounds can modulate the immune system by enhancing or suppressing its responses, depending on the context and the target cells.

Nzimande *et al*. (2022) isolated the endophytic fungi from *Hypoxis* spp and isolated the secondary metabolites further, showed that *A. alternata's* secondary metabolites have anti-HIV-1 activity and are anti-cytotoxic in TZM-bl cells, the mechanism of how the *A. alternata's* secondary metabolites inhibit HIV-1, which was not investigated. Therefore, this study aims to elucidates the mechanism of action of the fractionated crude extracts from *A. alternata* by conducting a time-of-addition assay. We further want to evaluate the immunomodulatory properties of the fractionated crude extracts.

3.3 Materials and methods

3.3.1. Cell lines

TZM-bl cells were obtained from the NIH AIDS Research and Reference Reagent Program. The TZM-bl cell line was derived from a HeLa cell clone that was engineered to express CD4, CCR5 and CXCR4 and to contain integrated reporter genes for firefly Luc and *E. coli* β-galactosidase under the control of an HIV-1 long terminal repeat. The Human Embryonic Kidney 293T cell line is a variant of 293T that harbours the SV40 large T antigen, which can bind to SV40 enhancers of expression vectors to increase protein production. Human peripheral blood mononuclear cells (PBMCs) are isolated from peripheral blood and identified as any blood cell with a round nucleus; Jurkat cells are $CD4^+/CD8^-$ T cell lymphoma that expresses an endogenous TCR.

3.3.2. Cell culture

The TZM-bl and 293T/17 cells were cultured in a monolayer in a sterile 75cm^2 culture flask using a complete culture medium (CCM) consisting of Dulbecco's Modified Eagle's Medium (DMEM) (Gibco, Thermofischer scientific, Waltham, MA) supplemented with 10% fetal bovine serum (FBS heat inactivated, gamma irradiated) (LTC Biosciences, Florida US), 25 mM HEPES (Gibco, Thermo fisher scientific, Waltham, MA), and 50 µl/ml gentamicin (Gibco, Thermofischer scientific, Waltham, MA) and was incubated at 37˚C (5% CO_2). Confluent cells were rinsed with PBS (Gibco, Thermofischer scientific, Waltham, MA), trypsinised to resuspend the cells using 0.25% Trypsin-EDTA (Gibco, Thermofischer scientific, Waltham, MA), counted using the trypan blue 0.4% (Invitrogen, Thermofischer scientific, Waltham, MA) method and subculture as required. Trypan blue stain is based on the principle that viable cells do not absorb the dye, whereas non-viable cells do. Jurkat cell line and peripheral blood mononuclear cells (PBMC) were maintained at 37 °C under 5% CO_2 in RPMI-1640 medium (Gibco, Thermofischer scientific, Waltham, MA) supplemented with 10% (v/v) heat-inactivated newborn calf serum (NCS).

3.3.3. Fractionation of *A. alternata (*PO4PR2) using Mixed Mode Solid phase extraction

The *A. alternata* (PO_4PR_2) crude extracts were previously extracted using methanol by Nzimande *et al*. (2022). Solid phase extraction (SPE) was performed following a method described by Abdah *et al.* (2014)*;* Cutignano *et al.* (2015)*;* Kiełbasa *et al.* (2019)*;* Stoszko *et al*. (2020) and Nzimande *et al*. (2022) with some modifications. Endophytic fungal crude extracts were dried at 40 °C followed by sequential fractionation steps. Briefly, lipids were removed by hexane/water extraction 50/ 50 (v/v), 4-mL total volume, and an aqueous phase was collected and dried. After drying, the crude extracts were reconstituted in 50% HPLC grade methanol (MeOH, Sigma Aldrich, South Africa), and 1 mL of this solution was spiked with 20 µL of phosphoric acid (Sigma Aldrich, South Africa) and loaded onto HLB (Hydrophilic-Lipophilic Balance), MCX (Mixed-mode, strong Cation-eXchange), and MAX (Mixed mode, strong Anion-eXchange) cartridges obtained from Waters Corporation, (Prague, Czech Republic). The adsorbed compounds were desalted and stepwise eluted with increasing (5%, 45%, and 95%) methanol (MeOH), providing sample variants, respectively. Eluted fractions of organic compounds were concentrated by evaporation to dryness at 40°C under a gentle stream of nitrogen. Dried fractions were reconstituted with 1 mL of acetonitrile (Sigma Aldrich, South Africa) and filtered with a 0.2 µm filter. The HLB column was based on N-vinylpyrrolidone–divinylbenzene copolymer. MCX was a cation-exchange sorbent representing the HLB material modified with SO3H− groups, and MAX was an anion-exchange cartridge. 0.2% DMSO was used as a solvent to dissolve the dried fraction and the crude extract. The dissolved fractions and the crude extract were screened for cytotoxicity and antiviral activity.

3.3.4. Screening of the cytotoxic effects of *Alternaria alternata* using MTT (3-[4,5-dimethylthiazol-2-yl]-2,5 diphenyl tetrazolium bromide) assay

Cell cytotoxicity and viability were determined using MTT assay according to the manufacturer's instructions (Thermo Fischer; South Africa) (Berridge *et al.,* 2005; Kuete *et al*., 2017). Briefly, TZM-bl cells were seeded (15,000 cells/ 200 µL DMEM/well) in a 96-well culture plate and incubated at 37°C, 5% CO_2. Then 25 µL of each fractionated crude extracts(MAX, MCX, HLB) with starting concentration of 285 µg/mL, and were serially diluted in DMEM containing 10% heat-inactivated fetal bovine serum, 50 µg/mL

Gentamycin, and 25 mM Hepes (LTC Biosciences, Florida US) buffer in a 96-well plate (The Scientific group, Maspeth, NY). Azidothymidine (AZT) at 300 µg/ml was used as the positive control, DMEM only was used as a blank and uninfected cell as the negative control. The plates were incubated for 24 h at 37°C, 5% CO_2. Following incubation, the supernatant (treatment medium) was removed, and 120µL of MTT solution comprising 100µl fresh DMEM and 20µL of MTT (Thermofischer; South Africa) (5mg/mL MTT salt in 0.1M PBS) was added to each well. The plate was then incubated for 4 hours (37℃, 5% CO_2). The MTT solution was discarded, and 100µL of dimethyl sulfoxide (0.2% DMSO) was added to each well and then incubated for 1 hour at 37℃ to dissolve the formazan crystals. The optical density was measured using a Victor Nivo multimode plate reader at 540 nm (PerkinElmer Inc. United States). The results obtained from the absorbance measurements were used to calculate the percentage cell viability by using the following equation: $(\%)\ Cell\ Viability = \frac{(Sample\ absorbance - Cell-free\ sample\ blank)}{(Mean\ media\ control\ absorbance)}\ x100.$

The MTT cell cytotoxicity assay was repeated three times in different days, in order to check the reproducibility and the trustworthy of the results.

The maximum cytotoxicity concentration resulting in 50% cell viability (CC_{50}) was obtained using GraphPad Prism version 5.01(San Diego, USA) by plotting a dose-response curve (concentration versus the percentage cell viability of the samples). `

3.3.5. Generation of viruses (pNL4.3) by transfection

The HIV-1 NL4.3 viruses were prepared by transfecting 293T cells (5×10^6 cells in 12 mL growth medium in a T-75 culture flask) with 12 µg NL4.3 plasmid DNA (wild type) (NIH AIDS Research and Reference Reagent Program, USA), using PolyFect transfection reagent FuGENE 6 reagent (Promega, Madison, Wisconsin, US) in the growth medium, as described by the manufacturer (Jacobsen et al., 2004). The transfection complexes were allowed to incubate for 30 minutes at room temperature (18°-25°C), after which they were added to 293T/17 cells in a T-75 culture flask and incubated for 48-72 hrs with one change of medium after 6 hrs. Virus-containing culture fluid was removed from the flasks, filtered through a 0.45 µm filter to eliminate cell debris, and stored at -80℃. The NL4.3 virus 50% tissue culture infective doses ($TCID_{50}$) were determined by performing 5-fold dilutions in quadruplicate in a growth medium in 96-well culture plates (The Scientific group, Maspeth, NY). Freshly trypsinised TZM-bl cells (10,000 cells in 100 µL volume) were added to each well in a DMEM containing an optimized concentration of DEAE-dextran (44 µg/mL) (Thermo Fisher, South

Africa). The culture plates were incubated for 48 hours. Culture fluid (100µL) was removed from each well and replaced with a Bright-Glo reagent per the manufacturer's recommendation (Promega, Madison, Wisconsin, US). After a 2-min incubation at room temperature to allow cell lysis, 150 µL of cell lysate was transferred to a 96-well black solid plate (Costar, Germany). The concentration that elicits a 50 000 relative luminescence unit (RLU) was determined using a luciferase assay using a Victor 2 luminometer (Perkin-Elmer Life Sciences, Shelton, CT). The experiment was repeated several times until the virus infectivity elicit more 50,000 RLU was obtained.

3.3.6. Luciferase-based antiviral assay

The viral inhibition of fungal extracts was evaluated by luciferase-based antiviral assay according to Sarzotti-Kelsoe *et al*. (2014); Cherne *et al*. (2019) and Nzimande *et al*. (2022). Briefly, 11µL of each drug sample was serially diluted (10-fold) in 140 µL of DMEM (supplemented with 10% heat-inactivated fetal bovine serum, 50 µg/mL gentamycin and 25 mM HEPES buffer), taking 50 µL each time serial dilutes in a 96-well flat bottom culture plates (Whitehead scientific, SA). The ranging starting concentration for HLB, MAX, MCX fractions was between 2-74 µg/mL, 285 µg/mL for the crude extract and 300 µg/mL for AZT (Nucleoside reverse transcriptase inhibitor) which was used as a positive drug control. The TZM-bl cells were infected with HIV-1 $NL_{4.3}$ virus $TCID_{50}$ (50 µL/well) except for the cell control (negative control). Then, the plate was covered and incubated for 1 hour at 37°C, 5% CO_2. A suspension of TZM-bl cells was prepared approximately 10-15 minutes prior to the use, at a density of 1×10^5 cells/mL in D-MEM containing DEAE dextran (44 µg/mL) (Thermo Fisher, South Africa). Then, 100µL of cell suspension was dispensed to all wells (10,000 cells/well). Then, the plates were covered and incubated for 48 hours at 37°C, 5% CO_2. Then 150 µL of culture was removed from each well and replaced 100µL of Bright-Glo luciferase reagent (Promega, Madison, Wisconsin, US). The plates were incubated in the dark at room temperature for 2 minutes to allow complete cell lysis. Then 150µL was transferred to a corresponding 96-well black plate (Costar, Germany), and the results were read immediately in a Victor Nivo multimode microplate reader at 540 nm (PerkinElmer; United States). The percentage inhibition was determined by calculating the difference in average RLU between the test wells (cells+ drug+ virus) and the cell control wells (cells only, column 1), dividing the results by the difference in average RLU virus control. The half-maximal inhibitory concentration (IC_{50}) was calculated using GraphPad Prism Software (v.5.00.288)

(San Diego, USA). Luciferase antiviral assay was repeated three times and the average was used.

3.3.7 The mode of action of the fractionated *A. alternata* crude extracts and its inhibitory mechanism in the HIV-1 life cycle using time of addition assay

(i). ELISA HIV-1 p24 Time-based assay to measure the p24 titre

Time of Addition (TOA) assay determined the stage of the HIV replication cycle with which this anti-HIV compound interferes. Time of Addition assay was performed according to Daelemans *et al*. (2011); Wang *et al*. (2011) and Corona *et al*. (2020), with some modifications. Briefly, the Jurkat cell line was seeded (50,000 cells/ 500 µL/well) in a 24-well culture plate (incubated at 37°C, 5% CO_2), then the cells were infected with HIV-1 $NL_{4.3}$ virus $TCID_{50}$ (100 µL/well). Different ARVs, *A. alternata* crude extract and 5% MCX (which had more anti-HIV activity compared to all the other fractions) were added (25 µL/well) to the infected cells at different time intervals (0, 1, 3, 8, 10, 14, 24 and 30h) during the replication cycle of the HIV-1 virus. The positive drug controls were maraviroc (CCR5 antagonist, 0-1h), azidothymidine (nucleoside reverse transcriptase inhibitor 3-8h), Raltegravir (integrase inhibitor 10-12h) and Amprenavir (protease inhibitor 16-24h). The HIV-1 p24 titer in the culture supernatant was measured using the Quicktiter Lentiviral Quantification kit (Cell Biolabs Inc., San Diego, CA, US) following the manufacturer's instructions. The multimode Victor Nivo microplate reader measured the p24 level at 480 nm (PerkinElmer; United States).

The viral titer was calculated as per the manufacturer's instructions as follows:

Calculate Viral Titer:

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

1 ng lentiviral RNA = (1x10-9) g / (8,000 bp x 660 g/bp) X 6 x 1023 = 1.1 x 10^8 VP

$$\text{Virus Titer (VP/mL)} = \frac{\text{Amount of lentiviral RNA (ng) X 1.1 x 108 VP X (20 µL/5 µL)}}{Viral\ sample\ volume\ (mL)}$$

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

(ii). Luciferase Time-based assay to determine the percentage inhibition

Time of Addition assay was performed according to Lara *et al*. (2014) with some modifications. Briefly, TZM-bl cells were seeded (10,000 cells/ 150 µL/well) in a 96-well culture plate (incubated at 37°C, 5% CO_2), then the cells were infected with HIV-1 $NL_{4.3}$ virus $TCID_{50}$ (50 µL/well). Different ARVs, *A. alternata* crude extract and fractionated extract using 5% methanol in MCX were added (15 µL/well) to the infected cells at different time intervals (1, 3, 5, 6, 8, 10, 16 and 20h) during the replication cycle of the HIV-1 virus. Then the plate was incubated at 37°C, 5% CO_2 for 48 hrs. The positive drug controls were maraviroc (CCR5 antagonist, 0-1h), azidothymidine (nucleoside reverse transcriptase inhibitor 3-8h), Raltegravir (integrase inhibitor 10-12h) and Amprenavir (protease inhibitor 16-24h). After 48 hrs, 150 µL of culture was removed from each well and replaced 100µL of Bright-Glo luciferase reagent (Promega, Madison, Wisconsin, US). The plates were incubated in the dark at room temperature for 2 minutes to allow complete cell lysis. Then 150µL was transferred to a corresponding 96-well black plate (Costar, Germany), and the results were read immediately in a Victor Nivo multimode microplate reader at 540 nm (PerkinElmer; United States). The percentage inhibition was determined by calculating the difference in average RLU between the test wells (cells+ drug+ virus) and the cell control wells (cells only, column 1), dividing the results by the difference in average RLU virus control.

3.3.8. Effect of the fractionated crude extracts from *A. alternata* on $CD4^+$ and $CD8^+$ T cell activation and exhaustion

(i) Flow cytometry staining

Peripheral Blood mononuclear cells (PBMC) from HIV-1 negative individuals were previously stored (FRESH cohort) in liquid nitrogen. Female Rising through Education, Support and Health (FRESH) cohort is an ongoing prospective cohort study in KwaZulu-Natal, South Africa, designed to diagnose acute HIV infection in young at-risk women, obtain blood and mucosal samples before and after infection, and enable the study of HIV pathogenesis and biological and behavioural risk factors for HIV acquisition, as well as research on vaccine and cure strategies. PBMCs were thawed and rested for 2 hours. Briefly, 8ml of pre-warmed R10 (R10: RPMI, 10% FBS, 1% glutamine, 1% Pen Strep, 1% HEPES) was aliquoted into a 15mL falcon tube, and the cryopreserved cells were thawed in 37℃

water bath until the pellet was half thawed. The thawed PBMC cells were pre-warmed in 8ml R10 media and centrifuged at 1500 rpm for 10 minutes (room temperature). The supernatant was discarded and resuspended with 10ml R10 and centrifuged at 1500 rpm for 10 minutes (room temperature). The supernatant was discarded, and the cells pellet was resuspended in 2mL R10 (For approximately 10 million cells) and rested at 37℃ in 5% CO_2 for at least 2 hours (falcon tube cap loosen).

Five HIV-1 infected PBMCs and five negative PBMCs were treated with the fractionated fractions, including the crude extracts. Untreated HIV-1 negative PMBC were also included, and AZT was used as a drug control. Furthermore, a suspension of PBMC was prepared at a density of 1×10^5 cells/well in R10 in a 24-well culture plate, followed by the addition of 20 µL of *A. alternata* crude extract and different fractions (MAX, HLB, MCX) and AZT was used as a positive control. Then 1500 µ*L* of cell suspension was dispensed to each well, followed by the addition of 100 µ*l* of NL4.3 ($TCID_{50}$) was dispensed in each well except for the negative control (uninfected PBMC).

The proportion of T cells expressing activation (HLA-DR and CD38) and exhaustion (PD-1) markers was determined in fresh PBMC as previously described (Chachage et al., 2014). Briefly, fresh PBMC samples were incubated for 30 min using the following fluorochrome-labelled monoclonal antibodies (mABs): CD3-BV650 (BD), CD4- APC (eBioscience, San Diego, US), CD8- FITC or CD56- BV510, CD45RO APC, HLA-DR PEcF and CD38 BV711 (BD bioscience, New jersey, US). The acquisition was performed on a FACS CANTO II (BD). Compensation was conducted with antibody capture beads (BD) stained separately with the individual antibodies used in the test samples. Flow cytometry data was analysed using FlowJo (version 9.5.3; Tree Star Inc.).

3.3.9. Statistical analysis

GraphPad prism V5.0 software (GraphPad Software Inc., La Jolla, USA.) was used for data analysis. The IC_{50}s or CC_{50}s were calculated using the inhibitor dose-response function (GraphPad Software, CA). Flow cytometry data was analysed using FlowJo (version 9.5.3, Tree Star Inc.) and GraphPad prism V5.0 software was used to draw the graphs using unpaired t-test and non-parametric tests (Mann-Whitney test).

3.4 Results

3.4.1. Cytotoxicity and Cell viability of the fractionated fractions of *A. alternata* using MTT assay

The MTT ((3-(4, 5-dimethylthiazolyl-2)-2, 5-diphenyltetrazolium bromide) assay was performed to determine cell viability and cell cytotoxicity for crude extract and different fractions (5% MeOH MCX, MAX, HLB) of *A. alternata* in TZM-bl cell line. The results show that the crude extracts and fractions had 80% cell viability when tested in TZM-bl cell line (Figure 3.1A) and the cell cytotoxicity (figure 3.1B and C). However, the viability remained above 80% for crude extract, fractions, 0.2% DMSO (solvent) and AZT (positive drug control) [Figure 3.1].

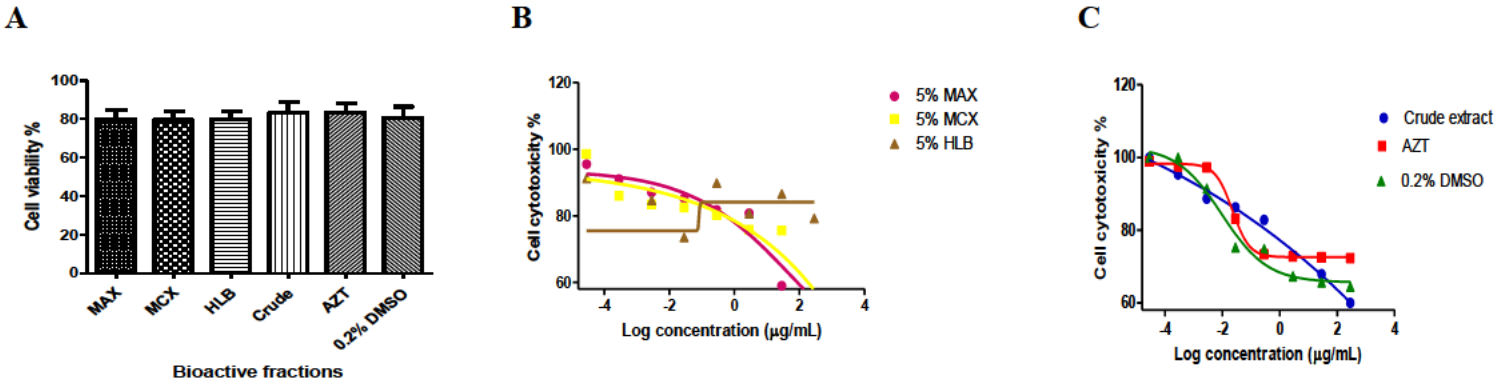


Figure 3. 1: Cell viability **(A)** and cytotoxicity **(B and C)** of the crude and *A. alternata* PO_4PR_2 fractions (MCX, HLB, MAX) were determined by MTT assay using the TZM-bl cells. The y-axis shows the cell viability percentage, and the x-axis represents the fractionated crude extract, AZT and solvent DMSO. **(A)** The percentage of cell viability in TZM-bl cell lines remained above 80% after the TZM-bl cells were treated with *A. alternata* crude extract and fractions (5% MAX, MCX, HLB) prepared using SPE. The dose-response curve was used to determine the CC_{50} value of *A. alternata* in TZM-bl cells. **(B and C)** shows the cell cytotoxicity at a 50% (CC_{50}) curve of 5% MAX, MCX, HLB, AZT, Crude extract and 0.2% DMSO. The y-axis shows the cell cytotoxicity in percentage, and the x-axis shows the log of concentration (µg/mL). **(B)** MAX fraction ($CC_{50=}$ 35.31 µg/mL), the yellow line represents the MCX fraction (CC_{50} =6231 µg/mL), and the brown line represents the HLB fraction (CC_{50} =81.81 µg/mL), the cell viability was between 50-80% in all the concentrations. **(C)** crude extract (CC_{50}= 285 µg/mL), the red line represents the AZT (CC_{50} =1041 µg/mL), and the green line represents 0.2% DMSO solvent (CC_{50} =901 µg/mL). The actual concentrations are 285;28.5; 2.85; 0.285; 0.0285; 0.00285; 0.000285; 0.0000285 µg/mL and they were transformed into log scale as follows; 2.45; 1.45;0.45; -0.55; -1.55; -2.54; -3.55;-4.55 so that the graph will be clear and the cell cytotoxicity at 50% concentration was determined. At

lowest concentration the cell viability is 100% (log-4) and highest concentration is 60% (log 2.45).

3.4.2. The anti-HIV-1 effects of *A. alternata* crude extract and fractionated fractions (MCX, MAX, HLB) using Luciferase-based antiviral assay

The virus inhibition of *A. alternata* crude extracts and fractions was evaluated using a luciferase-based antiviral assay on the TZM-bl cell line of the crude extract of *A. alternata* showed 100% HIV-1 inhibition at highest concentration (285 µg/mL) , and AZT (nucleotide reverse transcription inhibition) was a positive control with 100% HIV-1 inhibition (Figure 3.2). At 50% inhibition of crude extract, the inhibitory concentration was 2.2389 µg/mL and 0.1322 µg/mL for AZT. The 0.2% DMSO, used as a solvent, was also included. The HIV-1 inhibition from the 0.2% DMSO was only 17% with an IC_{50} of 26.2647 µg/mL. Figure 3.3 and Table 3.1 show the HIV-1 inhibition (%) versus the fractions prepared using SPE. The antiviral activity was also tested for 0%, 5%, 45% and 95% methanol for all three fractions (Figure 3. S1, S2 and S3).

The 0% methanol represents fractions passed through the column without methanol instead water was added, and (5,45 and 95%) represent fractions passed through increasing methanol gradient. The HIV-1 inhibition decreased with increasing methanol gradient in all the fractions (Table 3.1). The HLB fraction decreased from 64 to 32%, the MAX fraction decreased from 67 to 40 %, and the MCX fraction decreased from 77 to 53%. In addition, the selective index (SI) was calculated for 5% MeOH (MAX, MCX and HLB) fractions, AZT, and Crude extract to provide the most potent activity. The selective index for 5% MeOH (MAX, MCX and HLB) fractions, AZT, and Crude extract were 6.73, 17217, 0.0159, 7874 and 119, respectively. Lastly, we assessed the antiviral activity of the crude extract and MCX fraction (demonstrated good antiviral activity) against all the positive drug controls used to carry out the time of addition assay to get the concentration of an inhibitor where the response (or binding) is reduced by half (IC_{50}) (S1). We selected the MCX fraction, which showed the highest potency to inhibit HIV-1 in the TZM-bl cell line.

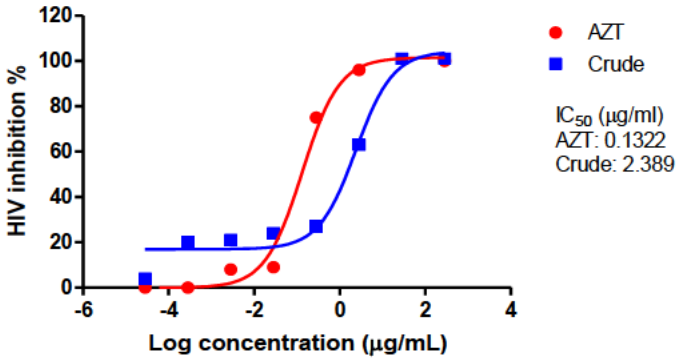


Figure 3. 2: The HIV-1 percentage inhibition curves of *A. alternata* crude extracts were tested with a luciferase-based antiviral assay using TZM-bl cell lines. TZM-bl cells were infected with HIV-1 (NL4.3) wild type and treated with the serial dilution of *A. alternata* crude extracts with AZT as positive control and incubated for 48 h at 37°C and 5% CO_2. The y-axis represents the percentage of HIV inhibition at different concentration, and the x-axis shows the log concentration in µg/mL. The actual concentrations are 285;28.5; 2.85; 0.285; 0.0285; 0.00285; 0.000285; 0.0000285 µg/mL and they were transformed into log scale as follows; 2.45; 1.45;0.45; -0.55; -1.55;-2.54; -3.55;-4.55. The red line represents the positive drug control AZT, and the blue line represents the fractionated fractions. The crude extract HIV-1 inhibition is comparable to AZT with an IC_{50} of 2.389 (µg/mL) and AZT of 0.1322 (µg/mL). Then 0.2% DMSO was used to dissolve the samples in 7.455e+006 (µg/mL) (not shown).

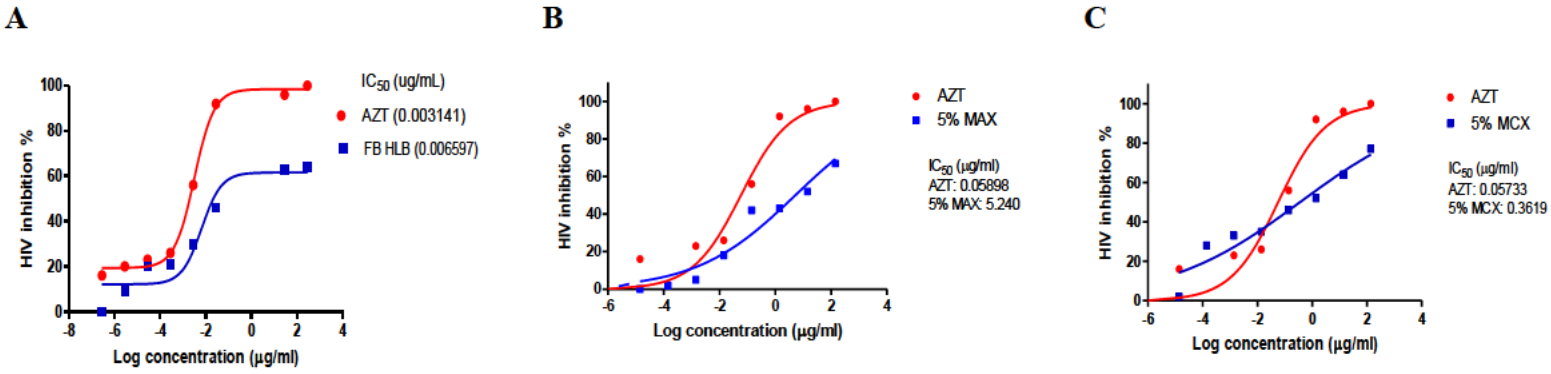


Figure 3. 3: Dose-dependent curves showing the HIV-1 percentage inhibition of *A. alternata.* A. crude extract, B. fractions with 5% methanol in MAX column fractions (acidic compounds), C. 5% methanol HLB fraction (neutral compounds) and D. 5% methanol MCX fractions (basic compounds). The y-axis represents the percentage of HIV inhibition, and the x-axis shows the log concentration in µg/mL. The red line represents the positive drug control AZT, and the blue line represents the fractions. The 5% MCX fraction shows high anti-HIV-1 (%) with an IC_{50} of 0.3619 (µg/mL) compared to 5% HLB 0.00659 (µg/mL) and 5% MAX fraction with an IC_{50} of 5.240 (µg/mL).

Table 3. 1: HIV-1 inhibition (%) and the IC_{50}s of all *A. alternata* fractionated fractions on TZM-bl cells.

Fractions	IC_{50} (µg/mL)	% HIV inhibition (highest concentration in ug/mL)
5% HLB	0.006597	64 (285)
45% HLB	0.1474	48 (285)
95% HLB	708.1	32 (285)
5% MAX	5.240	67 (143)
45% MAX	0.03843	63 (143)
95% MAX	0.03313	40 (143)
5% MCX	0.3619	77 (139)
45% MCX	36494	74 (139)
95% MCX	0.9103	53 (139)

Table 3. 2: Cell cytotoxic concentrations (CC_{50}), HIV inhibition concentration IC_{50}'s of *A. alternata* extracts on TZM-bl cells. The selective index was determined by dividing the cell cytotoxicity (CC_{50}) and the HIV-1 inhibitory concentration at 50% (IC_{50}). The average of three experiments was taken.

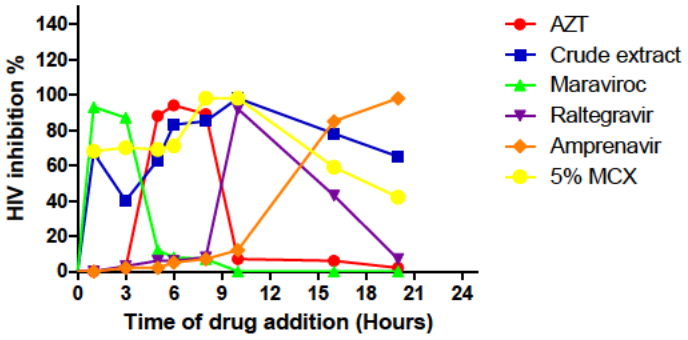
Extract	CC_{50} (µg/mL)	IC_{50} (µg/mL)	Selective index (CC_{50}/ IC_{50})
5% MAX	35.31	5.240	6.73
5% MCX	623.1	0.3619	17217
5% HLB	81.81	0.006597	12.4
Crude	285	2.389	119
AZT	1041	0.1322	7874

3.4.3. The mode of action of the active compound and its inhibitory mechanism in the HIV-1 life cycle using time of addition assay

To determine the active compound's mode of action and inhibitory mechanisms in the HIV-1 life cycle, the time of addition was carried out using Luciferase time-based assay (Figure 3.4A) and ELISA p24 time-based assay (Figure 3.4B). A time-of-addition experiment was conducted to determine the exact inhibition target of *A. alternata* crude extract fractionated fraction (5% MCX) because it showed more antiviral activity than the other fractions. In quantifying the p24 Quicktiter Lentiviral Quantification kit (Cell Biolabs Inc., San Diego, CA, US), Jurkat cell lines were infected with NL4.3 and treated with crude extract at different times. The crude extract showed high HIV-1 inhibition during HIV-1 entry (at 1 hour) because it was able to decrease the HIV-1 p24 titer from 75.187 pg/mL (virus control) to 11.22 (pg/mL) compared to the positive control drug (Maraviroc).

Additionally, the crude extracts decreased the HIV-1 p24 titer till integration. The HIV-1 p24 titer of 5% MCX inhibited the HIV-1 entry (18.53 pg/mL) and to reverse transcription (3-8 hours) to 9.06 and 11.89 (pg/mL). Concurrently in figure 4A, the luciferase time-based assay showed that 5% methanol in MCX has HIV-1 inhibition ranging from 68% and 98% between 0-8 hours (HIV-1 entry, reverse transcription, and integration) have more anti-HIV activity compared to the crude extract (67% and 83%) during the virus entry and reverse transcription. Nevertheless, the 5% MCX fraction showed to have high HIV-1 inhibition because it was able to decrease the concentration of p24 from 75.187 pg/mL (virus control) to 19.61 (pg/mL) compared to the crude extract (22.89 pg/mL) during HIV-1 integration (10-12 hours) and to 20.13 and 43.33 (pg/mL) during proteolysis (16-24 hours). Meanwhile, the TZM-bl cell line displayed that 5% MCX (68% and 98%) had more anti-HIV activity than the crude extract (67% and 83%) during the virus entry and reverse transcription. The crude extract (64%) has high anti-HIV activity during proteolysis compared to the 5% MCX fraction (59%). Both crude extract and the 5% MCX fraction have the same anti-HIV activity (98%) during integration.

A



B

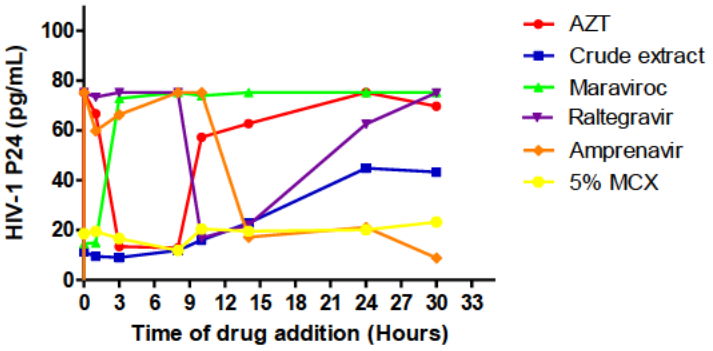


Figure 3. 4: Time of intervention in the HIV replication cycle of a fractionated *A. alternata* (A) Luciferase-based time of addition assay, TZM-bl cell lines infected with HIV-1 $NL_{4.3}$ at a high multiplicity of infection (0.5). Treatment was added at different time intervals (1, 3, 5, 6, 8, 10, 16, 20 hours). The y-axis represents the percentage of HIV inhibition, and the x-axis shows the time of drug addition in hours. **(B)** the time of addition assay using HIV-1 p24 ELISA, Jurkat cell lines were infected with HIV-1 $NL_{4.3}$ at a high multiplicity of infection (0.5). The y-axis represents HIV-1 P24 concentration (pg/mL), and the x-axis shows the time of drug addition in hours. Figure 4 B quantified the virus supernatant by monitoring the antigen of the virus-associated Gag core p24 (Quicktiter Lentiviral Quantification kit). The positive drug controls were maraviroc (CCR5 antagonist), azidothymidine (nucleoside reverse transcriptase inhibitor), Raltegravir (integrase inhibitor) and Amprenavir (protease inhibitor). The data shows that crude extract has minimal inhibition during different stages in an HIV life cycle. The most predominant is the integration, which shows 98% inhibition. The MCX fraction inhibits HIV-1 throughout the cycle.

3.4.4. Evaluation of the fractionated crude extracts from *A. alternata* effects on CD4^{+} and CD8^{+} T cell activation and exhaustion

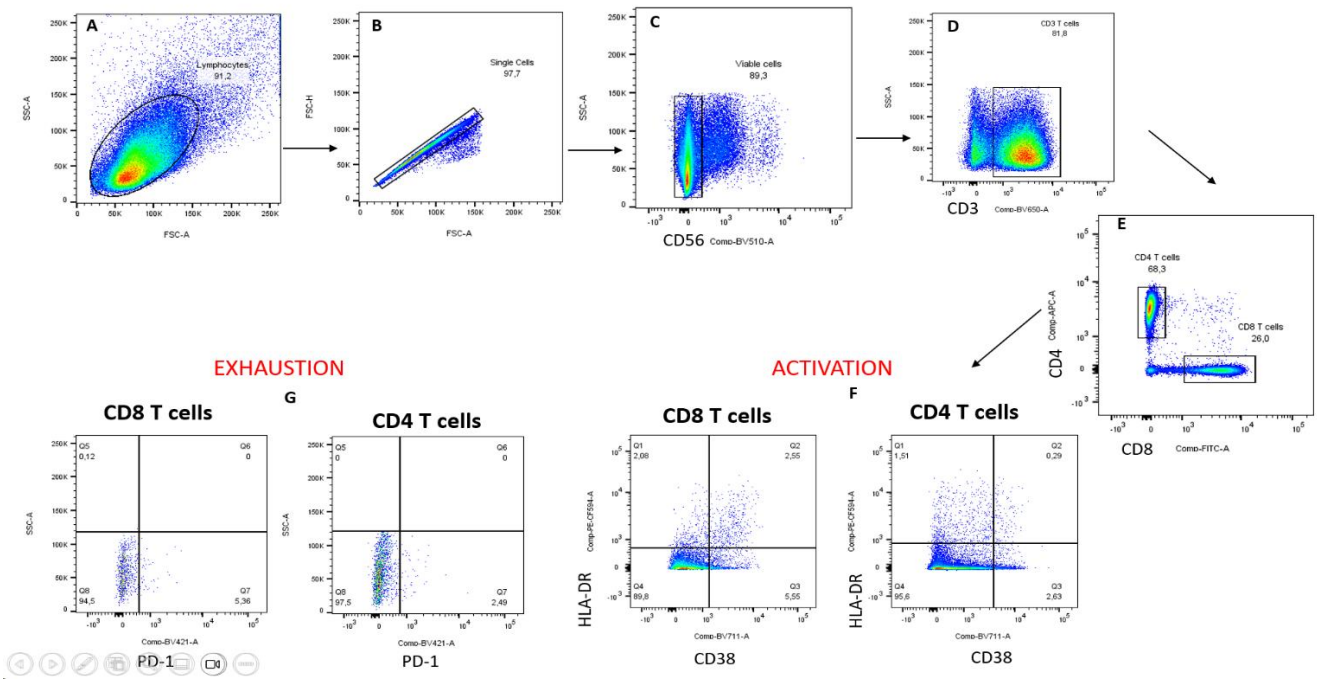


Figure 3. 5: The gating strategy for T cell subpopulation analysis: a) Lymphocytes were gated based on forward and side scatter area; b) From lymphocytes, we gated the single cells based on forward scatter height and side scatter area; c) Then from single cells, we gated viable cells based on side scatter area and CD56+ T cell population (BV510-A); d) Then from the viable cells we gated CD3+ T cell population based on side scatter area and CD3+ T cell population (BV650); e) From CD3+ T cell population we gated for CD4+ T cell population and CD8+ T cell population based on CD4+ T cell (APC) and CD8+ T cell (FITC); f) Then from CD4 T cell population and CD8+ T cell population we evaluated the activation based on HLA-DR (PE-CF) and CD38 (BCV711); g) We evaluated the exhaustion of CD4 T cell population and CD8 T cell population based on side scatter area and PD-1.

Flow cytometry

Flow cytometry staining was used to analyse T cell activation and exhaustion to investigate whether the crude extract and fractionated *A. alternata* PO4PR2 fractions (MCX, HLB, MAX) with antiviral activity may also possess immunomodulatory effects. Infected PBMCs and uninfected PBMCS were treated with crude extract and fractionated fractions (MCX, HLB, MAX), and AZT was used as a positive drug control. The PBMCs were stained using fluorochrome-labelled monoclinal fluorochrome-labelled monoclonal antibodies for activation (CD38 + HLA-DR) and exhaustion (PD-1). CD38+HLA-DR+CD4^{+} T cell

activation treated crude and fractionated fractions (MCX, HLB, MAX), and AZT was used as a positive control shows the low CD38+HLA-DR+$CD4^+$ T cell activation of the crude extract (Figure 3.6). There was a significant increase in CD38+HLA-DR+$CD4^+$ T cell activation when treated with fractionated *A. alternata* using MAX column in HIV-1 infected PBMCs with MAX (p-value = 0.0278) (Figure 3.6 D) and HLB fraction (-value = 0.0019) (Figure 3.6 C). There was a significant decrease in CD38+HLA-DR+$CD4^+$ T cell activation in HIV-1 infected PBMCs treated with the *A. alternata* MCX fraction (p-value = 0.0050) (figure 3.6B). No CD8+ T cell activation was observed in both treated, untreated, infected, and uninfected PBMCs (results not shown)

The infected PBMCs treated crude extract showed no significant difference in PD-1+$CD4^+$ T cell exhaustion compared to AZT (Figure 3.7 A). However, there was a significant increase in PD-1+$CD8^+$ T cell exhaustion in HIV-1 infected PBMCs treated with *A. alternata* crude extract compared to HIV-negative PBMC (p-value = 0.0112) (Figure 3.8 A). Moreover, the HIV-1 infected PBMCs treated with *A. alternata* MCX fraction had no significant increase in PD-1+$CD4^+$ and PD-1+$CD8^+$ T cell exhaustion (Figure 3.7.B and 3.8.B) (p-value = 0.0649). The HIV-1 infected PBMCs treated with HLB significantly increased PD-1+CD4+ T cell exhaustion (p-value = 0.0119) (Figure 3.7 C). However, there was a significant low PD-1+$CD8^+$ T cell exhaustion in HIV-1 infected PBMC treated with fractionated *A. alternata* HLB compared to HIV-1 negative (p-value = 0.0079) (Figure 3.8 C), AZT treated remained high. The HIV-1 infected PBMCs treated with fractionated *A. alternata* using MAX showed no significant increase in PD-1+CD4+ T cell exhaustion when compared with HIV-1 negative PBMC (Figure 3.7 D) and a no significant increase in the PD-1+$CD8^+$ T cell exhaustion (p-value = 0.278) (Figure 3.8 D).

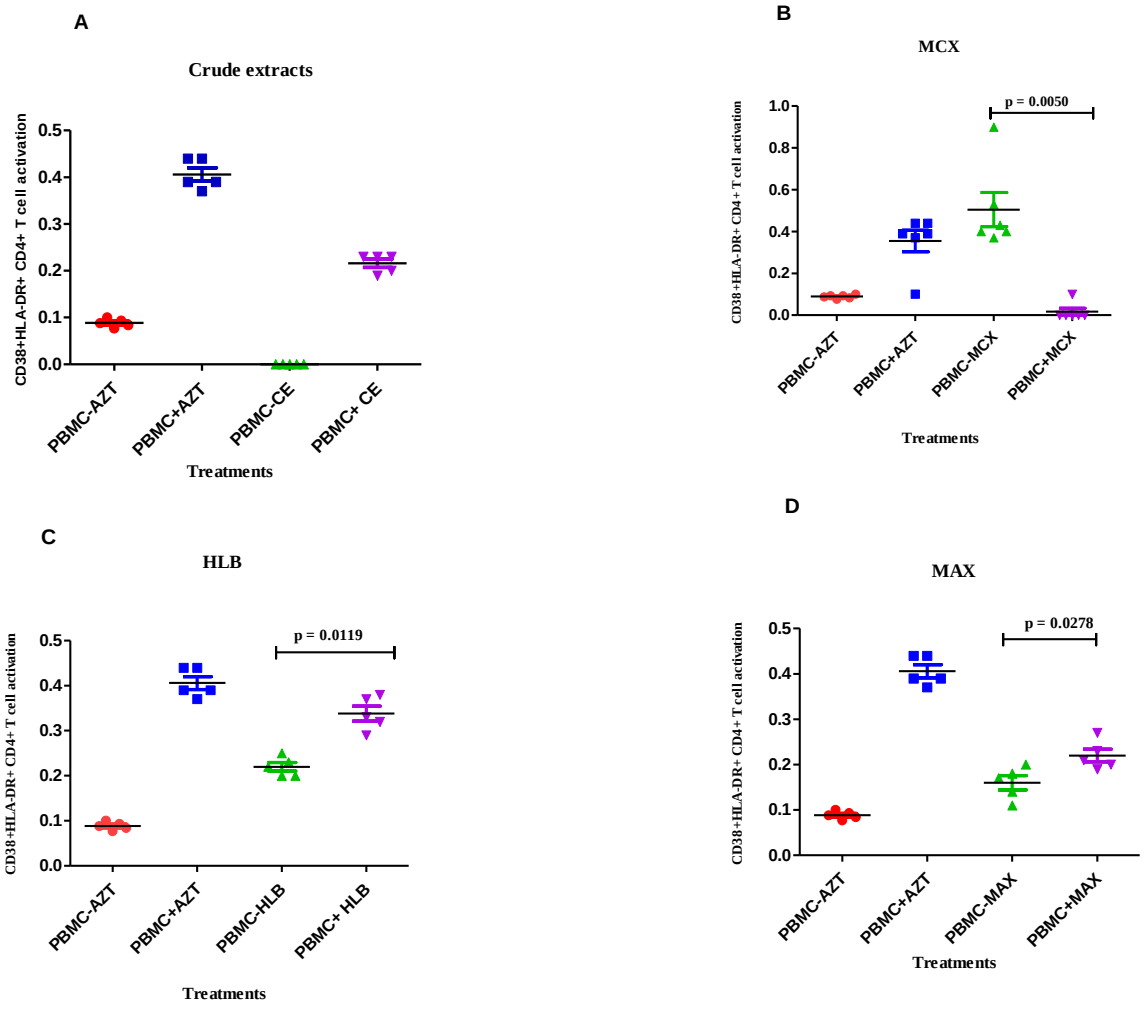


Figure 3. 6: The $CD4^+$ T and $CD8^+$ T cell activation of the crude and fractionated *A. alternata* PO_4PR_2 fractions (MCX, HLB, MAX) was determined on PBMCs using flow cytometry. The y-axis shows (%CD4 + CD38 + HLA-DR) T cell activation, and the x-axis indicates the treatments where PBMC represents uninfected PBMCs- and HIV-1 infected PBMCs by PBMC+. Azidothymidine (AZT) was used as a positive drug control. **(A)** The results show that the crude extracts had a non-significant low CD38+HLA-DR+$CD4^+$ T cell activation on both positive and negative PBMCs compared to AZT. **(B)**. The MCX fraction showed a significant increase in (%CD4 + CD38 + HLA-DR+) T cell activation on uninfected PBMCs compared to infected PBMCs (**, p-value = 0.0050). **(C)** The HLB fraction significantly increased (%CD4 + CD38 + HLA-DR+) T cell activation on infected PBMCs compared to uninfected (*, p-value = 0.0019), although it had low activation compared to AZT. **(D)** The MAX fraction showed a significant increase in (%CD4 + CD38 + HLA-DR+) T cell activation on infected PBMCs compared to uninfected PBMCs (*, p-value = 0.0278). However, it had low activation compared to AZT. There was no CD8+ T cell activation in both treated, untreated, infected, and uninfected PBMCs (results not shown).

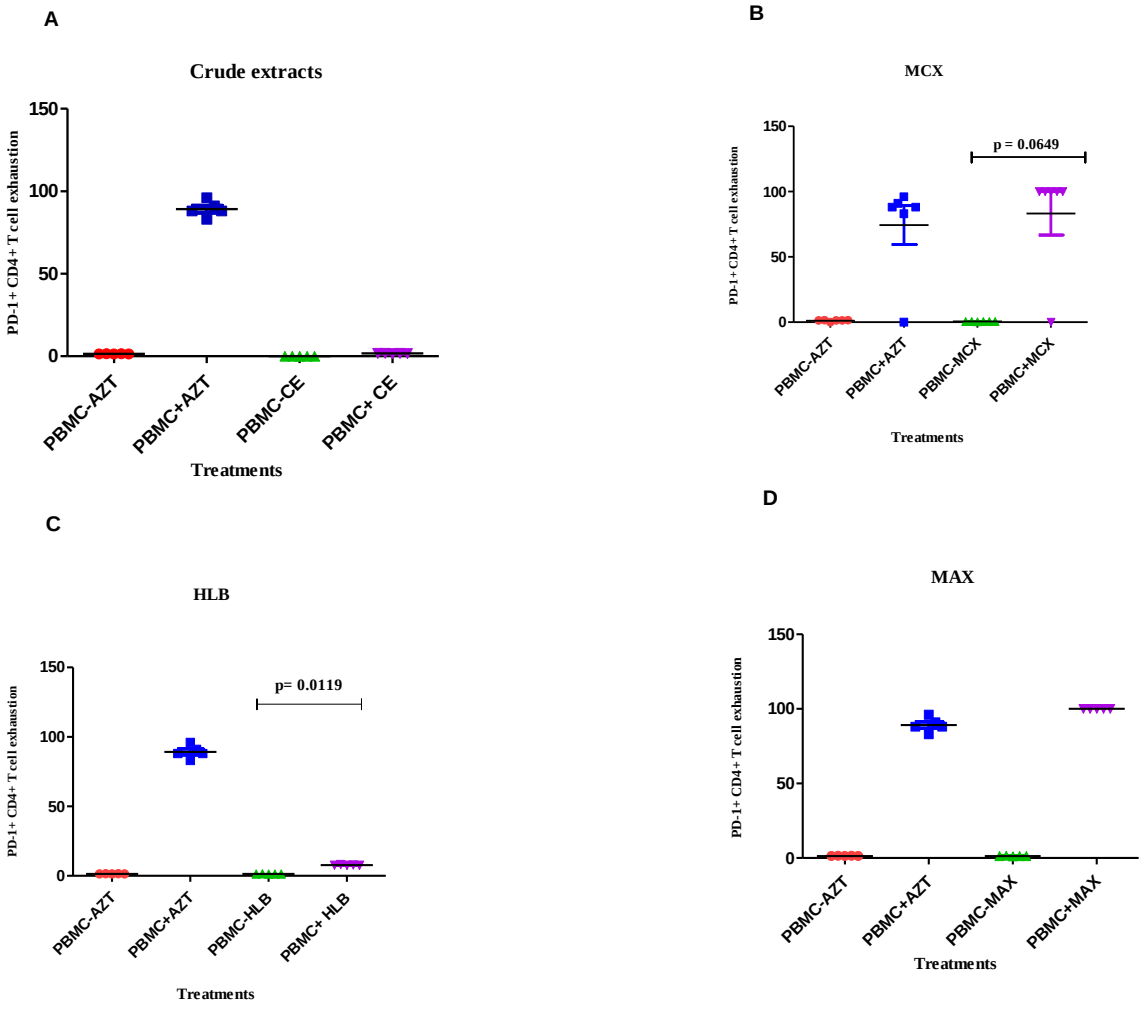


Figure 3. 7: The $CD4^+$ T cell exhaustion of the crude and fractionated *A. alternata* PO_4PR_2 fractions (MCX, HLB, MAX) was determined on PBMCs using flow cytometry. The y-axis shows (%CD4 + PD-1) T cell exhaustion, and the x-axis indicates the treatments where PBMC represents uninfected PBMCs- and infected PBMCs by PBMC+. Azidothymidine (AZT) was used as a positive drug control. **(A)** The results show that for the crude extract in both infected and uninfected PBMCs, there was no significant difference in the PD-1+$CD4^+$ T cell exhaustion. However, AZT had a high PD-1+$CD4^+$ T cell exhaustion on infected PBMCs. **(B)** The MCX fraction had a non-significant increase in PD-1+$CD4^+$ T cell exhaustion in infected PBMCs compared to uninfected PBMCs (ns, p-value = 0.0649), comparable to AZT. The PD-1+$CD4^+$ T cell exhaustion of MCX in infected PBMC is comparable to AZT. **(C)** The HLB fraction significantly increased PD-1+CD4+ T cell exhaustion in infected PBMCs compared to uninfected ones (*, p-value = 0.0119). **(D)** The MAX fraction showed no significant increase of PD-1+CD4+ T cell exhaustion in infected PBMCs compared to uninfected PBMCs. The PD-1+$CD4^+$ T cell exhaustion of the MAX

fraction is comparable to AZT.

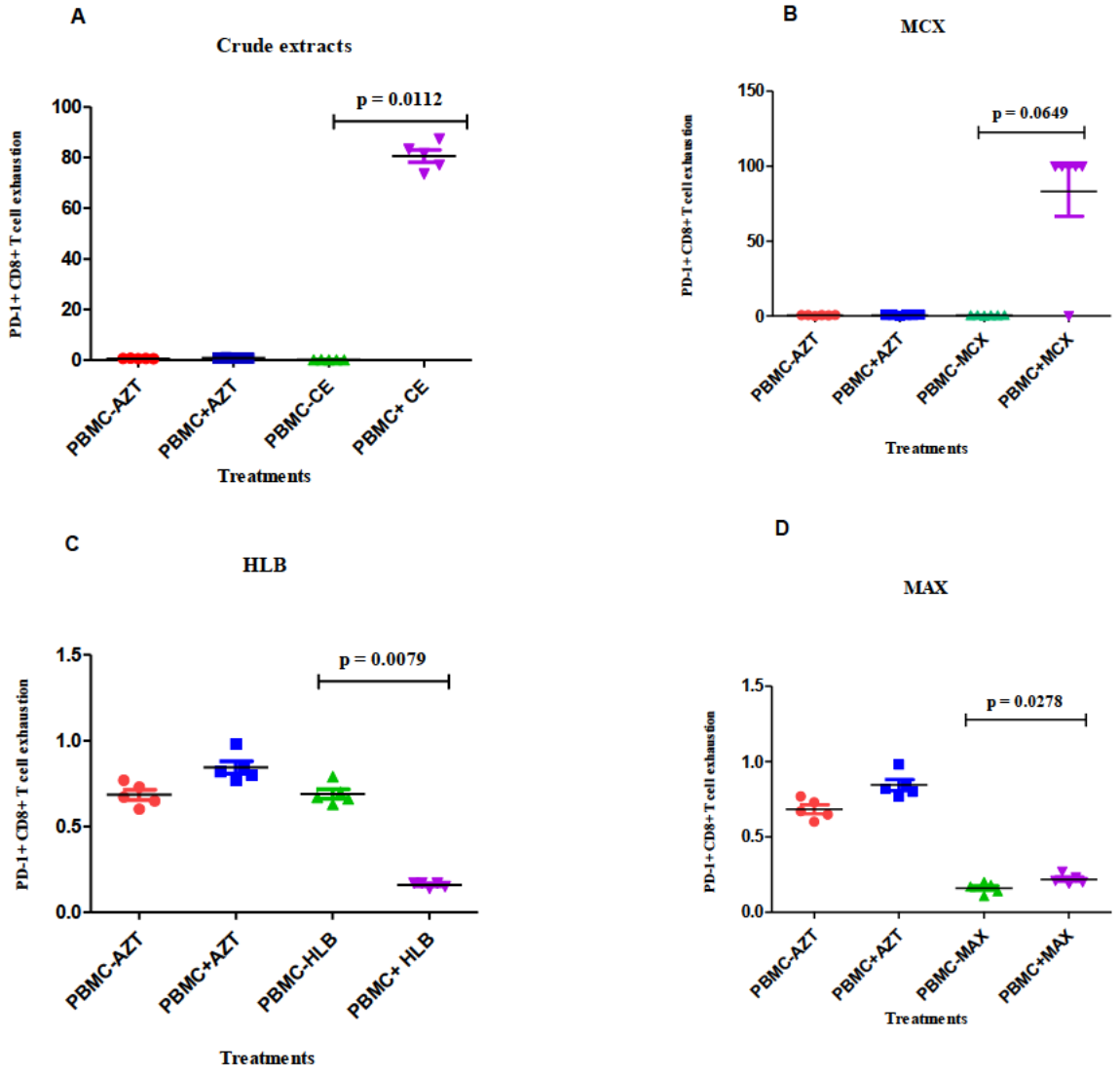


Figure 3. 8: The $CD8^+$ T cell exhaustion of the crude and fractionated *A. alternata* PO_4PR_2 fractions (MCX, HLB, MAX) was determined on PBMCs using flow cytometry. The y-axis shows (%CD8 + PD-1) T cell exhaustion, and the x-axis indicates the treatments where PBMC represents uninfected PBMCs- and infected PBMCs by PBMC+. Azidothymidine (AZT) was used as a positive drug control. **(A)** The crude extract significantly increased PD-1+$CD8^+$ T cell exhaustion in infected PBMCs compared to uninfected PBMCs (*, p-value = 0.0112). **(B)** The MCX fraction showed a non-significant increase in PD-1+CD8+ T cell exhaustion in infected PBMCs compared to uninfected ones (ns, p-value = 0.0649). There was no PD-1+$CD8^+$ T cell exhaustion in PBMCs treated with AZT compared to the crude extract and MCX fraction (A and B). **(C)** The HLB fraction significantly reduced PD-1+$CD8^+$ T cell exhaustion in infected PBMCs compared to infected PBMCs (**, p-value = 0.0079). **(D)** In the MAX fraction, there was a slightly significant increase in the PD-1+$CD8^+$ T cell exhaustion in the infected PBMCs compared to uninfected PBMCs (*, p-value = 0.278).

3.5. Discussion and conclusion

Endophytic fungi from medicinal plants are major reservoirs of bioactive compounds with antiviral, antimicrobial and antifungal (Adeleke and Babalola, 2021). The interaction between the host plant and endophytic fungi produces secondary metabolites, including terpenoids, alkaloids and many more *(*Khare *et al*., 2018). Endophytic fungi, *A. alternata,* isolated from *Hypoxis* spp, showed that it has potent anti-HIV-1 (Nzimande *et al.*, 2022). *Alternaria alternata's* crude extracts were fractionated using solid phase extraction and were not toxic to TZM-bl cells with high percentages of anti-HIV-1 activity. The fractionated MCX fraction of *A. alternata* showed high anti-HIV-1 activity during HIV-1 entry, reverse transcription and integration using p24 ELISA and Luciferase-based Time of addition assay. These results suggest that *A. alternata* secondary metabolites possess anti-HIV-1 compounds that can inhibit the virus in different stages of HIV-1 replication and act as an immune activator.

The results showed that SPE can successfully fractionate the crude extract of *A. alternata* using increasing methanol gradient approach. The SPE efficiently reduces hazardous organic solvents, has no foaming and emulsion formation, has a high recovery percentage, is easy to operate, and can pair with analytical devices (Hamidi, 2023). Using the different cartridges or columns (MCX, MAX, HLB) that attract acidic, basic, and neutral compounds may separate the active compound with strong antiviral activity. Stoszko *et al*. (2020) have implemented this method and discovered the latency-reversing agent gliotoxin (GXT) from endophytic fungi (Stoszko *et al*., 2020).

Assessing the cytotoxic response by measuring the mitochondrial functioning and cell viability is critical to determining if the crude extract and fractionated fractions (MCX, MAX, HLB) of *A. alternata* are safe and effective. In this study, high concentrations of *A. alternata* crude extract and fractions decreased cell viability (Figure 3.1). The decreased cell viability could be attributed to the production of NADPH, inhibition of succinate dehydrogenase or altered mitochondrial membrane potential (Zorova *et al*., 2018). However, the cell viability remained above 80% for all fractions and the crude extract. This means the cell viability or

mitochondrial membrane potential was not seriously affected after treating the TZM-bl cell line with these fractions. The maximum effects observed were less than 20% decrease in cell viability. It has been suggested that a decrease in cell viability by more than 30% is considered a cytotoxic effect in the International Organization for standardization (Berger *et al*., 2017). A crude extract is generally considered to have *In vitro* cytotoxic activity when the CC_{50} value is <30–40 µg/mL (Talib *et al*., 2010). Therefore, all the fractions and the crude extract showed no cytotoxicity effects on the TZM-bl cell line, with 50% cytotoxicity concentration (CC_{50}) ranging from 35.31 to 62.31 µg/mL.

To date, no studies have described the cytotoxicity of the fractionated *A. alternata* fractions (MCX, MAX, HLB). However, some studies have tested the crude extract of *A. alternata* on different cell lines (Abdou and Zakareia, 2020). A recent study has shown that a crude extract of *A. alternata* exerted moderate cytotoxicity against Hela cell lines with a CC_{50} value > 50 µg/mL (Abdou and Zakareia, 2020). Another study also shows that crude extract of *A. alternata* has no cytotoxicity effects on different cell lines such as Hela, MCF-7, H1975 and Hep G2 with a CC_{50} value ranging between 60.4 to 226.59 µg/mL (Ravi *et al*., 2022). All the fractionated *A. alternata* were non-toxic to human cell lines, suggesting that these extracts mya be used for further antiviral testing.

Alternaria alternata crude extract (PO_4PR_2) and fractionated fractions were tested for anti-HIV-1 activities using the Luciferase antiviral-based assay. The results demonstrated that the *A. alternata* crude extract and fractionated fractions possess anti-HIV activity, suggesting that endophytic fungi are a promising source of antiviral drugs mining; however, the bioactive compound needs to be further identified using NMR and liquid- chromatography mass spectrophotometry. The fractionated fractions (MCX, MAX, HLB) were prepared using increasing methanol gradient (0%, 5%, 45% and 95%, respectively) and did not lose their ability to inhibit HIV-1; however, the percentage inhibition decreases (Figure 3. S1, S2 and S3). The results suggest that fractionating the crude extract using different cartridges decreased the anti-HIV activity. Contrastingly, in Stoszko *et al*. (2020) the less active fractions became more active (high potency to reverse latency in J-Lat models) during purification.

The MCX (Mixed-mode, strong Cation-eXchange) fraction had high anti-HIV activity compared to all the other fractions MAX (Mixed mode, strong Anion-eXchange) and HLB

(Hydrophilic-Lipophilic Balance). So, this may suggest that the bioactive compounds with antiviral activity are acidic compounds since the MCX fraction captures acidic compounds. Meanwhile, Stoszko *et al*. (2020) study demonstrated that HLB (hydrophilic) fraction had high potency to reverse latency in J-Lat models compared to the acidic and basic fractions. These findings show that fractionation of endophytic fungi crude extracts using different columns results in distinctive biological activities. The data also shows that as the concentration of methanol increases in the fractions, the anti-HIV activity decreases. Even though methanol is a common solvent used for extracting bioactive compounds, it may also affect the compounds' stability and bioactivity (Dorokhov *et al*., 2018; Polanía *et al*., 2023). Methanol may also affect the expression and activity of some enzymes involved in the biosynthesis and metabolism of bioactive compounds, such as pectin methyl esterase (Dorokhov *et al*., 2018). This may be the reason why the anti-HIV activity decreases with increasing methanol concentration.

The GC-MS data by Nzimande *et al*. (2022) showed that there were several bioactive compounds in the MCX fraction, but only a few have been reported to have antiviral activity: Propargylamine and 1,2-cyclobutanedicarbonitrile and Coumarin (3,4-dihydro-4,5,7-trimethyl-2-chromanone which were also found on the crude extract. A selective index value greater than 10 indicated potential therapeutic use in cell toxicity (Pritchett *et al*., 2014). The MCX fraction showed the most significant HIV-1 inhibition and the highest selective index among all the tested fractions. Suggesting that the MCX fraction is theoretically more effective and safer than the other two tested fractions (Table 3.2). The selective index of MAX and HLB fractions was less than 10, indicating that these fractions were less potent and more toxic *In vitro* studies. Further studies can individually evaluate the MCX fraction and identify the active compounds.

Alternaria alternata crude extract and fractionated fraction (5% MCX) interfere with HIV-1 entry, reverse transcription, and integration during HIV-1 replication (Figures 3A and B). Daelamas *et al*. (2011) used the time of addition assay to determine the unknown anti-HIV-1 drugs. Time of addition is an assay performed to determine the stage of the HIV replication cycle with which *A. alternata* crude extract and fractionated fraction interfere (Daelemans *et al*., 2011; Lara, 2014). Daelemans *et al*. (2011) used MT-4 cells to conduct an ELISA p24 time-based assay. The MT-4 and Jurkat cell lines are human T-lymphocyte cell lines that

both express the CD4 receptor, which is the primary receptor for HIV entry into the cells, and both cell lines express CXCR4 and have 52- and 46-kDa factors (Xu *et al*., 1990; Kajiwara *et al*., 2006; Montano, 2014). The results demonstrate that then using the Jurkat cell line, the crude extract has high potent HIV-1 p24 reduction during entry and reverse transcription compared to a 5% MCX fraction, which showed to have high HIV-1 p24 after the infected cells were treated with a 5% MCX fraction compared to the crude. However, the 5% MCX fraction showed strong HIV-1 inhibition (%) during entry and reverse transcription and integration when using the TZM-bl cell lines, which suggests that the bioactive compounds present in the crude extract and MCX fraction target either the viral envelope glycoprotein gp120 or gp41, or the host cell receptors CD4, CCR5, or CXCR4 during entry (Qian *et al*., 2009). The bioactive compounds present in the two may also interfere with converting the viral RNA genome into DNA (reverse transcription) (Götte *et al*., 1999; Hu and Hughes, 2012). These compounds can target different aspects of reverse transcription, such as the viral enzyme reverse transcriptase (RT), the RNA/DNA hybrid intermediate, or the RNA secondary structure TAR that binds the viral trans activator protein Tat (Parniak and Sluis-Cremer, 2000; Hu and Hughes, 2012).

During protease, the 5% MCX fraction shows high HIV-1 inhibition during proteolysis and integration in the Jurkat cell line compared to the crude extract, which has high HIV-1 inhibition in TZM-bl cell lines. The crude extract and 5% MCX fraction have the same HIV-1 inhibition (98%) during integration when using the TZM-bl cell line. The bioactive compounds present may also interfere with HIV replication and infection of new cells by blocking HIV enzyme integrase and protease. So, in summary, the crude extract and MCX fraction from *A. alternata* were shown to inhibit HIV-1 during entry and act as reverse transcriptase inhibitors, protease inhibitors and integrase inhibitors. These results support the study by Melappa *et al*. (2015) where they revealed that partially purified coumarins isolated from *Alternaria* sp. showed high HIV-1 inhibition on three viral enzymes: integrase enzyme (98%), reverse transcriptase enzyme (82.81%) and protease activity (78%) (Melappa *et al*., 2015).

Another study also demonstrated that L-50 (which is cyclic peptides that block Tat-TAR interactions) was identified as an inhibitor of HIV-1 transcription (tested using time of addition assay) since it blocks viral replication with a time course that closely resembled the DRB. This drug prevents RNAP II transcriptional elongation by selectively inhibiting the

protein kinase component of P-TEFb, CDK9 (Lalonde *et al*., 2011). Furthermore, to better understand the mechanism of action of *A. alternata,* we can also apply bioinformatics approaches such as molecular docking.

The immunomodulatory effects of fractionated *A. alternata* fractions (MAX, MCX, HLB) were investigated by evaluating the T cell activation and exhaustion of HIV-1 infected PBMCs. ARVs can reduce the viral load and the level of immune activation in HIV-infected individuals, which may help preserve or restore the T cell function and prevent or reverse T cell exhaustion (Li *et al*., 2019b). The current study demonstrates that HIV-1 infected PBMCs showed an increase in $CD4^+$ T cell activation (CD38 + HLA-DR+) when treated with fractionated fractions. So, in response to HIV infection, the immune cells release signalling molecules called cytokines (Freeman *et al*., 2016). These cytokines act as messengers and stimulate the activation and proliferation of $CD4^+$ T cells (Freeman *et al*., 2016). Hence, we observed an increase in $CD4^+$ T cell activation on PBMCs after infecting with HIV (results not shown). An increase in $CD4^+$ T cell activation on PBMCs after infecting with HIV means that the immune system is trying to respond to the virus but also losing more $CD4^+$ T cells in the process.

The fractionated fractions (5%; MCX, HLB, MAX) and crude extract of *A. alternata* decrease the $CD4^+$ T cells (CD38 + HLA-DR+) T cell activation compared to treating with AZT (Figure 3.6). A decline in $CD4^+$ T cell activation on HIV-1 infected PBMC after treatment with crude and fractionated fractions could imply that the $CD4^+$ T cells in the PBMCs that are infected with HIV-1 have reduced levels of activation markers, such as HLA-DR and CD38, after being exposed to treatment and treatment is effective in preventing HIV from replicating and activating $CD4^+$ T cells (Schultz *et al*., 2017). The 5% MCX (Mixed-mode, strong Cation-eXchange) fraction showed to have the lowest $CD4^+$ (CD38 + HLA-DR+) T cell activation when compared to the crude extract, AZT, and fractions 5% MAX (Mixed mode, strong Anion-eXchange) and 5% HLB (Hydrophilic-Lipophilic Balance). The findings support the *A. alternata* 5% MCX fraction, associated with low CD4+ T cell activation, which can benefit the immune system. However, it may not be a definitive indicator of the drug's efficacy or safety, and other factors, such as CD4 T cell count and viral load, should also be considered.

In this study, we also assessed the activation of CD8+ T cells by the *A. alternata*, the crude extract, and the fractionated fractions using HLB, MAX, MCX. The results demonstrated no or low activation of $CD8^+$ T cells in both treated, untreated, infected, and uninfected PBMCs. This may suggest that the antiviral compounds tested effectively suppressed the virus, so there was no need for CD8+ T cell activation (Qiu *et al*., 2022). However, since the mechanism behind no CD8+ T cells activation is unknown, further characteristisation of *A. alternata* fractions (MAX, MCX, HLB) may provide more information.

In addition, the continuous response of $CD8^+$ T cells towards various antigenic stimuli during HIV infection leads to increased $CD8^+$ T cell exhaustion. The exhausted cells are phenotypically characterized by the expression of PD-1, an inhibitory receptor belonging to the CD28 family (Wherry and Kurachi, 2015; Bănică *et al*., 2021). Although T-cell exhaustion was first identified in CD8 T cells, it is now accepted that CD4 T cells are also subject to exhaustion, leading to reduced production of IL-2, IFN-γ, and TNF-α along with reduced CD4 T-cell help (Antoine *et al.*, 2012; Fenwick *et al*., 2019). The results showed that the crude extracts and the 5% HLB (Hydrophilic-Lipophilic Balance) decreased PD-1+CD4+ T cell exhaustion in infected PBMCs. At the same time, there was high exhaustion on PD-1+$CD4^+$ T cells treated with MCX and MAX fractions (Figure 3.7). High T cell exhaustion can be characterized by the loss of effector functions, such as cytokine production and the increased expression of inhibitory receptors, such as PD-1, LAG-3, and TIM-3, that suppress T cell activation (Zheng *et al.*, 2020). We observed a low PD-1+CD8+ T cell exhaustion in the HLB and MAX fraction compared to the MCX fraction and the crude extract (figure 3.8), suggesting that bioactive compounds present in HLB and MAX fractions are effective at preventing $CD8^+$ T cell exhaustion and thus assisting in maintaining the immune system's ability to fight off the virus.

High $CD8^+$ T cell exhaustion observed in MCX fraction (acidic compounds) means that there are compounds in it that affect the major functions of $CD8^+$ T cells, resulting in the loss of their primary function of secreting the cytokines like IFN-g, Il2, granzyme and perforin. It is important to further characterized the bioactive compound in MCX fractions so that anti-HIV-1 can be identified. The results imply that the crude extracts, MAX and HLB fractions may possess compounds with immunomodulatory effects with moderate antiviral activity. Nonetheless, the mechanism of how these immunomodulators decrease T cell exhaustion on $CD4^+$ T cells and $CD8^+$ T cells still need to be explored. Although the GC-MS data by Nzimande *et al*. (2022) display all possible present bioactive compounds in *A. alternata,* it is

still necessary to extract and identify those bioactive compounds with antiviral and immunomodulation activity using techniques liquid chromatography mass/mass spectrophotometry, and nuclear magnetic resonance (NMR).

3.6 Ethics

Ethical approval from the Biomedical Research Ethics Administration (BREC). An examination from ethics was granted, Protocol reference number: BREC/00004492/2022.

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3.8 Supplementary figures

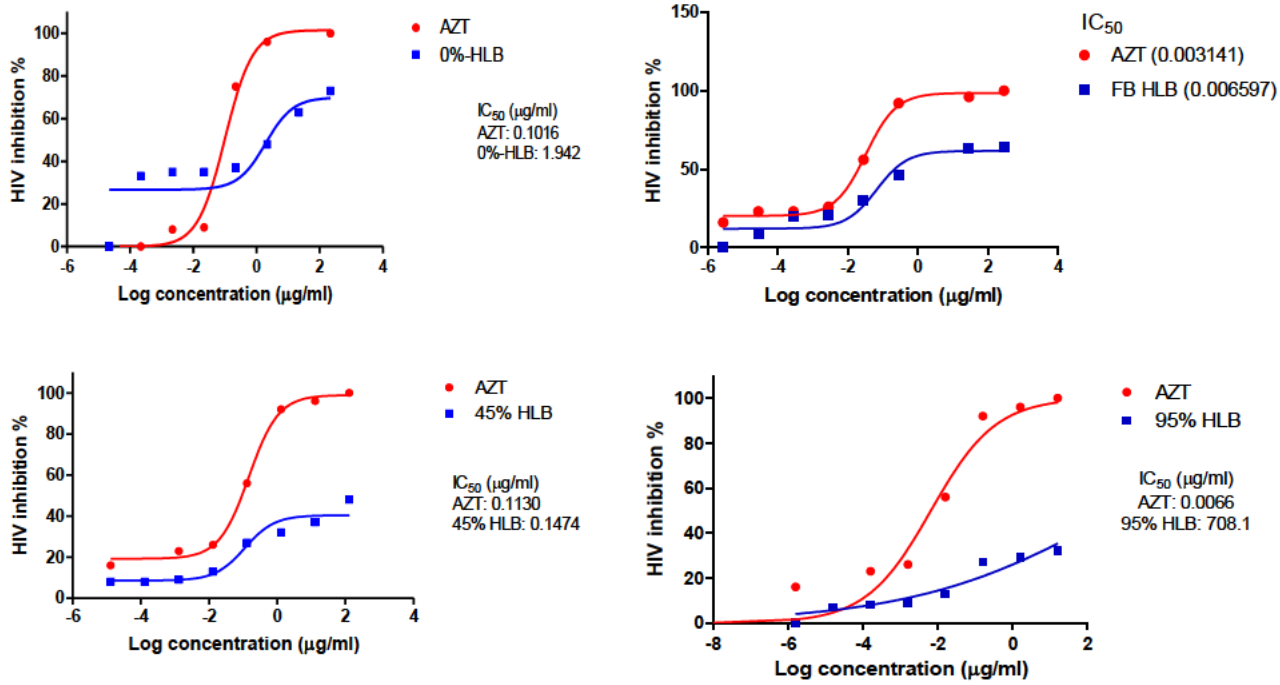
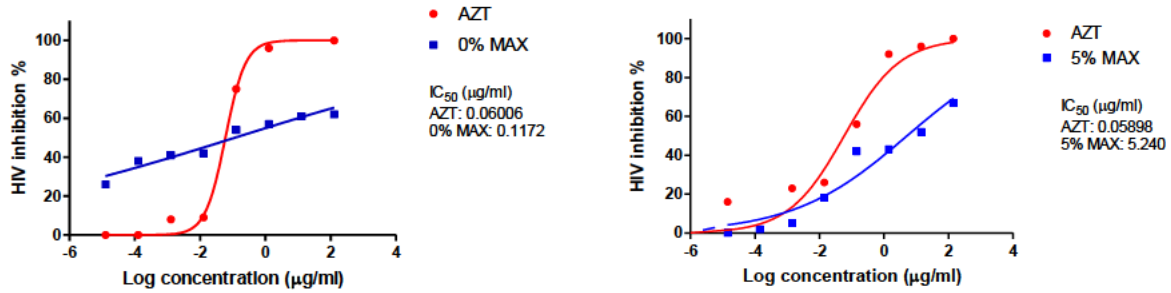


Figure S3. 1: TZM-bl cell lines were used in a Luciferase-based antiviral assay to examine the HIV-1 % inhibition curves of *A. alternata* crude extract and HLB fractions (neutral chemicals). *A. alternata* HLB fractions and crude extract were serially diluted, and AZT was used as a positive control. The x-axis displays the log concentration in µg/mL, while the y-axis depicts the percentage of HIV inhibition. The blue line shows the fractions, while the red line shows the positive drug control AZT. In comparison to the 45% and 95% fraction, which had an IC_{50} of 1.942 and 5146 (µg/mL) after SPE, the 0% HLB and 5% HLB fractions exhibit high anti-HIV-1 (%) with IC_{50} of 0.1474 and 708.1 (µg/mL).



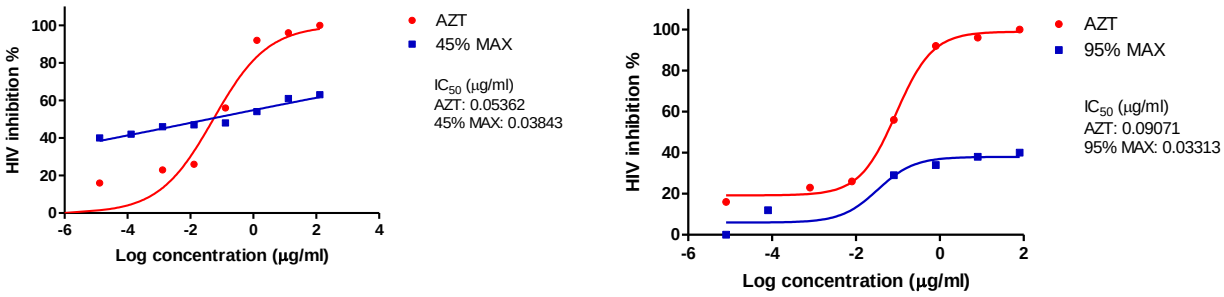


Figure S3. 2: The HIV-1 % inhibition curves of *A. alternata* MAX fractions (acidic compounds) were investigated using TZM-bl cell lines in a Luciferase-based antiviral assay). The x-axis displays the log concentration in µg/mL, while the y-axis depicts the percentage of HIV inhibition. The blue line shows the fractions, while the red line shows the positive drug control AZT. In comparison to the 0%, 45%, and 95% fractions, which had IC_{50}s of 0.1172, 0.03843, and 0.03313 (µg/mL), the 5% MAX fraction has high anti-HIV-1 (%).

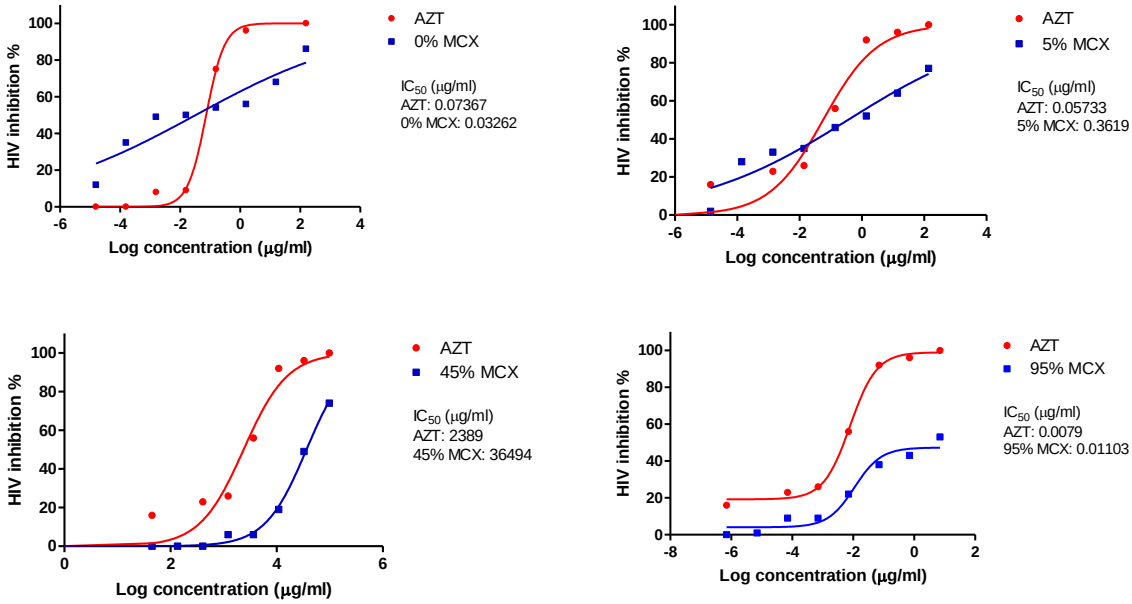


Figure S3. 3: The HIV-1 percentage inhibition curves of *A. alternata* MCX fractions (basic compounds) were tested with Luciferase-based antiviral assay using TZM-bl cell lines. The y-axis represents the percentage of HIV inhibition, and the x-axis shows the log concentration in µg/mL. The red line represents the positive drug control AZT, and the blue line represents the fractions. The 0% MCX, 5% MCX and 45% MCX fraction shows high anti-HIV-1 (%) after SPE with an IC_{50} of 0.03262, 0.3619 and 3.6494 (µg/mL) compared to 95% MCX fraction with an IC_{50} of 0.011103 (µg/mL).

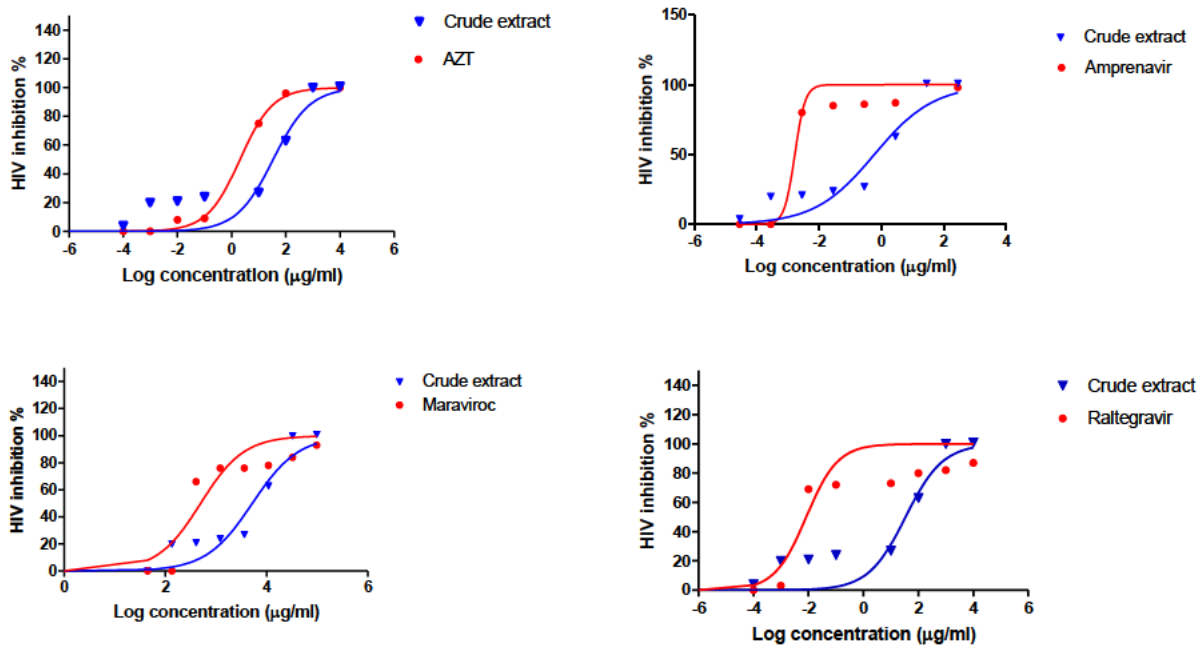


Figure S3. 4: The HIV-1 percentage inhibition curves of the positive drug controls were tested with Luciferase-based antiviral assay using TZM-bl cell lines. Positive controls (AZT, Maraviroc, Raltegravir and Amprenavir). The y-axis represents the percentage of HIV inhibition, and the x-axis shows the log concentration in µg/mL. The red line represents the positive drug controls, and the blue line represents the crude extract. Maraviroc showed inhibition of 93% with an IC_{50} of 499.9 (µg/mL), Zidovudine was 100% with an IC_{50} of 2.0550 (µg/mL), Raltegravir was 87% with an IC_{50} of 0.008331 (µg/mL), Amprenavir was 98% with an IC_{50} of 0.00168 (µg/mL), and the crude extract was 101% with an IC_{50} of 34.38 (µg/mL).

CHAPTER 4: SYNTHESIS

4.1 General discussion and conclusion

Endophytic fungi can serve as an alternative source for valuable active plant compounds in searching for bioactive molecules as pro-drug compounds or in developing medicines. Previous studies showed that endophytic fungi from desert plants are a promising source in identifying potent inhibitors in the replication of HIV-1 (Wellensiek *et al*., 2013; Bashyal *et al*., 2014). Thus, the present study showed the fractionated crude extracts from endophytic fungi *A. alternata* isolated from *Hypoxis* spp as potential anti-HIV compounds and/ or immunomodulatory agents against HIV-1.

The previous study by Nzimande *et al*. (2022) showed that *A. alternata* PO_4PR_2 crude extract had 100% HIV-1 inhibition in the TZM-bl cell line. It also demonstrated that this crude extract inhibited HIV-1 replication on PBMCs and $CD4^+$ T. The study has successfully proven that the fractionated crude extracts from *A. alternata* (PO_4PR_2) contain anti-HIV and/ or immunomodulatory properties against HIV-1. It is unfortunate that this study did not plan to identified the bioactive compounds from these fractions. However, this study determined the anti-HIV activity of the fractionated crude extracts of *A. alternata* PO_4PR_2 prepared using SPE. The SPE provided different columns, which are MAX (Mixed mode, strong Anion-eXchange), MCX (Mixed-mode, strong Cation-eXchange) and HLB (Hydrophilic-Lipophilic Balance). One advantage of using different columns or cartridges during SPE is that they can have different stationary phases, such as reversed phase, normal phase, ion exchange, or mixed mode. These phases have different properties and interactions with the analytes and the matrix components, allowing for selective retention and elution of the desired compounds (basic, acidic, or neutral).

The results showed that SPE can successfully fractionate the crude extract of *A. alternata* using increasing methanol gradient approach. The cytotoxicity and cell viability of the crude extract and the fractionated extracts were tested in the TZM-bl cell line to determine its safety efficacy and to optimize the concentration, delivery, and formulation. Cell viability and cytotoxicity assays can also provide insights into the mechanisms of action and the modes of cell death induced by different agents (Özlem Sultan, 2017). However, They may not be able

to reflect the complex interactions and responses of cells in a living organism, such as tissue architecture, blood flow, immune system, metabolism, and pharmacokinetics (Özlem Sultan, 2017). Therefore, cell viability and cytotoxicity assays should be used cautiously and in combination with other methods, such as *in vivo* studies and clinical trials, to validate and confirm the results. The cell viability remained above 80% for all fractionated fractions and the crude extract, which means that the cell viability or mitochondrial membrane potential was not seriously affected after treating the TZM-bl cell line.

The crude extract showed high potency to inhibit HIV-1 compared to the fractionated fractions (MAX, MCX, HLB). Fractionated of the *A. alternata* crude extracts is very important so that single active compound with anti-HIV properties can be identified. The results suggest that purifying the crude extract using different cartridges decreased the anti-HIV activity. Moreover, between the fractionated fractions, the MCX (Mixed-mode, strong Cation-eXchange) had high anti-HIV activity compared to other fractions MAX (Mixed mode, strong Anion-eXchange) and HLB (Hydrophilic-Lipophilic Balance). So, this may suggest that the bioactive compounds with antiviral activity are acidic compounds since the MCX fraction captures acidic compounds. Chromatographic assays with high performance are recommended to identify these compounds.

These findings show that fractionation of endophytic fungi crude extracts using different columns results in distinctive biological activities. Meanwhile, Stoszko *et al*. (2020) study demonstrated that HLB (hydrophilic) fraction had high potency to reverse latency in J-Lat models compared to the acidic and basic fractions. I further evaluated the mechanism of action of the crude extract and MCX fraction using the time of addition assay. The results showed that the fractionated crude extract from *A. alternata* (MCX) has strong HIV-1 inhibition potential during reverse transcription and integration.

Similarly, the study by Melappa *et al*. (2015) where revealed that partially purified coumarins isolated from *Alternaria* sp. showed high HIV-1 inhibition on three viral enzymes: integrase enzyme (98%), reverse transcriptase enzyme (82.81%) and protease activity (78%). The results also imply that the crude extracts, MAX and HLB fractions may possess compounds with immunomodulatory effects with moderate antiviral activity. Nonetheless, the

mechanism of how these immunomodulators decrease T cell exhaustion on $CD4^+$ T cells and $CD8^+$ T cells still need to be explored. To conclude, the results obtained align with the hypothesis which demonstrated that the *A. alternata* crude extract and fractionated fractions possess anti-HIV activity. Moreover, it is associated with immune activation, suggesting that *A. alternata* is a promising source of antiviral drug mining.

4.2 Limitations and Recommendations

Although, the time of addition revealed the stages of HIV-1 where the crude extract of the endophytic fungi inhibits HIV-1. Further studies are still required to understand the mechanism of action better. Some assays that could be utilised to improve the knowledge of the mechanism of action include cell-based assays such as HIV-1 integrase activity assay, reverse transcription assay and computer-based approaches such as molecular docking. Other important factors, including the pharmacokinetic, must be considered to further evaluate the potential as an antiviral compound. Future studies could also explore the latency-reversing activity of these endophytic fungi.

The GC-MS data showed several compounds secreted by the endophytic fungi where; some were reported in the literature to have antiviral activity, and some were not reported to have any biological activity. The disadvantage of using the GC-MS is that it target the volatile compound. Another limitation of the study is that we could not identify the compound to which the antiviral activity is attributed. Further studies must consider the development of metabolomics and genomic tools on how endophytes colonise the plant and plant-microbe interaction and identify the active compound using techniques such as NMR and high resolution LC-MS. There is still a need to study the compounds produced by these endophytic fungi and their activities in reducing other diseases.

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