Activation of silent biosynthetic gene clusters and profiling of secondary metabolites secreted by endophytic fungi for use as potential anti-HIV agents

By

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PREFACE

The experimental work described in this dissertation was carried out in the Department of Medical Microbiology, College of Health Sciences, Nelson R. Mandela School of Laboratory Medicine and Medical Science. University of KwaZulu-Natal, Durban, South A from February 2020 to November 2022, under the supervision of Dr. S. Ndlovu and the co-supervision of Dr. N. Mkhwanazi, respectively. These studies represent original work by the author and have not otherwise been submitted in any form for any degree or diploma to any tertiary institution. Where use has been named of the work of others it is duly acknowledged in the text.

DECLARATION

I .....Makhwitine John Phuti..... declare that

(i) The research reported in this dissertation/thesis is my original research, except where otherwise indicated.

(ii) This dissertation/thesis has not been submitted for any degree or examination at any other university.

(iii) This dissertation/thesis does not contain other persons' data, pictures, graphs, and other information unless specifically acknowledged as being sourced from other persons.

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02/08/2022

J.P Makhwitine (Student) Date

As the candidate's supervisor I agree to the submission of this thesis:

02 August 2022

Dr. S.I Ndlovu (Supervisor) Date

02 August 2022

Dr. N.P Mkhwanazi (Co-supervisor) Date

DEDICATION

I dedicate this work to my grandmother Miss Tlou Flora Mahwiting, words cannot begin to express my gratitude, thank you for your endless love, support and encouragement and in the loving memory of my late mother Miss Matome Francinah Makhwitine.

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RESEARCH OUTPUTS

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   Section 2.6 of Chapter 2 is extracted to the review paper.

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TABLE OF CONTENTS

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

3TC	–	Lamivudine
A	–	Adenylation
AIDS	–	Acquired immunodeficiency syndrome
ARVs	–	Antiretroviral therapy
AT	–	Acyltransferas
AZT	–	Azidothymidine/zidovudine
BGCs	–	Biosynthetic Gene Clusters
BIC	–	Bictegravir
BLAST	–	Basic Local Alignment Search Tool
cART	–	Combination Antiretroviral Therapy
CCR5	–	CC chemokine receptor 5
CXCR4	–	C-X-C chemokine receptor type 4
DMATSs	–	Prenyltransferases
DNA	–	Deoxyribonuclease acid
DNMT	–	DNA methyltransferase
FDA	–	Food and drug administration
GC-MS	–	Gas chromatography mass spectrometry
gp120	–	Glycoprotein 120
gp41	–	Glycoprotein 41
HAART	–	Highly active antiretroviral therapy
HDAC	–	histone deacetylase
HIV	–	Human immunedefieciency virus
INSTIs	–	Integrase strand transfer inhibitors
ITS	–	Internal transcribed sequence
KS	–	Ketosynthase
MEA	–	Malt extract agar
NAD^+	–	Nicotinamide adenine dinucleotide

NNRTIs	–	Non-nucleoside reverse transcriptase inhibitors
NRPSs	–	Non-ribosomal peptide synthases
NRPSs-PKs	–	Non-ribosomal peptide synthases- Polyketide synthases
NRTIs	–	Nucleoside Reverse Transcriptase Inhibitors
OSMAC	–	One strain many compounds one-strain many compounds
PCP	–	Peptidyl carrier protein
PDA	–	Potato dextrose agar
PKSs	–	Polyketide synthases
RNA	–	Ribonucleic acid
TCs	–	Terpene cyclases
TE	–	Thioesterase
UKZN	–	University of KwaZulu-Natal
UNAIDS	–	United Nations Programme on HIV/AID

CHAPTER 1

GENERAL INTRODUCTION

1.1. Background of the study

The Human Immunodeficiency Virus (HIV) was first discovered in 1981 by two research groups led by scientists, Robert Gallo, Barré-Sinoussi, and Luc Montagnier (Gallo *et al.*, 1983; Barré-Sinoussi *et al.*, 1983). The prevalence of HIV/AIDS has been estimated at 37.7 million infection cases and 36.3 million deaths since its discovery in 1981 (UNAIDS, 2021). Due to a combination of antiretroviral therapy (ART) and preventive measures, the prevalence of HIV infection has become manageable, allowing people living with HIV to live long and healthy lives (Sterne, 2009; McLaren and Fellay, 2021). However, antiretroviral drugs (ARVs) do not cure HIV but inhibit its replication in the host cell, reducing viral loads and improving the life expectancy of people living with HIV (Andersen *et al.*, 2018). In addition, the existing ARTs are gradually becoming ineffective due to the emergence of HIV drug resistance and adverse side effects such as increased cytotoxicity to the host cells (Andrae-Marobela *et al.*, 2013). Therefore, there is an urgent need for alternative therapeutic options for the treatment and management of HIV infections.

Previous study has screened bioactive compounds from numerous traditional medicinal plants based on ethnobotanical knowledge for antiviral activity during the last 25 years (Jassim and Naji, 2003). Due to the increasing demands for herbal remedies and pharmaceuticals, the global trend of medicinal plant utilization has been increasing (Palanichamy *et al.*, 2018). However, utilizing plants for medicinal purposes is facing numerous obstacles due to overharvesting without replanting, which poses a risk of the extinction of indigenous plants (Palanichamy *et al.*, 2018). Bioactive compounds of fungal origin have proven to be highly appropriate in the discovery of new drugs. As a result, fungi that inhabit medicinal plants are increasingly being studied for their biological activities (Kaul *et al.*, 2012; Strobel *et al.*, 2005; Kumar *et al.*, 2014). According to research, approximately 18% of plant-derived metabolites can also be obtained from associated fungus (Chowdhary *et al.*, 2012). Taxol, for example, is a bioactive compound derived from the medicinal plant *Taxus* and its endophytic fungus, *Taxomyces andreanae* (Stierle *et al.*, 1995). Fungi potentially contain or produce several antiviral compounds that could be utilized to treat other hosts (Linnakoski *et al.*, 2018).

One of the promising alternative sources of therapeutic agents with anti-HIV properties that are regaining impetus in the research community are microbial-derived natural products (also known as secondary metabolites). In drug discovery research, natural products have largely been explored as a source of antimicrobials, and these initiatives were successful (Cragg and Newman, 2013). However, the early phenotypic studies were mainly focused on antibacterial agents, with little focus on antifungals and antiviral agents. There have been minimal reports on searches for natural products with anti-HIV activities, and no drug from these sources is currently on the market (Linnakoski *et al.*, 2018). The primary reasons are lack of screening platforms and the need for specialized laboratory facilities (Linnakoski *et al.*, 2018). The required facilities come at a high price and require a high degree of oversight due to their cost and difficulty to build and maintain (Adamson *et al.*, 2021). Their limited global distribution restricts opportunities for testing compounds against live viruses (Adamson *et al.*, 2021). These predicaments have to be addressed, in order to provide a more extensive pipeline of potential antivirals.

Endophyte-derived natural products have been explored as alternative sources of drug-like candidates (Strobel *et al.*, 2004). In bioprospecting studies of endophytic fungi, only a few strains have been studied in depth, while others indicate the potential existence of significant novel candidates (Berdy, 2005; Newman and Cragg, 2020). A number of compounds are known to exhibit antiviral activities, including alkaloids, lignans, phospholipids, quinines, depsidones, iridoids, xanthones, peptides, lactones, triterpenes, and saponins (Ng *et al.*, 1997; Vlietinck *et al.*, 1998; Rangari *et al.*, 2009; Yang *et al.*, 2001). The chemical diversity of natural products makes them an excellent source for screening novel antiviral agents such that more research is required on them for anti-HIV drug discovery.

Fungal natural products have also been found to inhibit enzymes and proteins crucial to the HIV life cycle, including reverse transcriptase, integrase, and protease enzymes (De Clercq and Field, 2006; Cos *et al.*, 2004). To highlight a few targets, reverse transcriptase is a critical enzyme in the HIV life cycle that generates complementary deoxyribonucleic acid (DNA) from a ribonucleic acid (RNA) template (Jochmans, 2008; Roy, 2017). These include nucleoside reverse transcriptase inhibitors (NRTIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs) (Mbuagbaw *et al.*, 2016; Roy, 2017). Despite their success in inhibiting reverse transcriptase, they are associated with hypersensitivity, hepatotoxicity, and mitochondrial toxicity, which decreases treatment adherence, favouring the emergence viral strains that are

resistant to therapy (Montessori *et al.*, 2004; Esser *et al.*, 2007). Efforts have been made to discover anti-HIV compounds that may have fewer or no side effects from alternative sources, including endophytic fungi. A study by Bashyal *et al.* (2014) discovered altertoxins produced by *Alternaria tenuissima,* isolated from *Quercus emoryi* in the Sonoran desert (Bashyal *et al.*, 2014). Altertoxins demonstrated significant anti-HIV activity, which resulted in the inhibition of the HIV-1 reverse transcriptase enzyme. However, the molecular mechanisms underlying the anti-HIV effects of altertoxins compounds still need to be elucidated (Bashyal *et al.*, 2014; Wellensiek *et al.*, 2013).

The protease enzyme is also a target in HIV drug development and treatment as it is responsible for processing viral polyproteins and converting them into functional proteins (Monini *et al.*, 2003). Antiretroviral drugs developed to target protease enzymes are well known as protease inhibitors. However, the limitation is that the protease inhibitors are associated with lipodystrophy and metabolic disturbances (Menendez-Arias, 2002; Gardner *et al.*, 2009). Endophytic fungi have also been shown to produce active compounds that can target the protease enzyme in HIV replication (Roy, 2017). Vora *et al.* (2021) revealed a protease inhibitor, Mulberroside C compound, produced by unidentified endophytic fungi isolated from the *Morus alba* plant. This compound inhibited the protease enzyme in a dose-dependent manner (Vora *et al.*, 2021). Further studies are still required to elucidate the mechanisms of these compounds against HIV and any associated side effects before it is considered for further development stages such as preclinical trials. These discoveries further indicate that endophytic fungi are a potential source of natural products (Strobel and Daisy, 2003).

The renewed interest in microbial natural products, particularly fungal natural products, is also amplified by the advent of high throughput genome sequencing technologies coupled with bioinformatics tools (Rutledge and Challis, 2015). These technological advances have revealed that the conventional phenotypic screens used in the search for active compounds are limited and only capture a very small percentage of active compounds (Reen *et al.*, 2015). Most of the genes encoding bioactive compounds are silent or transiently expressed when microorganisms are cultivated under laboratory conditions (Reen *et al.*, 2015; Netzker *et al.*, 2018). In fungi, the silencing is partly due to the positioning of the biosynthetic gene clusters (BGCs) encoding these secondary metabolites or natural products near the telomers in the chromosome. In these positions, the BGCs are highly regulated by global and pathway-specific regulators (Pfannenstiel and Keller, 2019).

Non-ribosomal peptide synthase (NRPSs) and polyketide synthase (PKSs) gene clusters have been identified as the most prolific in fungi by genome prediction studies, while hybrid NRPS-PKS enzymes, prenyltransferases (DMATSs), and terpene cyclases (TCs) are also commonly found in fungi (Bergmann *et al.*, 2007; Khaldi *et al.*, 2010; Alvin *et al.*, 2014). These "backbone" enzymes are capable of synthesizing secondary metabolite core structures such as non-ribosomal peptides (NRPs), polyketides (PKs), non-ribosomal peptides- polyketides NRPS-PKS hybrids, indole alkaloids, and terpenoids (Hoffmeister and Keller, 2007; Linnakoski *et al.*, 2018). Before the final product is transferred outside the fungal cell, the produced core structures and product intermediates are often further processed by tailoring enzymes (Andersen *et al.*, 2013; Brakhage, 2013). These BGCs represent a potential source for new scaffolds for the discovery of novel antimicrobials (Tyurin *et al.*, 2018).

Fermentation of endophytic fungi with the potential for the production of bioactive compounds offers various advantages, such as reproducible and dependable productivity (Kumar *et al.*, 2017). The producing strains can be optimized by channelling metabolic fluxes and cultivated in fermenters to provide an unlimited supply of bioactive compounds and thus be profitably utilized (Fernández-Cabezón and Nikel, 2020). Direct modifications in culture conditions can be examined as a strategy for optimizing various biosynthetic pathways that contribute to the development of new derivatives and analogs (Rutledge and Challis, 2015). Innovative cultivation approaches such as co-culture, one strain many compounds (OSMAC) and the use of chemical elicitors such as histone deacetylase (HDACs) and DNA methyl transferase (DNMTs) inhibitors are examples of successful culture-based strategies (Okada and Seyedsayamdost, 2017). The use of small-molecule elicitors to modify chromatin has emerged as an effective method for inducing bioactive metabolites in endophytic fungus (Magotra *et al.*, 2017; Cichewicz, 2010). In *Alternaria alternata* cultures, the addition of trichostatin A, an HDAC inhibitor, resulted in the production of sterigmatocystin (Smith and Edlind, 2002). Treatment of endophytic fungal cultures with epigenetic modifiers have resulted in the production of several compounds, including penicillin and isosulochrin (Shwab *et al.*, 2007; Henrikson *et al.*, 2009). These observations provide plausible evidence for the possible use of epigenetic modifiers to trigger silent BGCs for bioactive compound secretion.

The advances in phenotypic screening platforms for naturally occurring bioactive compounds has enhanced this area of research and has been gaining impetus in the recent years (Adeleke

and Babalola, 2021). The plaque reduction assay, cytopathic effect (CPE) assay, and immunofluorescence assay are the most commonly used methods for screening fungal extracts for antiviral activity (Zhu *et al.*, 2004; Faccin *et al.*, 2007; Rincão *et al.*, 2012; Liu *et al.*, 2004; Zhang *et al.*, 2011). These assays are also used to conduct time-of-addition experiments and explore therapeutic compounds direct virucidal activity (Liu *et al.*, 2004; Faccin *et al.*, 2007). As indicated earlier, these approaches only confirm the antiviral capability of bioactive compounds and do not provide any insights on their mechanisms of action (Linnakoski *et al.*, 2018). Only a few research studies have gone on to assess the underlying molecular targets. Several approaches were applied to analyse diverse viral or cellular targets of drug activity. More specific assays have also been employed to evaluate bioactive compounds against virus-specific proteins and enzymes such as integrase and proteases. These assays have been designed to detect specific viruses such as HIV or human cytomegalovirus (CMV) (Guo *et al.*, 2000; Singh *et al.*, 2004). Despite relatively high success rates of these assays, their use remains limited since antiviral screening is an emerging field and therefore there is a need of funding for specialized laboratory facilities and skills for successful antiviral screening.

The current study report on the activation of silent BGCs in endophytic fungi isolated from *Albizia adianthifolia* using small epigenetic modifiers to increase the secretion of secondary metabolites for use as anti-HIV compounds. To achieve this, extracts were prepared from endophytic fungi cultured in the presence of sodium butyrate and were tested against the HIV-1 pseudo strain. This was followed by fungal molecular identification and bioassay-guided isolation. This study suggests that activation of silent BCGs by small epigenetic modifiers in endophytic fungi may strengthen the ability to develop effective anti-HIV compounds from endophytic fungi secondary metabolites.

1.2. Rationale

The Human Immunodeficiency Virus continues to be a public health concern due to the emergency of drug-resistant HIV strains that escape antiviral drug regimens, side effects that result in non-adherence, and failure of currently available drugs to eliminate proviral particles (Andersen *et al.*, 2018). The development of safe, effective anti-HIV drugs at low cost is one of the top global priorities of drug development since HIV is presently not curable (Esté and Cihlar, 2010). One of the alternative sources of potential anti-HIV agents that is gaining impetus are microbial-derived natural products, mainly from unexplored biodiversity such as endophytic fungi associated with medicinal plants (Rege *et al.*, 2015). It is proposed that the

symbiotic relationship between plants and fungi facilitates the sharing of genes encoding secondary metabolites (Ul-Hassan *et al.*, 2012). Although endophytic fungi have a high potential as a source of diverse compounds, the remaining challenge is that these compounds are encoded by silent biosynthetic pathways, which limits their exploitation under laboratory cultivation conditions (Reen *et al.*, 2015). Several innovative cultivation techniques have been recently shown to elicit the expression of most of these biosynthetic genes, yielding a plethora of active properties, including viral inhibiting properties (Reen *et al.*, 2015; Ibrahim *et al.*, 2016). Epigenetic elicitation of fungal biosynthetic genes with small molecular modifiers has successfully induced various fungal secondary metabolite gene clusters (Deepika *et al.*, 2016; Chen *et al.*, 2013). The successes achieved with the epigenetic elicitors provide a rationale for exploring genetically uncharacterized fungal isolates from unique environments, such as in the relationship of endophytic microorganisms with plants (González-Menéndez *et al.*, 2016). In these efforts, small molecular epigenetic modifiers can be used to elicit the expression of silent biosynthetic pathways to reveal a treasure trove of bioactivities, including anti-HIV properties.

1.3. The main aim of the study

To investigate the impact of small epigenetic modifiers in activating silent BGCs from endophytic fungi and profile the produced secondary metabolites for potential use as anti-HIV agents.

1.4. Specific objectives

- To activate silent BGCs using epigenetic modifiers and profile the fungal crude extracts of both induced and non-induced cultures for anti-HIV activity.
- To identify fungi exhibiting anti-HIV properties using the molecular technique: sequencing of the 18S rDNA internal transcribed spacer (ITS) region.
- To follow a bioassay-guided approach in isolating and purifying the active compound exhibiting anti-HIV activity.

1.5. Research question

Can epigenetic modifiers induce silent BGCs to produce secondary metabolites with the potential as antiviral agents?

1.6. Hypothesis

Treatment of endophytic fungi with epigenetic modifiers can induce the expression of silent secondary metabolites with varying bioactivities, including antiviral activities.

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CHAPTER 2

LITERATURE REVIEW

Section 2.6 of chapter 2 is extracted to the review paper

2.1. HIV/AIDS

2.1.1. Global statistics and classification.

Human Immunodeficiency Virus-1 (HIV-1) was discovered in 1981 by French scientists, Luc Montagnier and Barre Sinousi and American scientist, Robert Gallo. In 1983, it was verified that HIV-1 was the source of acquired immunodeficiency syndrome (AIDS) (Gallo *et al.*, 1983; Barré-Sinoussi *et al.*, 1983). Human Immunodeficiency Virus is still a significant pandemic, with 37.7 million people living with HIV globally, while around 680 000 people have died from AIDS-related illnesses worldwide and 28.2 million people were accessing antiretroviral therapy (**Figure 2.1**) (UNAIDS, 2021). Due to its increasing spread and the associated high mortality rate, AIDS represents a global health threat, and fighting this disease remains one of the main public health challenges (Roy, 2017).

Human Immunodeficiency Virus is a retrovirus, specifically a lentivirus, which cannot self-replicate and is divided into two major classes, HIV-1 and HIV-2 (Hemelaar, 2012). Human Immunodeficiency Virus -1 is the most prominent, accounting for 95% of infections. It can be divided into four groups: M, N, O, and P, with group M being the most wildly spread. Group M is further classified into nine subtypes: A, B, C, D, F, G, H, J, and K (Hemelaar, 2012). Exploring the structure of HIV-1 and its pathogenesis would reveal sites that are targeted by newly established therapeutics.

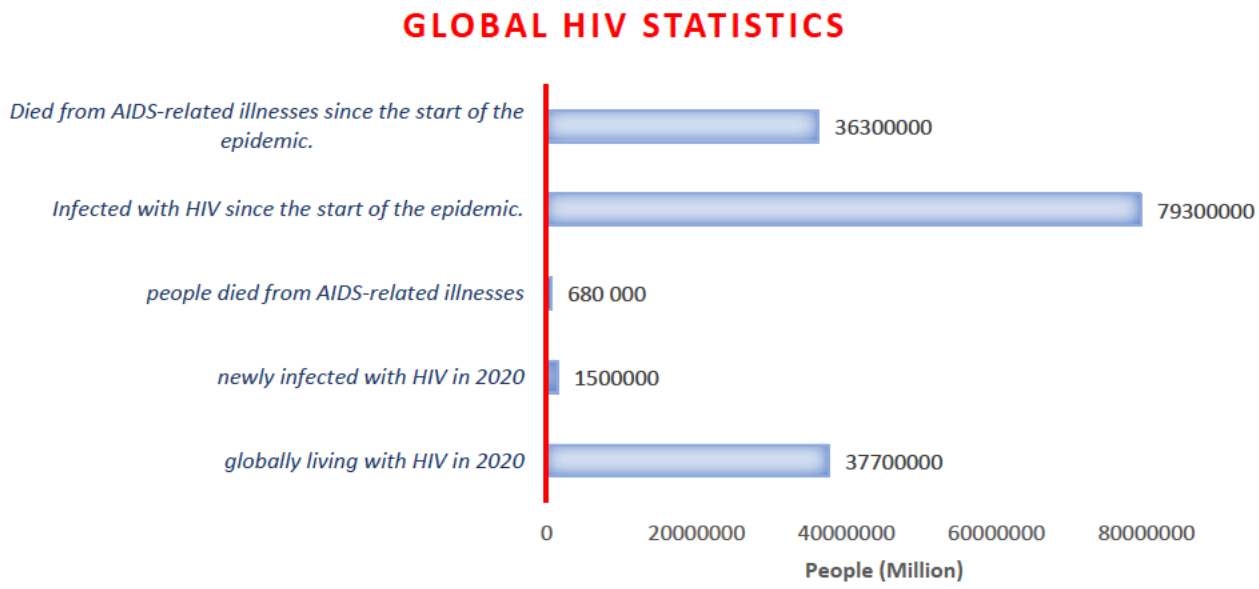

Figure 2.1: Global statistics of HIV/AIDS Adapted from (UNAIDS, 2021).

2.1.2. HIV-1 genome organization

The HIV genome consists of two identical single-stranded ribonucleic acid (RNA) molecules enclosed within the virus particle's core. Human Immunodeficiency Virus -1 comprises nine genes that encode 15 proteins (**Figure 2.2**) (Frankel and Young, 1998; Coffin *et al.*, 1997). These genes are group-specific antigen (*gag*), polymerases (*pol*), an envelope protein (*env*), trans-activator of transcription (*tat*), a regulator of expression of viral proteins (*rev*), negative regulatory factor (*nef*) , viral infectivity factor (*vif*), viral protein R (*vpr*) and viral protein U (*vpu*) (**Figure 2.2**) (Freed, 1998, 2001). The Gag polyprotein precursor is proteolytically processed to generate the matrix (MA), capsid (CA), nucleocapsid (NC), and p6 proteins. The *gag-pol* polyprotein contains protease (PR), reverse transcriptase (RT) and integrase (IN). The *env* gene encodes a 30-amino-acid signal peptide (SP), gp120, and gp41. Other sequences encode auxiliary proteins *gag* encode MA (P17), CA (P24), and TF(P6), and *pol* encodes protease (PR; P11), reverse transcriptase (RT; P65/P51), and integrase (IN; (P32). Both ends of the RNA genome are flanked by a long terminal repeat (LTR) promoter region (Coffin *et al.*, 1997). Inside virions, HIV genomic RNA is found as a non-covalent dimer, is 5′ capped and 3′ polyadenylated, and is annealed to a host tRNALys3 molecule (Coffin *et al.*, 1997; Watts *et al.*, 2009). **Figure 2.2** illustrates the organization of HIV-1 genome (Nkeze *et al.*, 2015).

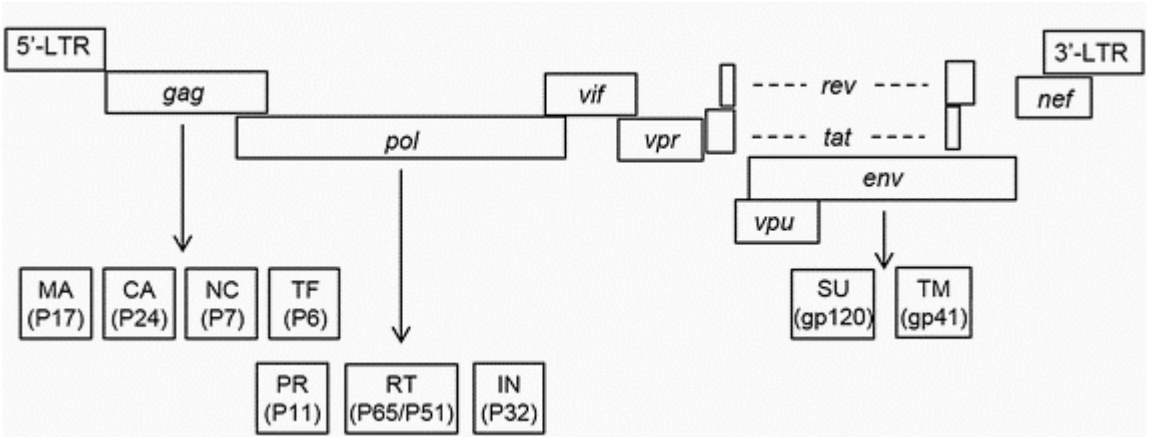

Figure 2.2: Schematic presentation of HIV-1 genome organization. Arrows point to cleaved protein products. MA, CA, NC, and TF from the *gag* gene, PR, RT, and IN from the *pol* gene, and SU and TM from the env gene. Dashed lines represent RNA splicing for rev and tat genes. The number in parenthesis is the molecular weight of each protein, and 5'-LTR and 3 indicate the ends' LTR (Nkeze *et al.*, 2015).

2.1.3. HIV/AIDS replication cycle

The basic steps of the HIV replication cycle consist of the $CD4^+$ T antigens as the primary receptor for viral entry. $CD4^+$ T antigens are present on $CD4^+$ T lymphocytes, monocytes, dendritic cells, and brain microglia (Male *et al.*, 2006). Human Immunodeficiency Virus infection begins when the glycoprotein 120 (gp120) binds to a $CD4^+$ T receptor on the host cell surface. During binding of gp120 to $CD4^+$ T receptors, conformational changes expose chemokine receptor binding sites (CC chemokine receptor 5 (CCR5) or C-X-C chemokine receptor type 4 (CXCR4)), which serve as core receptors for viral entry. (**Figure 2.3**) (Kwong *et al.*, 2000; Xiao *et al.*, 2021). Human Immunodeficiency Virus -1 infectivity is intermittently compromised in individuals with mutant (non-functioning) CCR5 proteins and those agents that bind to CCR5 and CXCR4, reducing viral infectivity (Male *et al.*, 2006; Eckert and Kim, 2001). When the virus successfully attaches to the host cell, the virus is then able to pass through the host cell membrane by fusing the viral envelope to the host cell's plasma membrane (**Figure 2.3**) (Ng *et al.*, 1997).

Upon entry of HIV into the host cell, the single-stranded RNA genome is converted into double-stranded deoxyribonuclease acid (DNA) by the reverse transcriptase enzyme (Roitt and Delves, 2001; Singh *et al.*, 2005). Reverse transcription is a crucial step in the HIV-1 life cycle since it prepares the viral genome for successful integration into the host chromosome (Hu and Hughes, 2012). Once the reverse transcription is accomplished, the viral genome, which is now a double-stranded DNA molecule and the proteins associated with reverse transcription, can then enter the nucleus of the host cell, and the viral genome which is incorporated within the

host chromosome is termed a provirus (**Figure 2.3**) (Hu and Hughes, 2012). Integration is a process by which the integrase enzyme integrates HIV-1 genome into the host cell's chromosome (Roitt and Delves, 2001).

As a result, provirus DNA can start manufacturing infectious viral particles or become latent. This is facilitated by vital host factors determining whether the host cell is activated or inactivated (Roitt and Delves, 2001). In an activated cellular arrangement, the provirus DNA is copied by the host cell's RNA polymerase II to create viral RNA. These RNA transcripts are spliced into messenger RNAs for translation into HIV regulatory proteins (*tat* and *rev*) or precursor proteins encoded by the *gag*, *pol*, and *env* genes. However, the core protein precursor is cleaved only by the HIV protease enzyme (Male *et al.*, 2006). Two copies of RNA, four proteins (P24, P17, P9, P7), and three pol enzymes (Reverse transcriptase, Integrase, and Protease) assemble together to form an infective virion particle. The infective virion particles are then transferred to the cell membrane to acquire envelope proteins (gp120 and gp41), which is subsequently released into the plasma membrane to infect healthy cells (Roitt and Delves, 2001; Botes *et al.*, 2007).

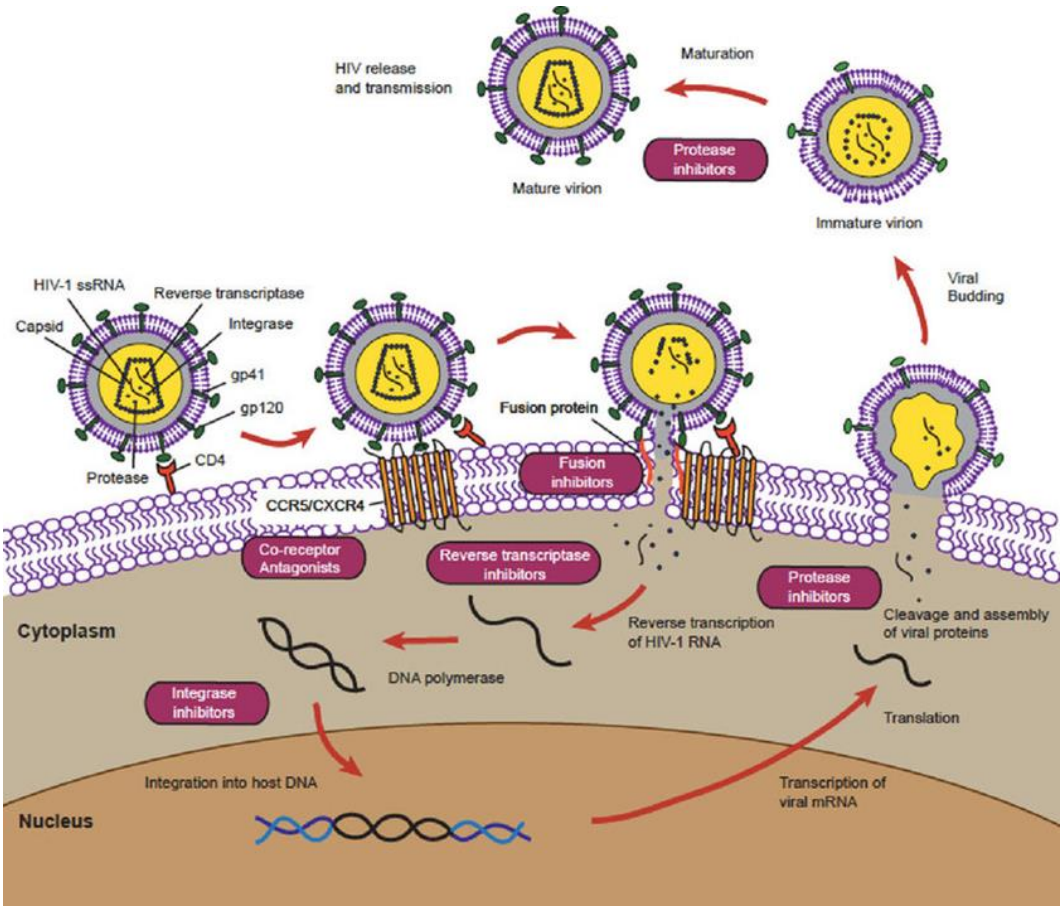

Figure 2.3: HIV replication cycle and steps targeted by antiretroviral therapy (Yavuz *et al.*, 2018). The diagram indicates replication cycle steps illustrated by arrows starting with viral attachment, fusion, RNA reverse transcription, DNA integration, mRNA transcription and translation, and lastly, cleavage and assembly of viral proteins, budding and viral maturation.

2.1.4. Current HIV therapies

Although HIV/AIDS is incurable, treatments have improved life expectancy and ensured many people do not develop AIDS (De Clercq, 1995; Broder, 2010). Due to successful efforts in drug discovery by the pharmaceutical industry and researchers, HIV has become the chronic clinically manageable disease. In 1987, Azidothymidine (AZT), an analog of nucleoside, was approved as the first antiretroviral drug (ARV) (De Clercq, 2009; Gulick, 1997). The drug interferes with the enzyme reverse transcriptase, which converts viral RNA to DNA. Azidothymidine competes with thymidine triphosphate, the natural nucleoside substrate for binding to the active site of reverse transcriptase and incorporation into viral DNA (Jochmans, 2008; De Clercq, 2013). However, Azidothymidine is rarely used, primarily because of poor tolerance and substantial risk of long-term adverse effects. Azidothymidine has been linked to a variety of side effects, including tiredness, gastrointestinal disturbance, lipoatrophy, bone marrow suppression, and myopathy (De Clercq, 2013). Since the AZT discovery, several drugs have been discovered, It is further reported that anti-HIV medication taken alone is not as

effective as combined ARV treatment as a single treatment allows the virus to mutate over time and build up resistance to the medication (Roy, 2017; Bhatti *et al.*, 2016).

The advent of combination antiretroviral therapy (cART), also known as highly active antiretroviral therapy (HAART), for treating HIV-1 infection was seminal in reducing the morbidity and mortality associated with HIV-1 infection and AIDS (McLaren and Fellay, 2021; Cihlar and Fordyce, 2016). To date, there are 26 Food and Drug Administration (FDA)-approved drugs available for the treatment of HIV-1 infections (**Table 2.1**). These drugs are mainly directed against three key viral enzymes required for replication, namely reverse transcriptase, protease and integrase. Reverse transcriptase targeted by nucleoside-analog reverse transcriptase inhibitors (NRTIs) and non–nucleoside reverse transcriptase inhibitors (NNRTIs). Protease inhibitors interfere with the function of protease enzyme while integrase inhibitors target the integrase enzyme and fusion and entry inhibitors target viral envelope glycoprotein gp41, and a coreceptor (Cos *et al.*, 2004; Meintjes and Maartens, 2012; Meintjes *et al.*, 2017). All classes of ARVs have successfully inhibited HIV-1. For example, the NRTIs recommended for initial regimens include two drugs such as abacavir (ABC) with lamivudine (3TC) or tenofovir disoproxil fumarate with emtricitabine (Günthard *et al.*, 2014; Günthard *et al.*, 2016). Protease inhibitors available are Atazanavir (ATV) and Darunavir (DRV) (Meintjes *et al.*, 2017) , and there are only three approved drugs as integrase inhibitors, namely raltegravir (RAL), elvitegravir (EVG), and dolutegravir (DTG) (Pommier *et al.*, 2005). **Table 2.1** presents all classes of ARVs with their mechanisms and example of drugs approved by the FDA.

Table 2.1: Antiretroviral drugs approved by the FDA (Lipsky, 1996; Meintjes *et al.*, 2012).

Class of ARVs	Drug compound	Mechanism
Chemokine receptors antagonist (CCR5)	Maraviroc (MVC)	Block CCR5 receptors on CD4 cells
Fusion Inhibitors	Enfuvirtide (ENF)	Block entrance of HIV into CD4 Cells
Integrase single transfer Inhibitors (INSTIs)	Raltegravir (RAL) Elvitegravir (EVG) Dolutegravir (DTG)	Blocks the incorporation of HIV's DNA into the host cells by inhibiting integrase enzyme
Nucleoside reverse transcriptase inhibitors (NRTIs)	Abacavir (ABC) Didanosine (DDI) Emtricitabine (FTC) Lamivudine (3TC) Stavudine (d4T) Tenofovir (EFV) Zidovidine(AZT, ZDV	Inhibit reverse transcriptase through chain termination
Non-nucleoside reverse transcriptase inhibitors (NNRTIs)	Delavirdine (DLV) Efavirenz (EFV) Etravirine (ETR) Nevirapine (NVP)	Inhibit reverse transcriptase through direct binding and inactivation

	Nevirapine extended-release (NVP XR) Rilpivirine (RPV)	
Protease Inhibitors (PIs)	Atazanavir (ATV) Darunavir (DRV) Fosamprenavir (FPV) Indinavir (IDV) Lopinavir/ritonavir (LPV/r) Nelfinavir (NFV) Ritonavir (RTV/r) Saquinavir hard gel caps (SQV) Tipranavir (TPV)	Inhibit HIV protease, an enzyme necessary for catalytic cleavage of proteins needed for viral replication

Despite the cART being potent and life-prolonging, a significant drawback is that it is not curative and does not eradicate HIV-1 infection since the HIV-1 provirus remains integrated into the host genome primarily in the $CD4^+$ T cells (Hamlyn *et al.*, 2012). Antiretroviral therapy disruption of the stable viral reservoir inevitably leads to a rapid rebound of viremia, and therefore elimination of this reservoir is crucial to achieve sterilizing cure (Monforte *et al.*, 2000; Sengupta and Siliciano, 2018; Cihlar and Fordyce, 2016). Antiretroviral therapy is a lifelong commitment, and there is an urgent need for curative strategies to reduce the burden of administrating drugs daily. Of note, long-term toxicity and sustained immune activation, particularly monocyte activation, are associated with lifelong ART (Kuritzkes, 2016; Kelesidis *et al.*, 2012). These reports have created an urgent need for a continuous search for HIV cure strategies and the development of effective anti-HIV drugs with novel modes of action.

Various strategies for an HIV-1 cure have been studied, such as “shock and kill”, “Block and lock”, gene editing, and RNA interference (Moranguinho and Valente, 2020; Abner and Jordan, 2019; Ognenovska *et al.*, 2018; Atkins *et al.*, 2021). These strategies mainly focus on removing the provirus from the host cell genome, eliminating cells containing the provirus, or blocking key HIV-1 or host factors required for replication (Sadowski and Hashemi, 2019). There has been a lot of attention given to the concept of "Shock and kill" which employs latency reversing agents (LRA) to reactivate or shock latent HIV (Abner and Jordan, 2019). Despite their significant success in activating proviral DNA in latent cells, both *in vitro* and *in vivo*, LRAs have not been able to result in any significant reduction of the latent viral reservoir of memory T cells (Tyagi and Bukrinsky, 2012; Kim *et al.*, 2018). In the process of reactivating latent viruses, the main hurdle is that the cells are quiescent and do not express activating factors that contribute to host gene expression and efficient HIV transcription (José *et al.*, 2014). There is need for cheap, and effective compounds with less or no toxicity.

Natural products from endophytic microbes, such as fungi and bacteria isolated from plants, are one alternative strategy that has recently gained interest in drug discovery (Strobel *et al.*, 2004). Particularly, endophytic fungi within plants synthesize diverse and unique secondary metabolites; therefore, they have been the subject of research on anticancer, antibacterial, antifungal, and antiviral natural products (Staniek *et al.*, 2008; Aly *et al.*, 2013; Newman *et al.*, 2003; Newman and Cragg, 2020). Due to the availability of well-established industrial production parameters that utilize fungal species, the production of fungal secondary metabolites can be scaled up at a reasonable cost and time (Roy, 2017; Venugopalan and Srivastava, 2015). It has been proposed that fungal compounds often exhibit innovative mechanisms of action that can circumvent previously identified mechanisms of drug resistance in pathogens (Alvin *et al.*, 2014).

Whilst endophytic fungal secondary metabolites with antiviral properties have received less attention, some compounds have been shown to inhibit HIV in screening programs successfully. For example, gliotoxin compound from endophytic fungi *Aspergillus fumigatus*, have been found to reverse HIV-1 latency (Stoszko *et al.*, 2020). Recent reviews by Roy. (2017) and Linnakoski *et al.* (2018) have documented some of these compounds. To date, no fungal metabolite has been approved for the treatment of viral infections mainly because of lack specialized laboratory facilities since live viruses are highly infectious and that also point to a need for high skill training for laboratory personnel (Roy, 2017; Linnakoski *et al.*, 2018). To further optimize therapeutic options, there is a need to develop new drugs from alternative sources that will not be susceptible to ongoing resistance mechanisms and that will be directed against alternative targets in the virus lifecycle.

2.2. Natural products from endophytic fungi

Natural products (also referred to as secondary metabolites) derived from microorganisms, plants, or animals have been well investigated as a potential source of therapeutic compounds (Strobel and Daisy, 2003). Tiwari *et al.* (2015) have demonstrated that natural products have so far had an inexplicable effect on clinical applications, with the pharmaceutical sector employing high-throughput techniques to screen secondary metabolites as the prospective hit to lead molecules (Tiwari *et al.*, 2015). These products of natural origin have been studied in the context of other diseases, such as cancer, antibacterial and antifungal agents, and several chemotherapeutic drugs have resulted from these studies (Newman *et al.*, 2003; Shu, 1998).

Approximately 60% of commercially available anti-cancer and anti-infective drugs are of natural origin or direct derivatives of the natural chemical scaffolds (Cragg *et al.*, 1997).

A substantial amount of evidence points to microbial natural products for therapeutic drug development and discovery due to their chemical diversity, uniqueness, and biological capabilities (Strobel and Daisy, 2003; Gunatilaka, 2006; Chapla *et al.*, 2014). Natural products derived from fungal endophytes exhibiting antiviral properties encompass alkaloids, steroids, terpenoids, flavonoids, glycosides, xanthones, coumarins, quinones, phenylpropanoids, lignans, aliphatic metabolites, and lactones (Zhang *et al.*, 2006; Aly *et al.*, 2013). It is vital to discover novel and cheaper anti-HIV agents from natural sources that may have fewer side effects with novel modes of action.

2.3. Endophytic fungi

2.3.1. Definition

The term "endophytes" refers to a group of microorganisms, often fungi and bacteria, which live in association with plants. In 1866, Anton de Bary proposed the term endophytes to describe microorganisms that live inside their host plant tissues - as opposed to epiphytes, a term assigned to microorganisms that live on the surface of plants (de Bary, 1866; Dutta *et al.*, 2014; Gakuubi *et al.*, 2021). By definition, endophytic fungi colonize the internal organs of plants without damaging them during any stage of their lifecycle (Bacon and White, 2000; Hyde and Soytong, 2008). Many fungal endophytes have been isolated from trees, shrubs, herbaceous plants, grasses, mosses, ferns, and marine plants (Mishra *et al.*, 2014).

2.3.2. The prevalence of endophytic fungi

Fungal diversity is exceedingly high, with approximately 5.1 million species reported in the literature (Blackwell, 2011). An estimated 300,000 species of plants are on the planet, yet very few are explicitly studied in terms of endophytic communities (Strobel and Daisy, 2003). Usually, endophytic fungi belong to the *Ascomycete* order and sometimes to the *Basidiomycete* or *Zygomycete* (Sun and Guo, 2012).

Endophytic fungi have previously been classified into two groups based on evolutionary relatedness, taxonomy, plant hosts, and ecological functions (Rodriguez *et al.*, 2009). Clavicipitaceous endophytes which infect some grasses, and non-clavicipitaceous endophytes which can be recovered from asymptomatic tissues of nonvascular plants, ferns and allies,

conifers, and angiosperms are the groups of endophytic fungi. Class 1 endophytes frequently increase plant biomass, promote drought resistance, and produce toxic compounds that reduce herbivory (Uzma *et al.*, 2019; Clay, 1988). Based on host colonization patterns, mode of transmission between host generations, in planta biodiversity levels, and ecological function, non-clavicipitaceous endophytes can be classified into three functional classes. All three classes of endophytes have diverse host ranges, Class 2 endophytes can thrive in both above- and below-ground tissues. Class 3 and 4 endophytes, on the other hand, are restricted to above-ground tissues and roots, respectively. Colonization of host tissues varied as well: Class 3 endophytes cause extremely localized infections, but Class 2 and 4 endophytes can colonize large areas of tissue (Hardoim *et al.*, 2015; Rodriguez *et al.*, 2009) .

2.3.3. Endophyte-plant interactions

Endophytic fungi colonize the inside of host tissues, facilitating the production of plant hormones and bioactive compounds (Khan *et al.*, 2016; Satheesan and Sabu, 2020). The plant benefits the fungal endophyte in a mutual relationship by providing nutrients, shelter, and seed dispersal. In contrast, the fungal endophytes convert the host-bioactive plant's compounds into multifunctional products for plant protection (Schouten, 2019). The endophyte accomplishes this protection by releasing secondary metabolites to attack the antagonists or cells affected by lysing, thereby inducing host defence mechanisms and promoting plant growth (Alvin *et al.*, 2014). Kusari *et al*. (2012) reported that this interaction between endophytes and plants triggers the biosynthesis of bioactive compounds in endophytic fungi (Kusari *et al.*, 2012).

A shared biosynthetic pathway between endophytes and their hosts was first identified in the discovery of paclitaxel (Taxol), one of the most recognized anticancer agents. Taxol was originally discovered in the bark of *Taxus brevifolia*, and remains one of the most potent anti-cancer agents (Stierle *et al.*, 2003; Stierle *et al.*, 1995). First, *Taxomyces andreanae* was found to produce paclitaxel isolated from *T.brevifolia*, but later, other paclitaxel-producing fungi were found from other Taxus species (Xiong *et al.*, 2013; Roopa *et al.*, 2015). The fact that fungal endosymbionts of this plant could also yield this drug has inevitably led to renewed interest in the quest for novel therapeutics in fungal species (Stierle *et al.*, 2003; Stierle *et al.*, 1995; Aly *et al.*, 2013).

In published research by Kumar et al. (2017); Guo *et al.* (2000) and Wellensiek *et al.* (2014) have reported xanthoviridicatins, cytonic acid A and B, and altertoxins as antiviral compounds (Guo *et al.*, 2000; Kumar *et al.*, 2017; Bashyal *et al.*, 2014). Despite the successful efforts in

search effective therapeutic compounds from fungi-associated plants, our knowledge of the host-endophyte (fungal) relationship in biochemistry and physiology is limited and needs further elucidation.

2.3.4. Organization of biosynthetic gene clusters in fungi

The genome of each fungus contains remarkable biosynthetic gene clusters (BGCs) encoding various secondary metabolites (Chen *et al.*, 2013; Inglis *et al.*, 2013; Khaldi *et al.*, 2010). The clustering of the biosynthetic genes is suggested to guarantee coordinated regulation of secondary metabolism (Shwab *et al.*, 2007). These BGCs belong to a few categories of biosynthetic pathways encoded by enzymes often described as backbone and core enzymes. Despite the diversity of structural characteristics of secondary metabolites, a few common biosynthetic pathways facilitate their production (Keller, 2019; Linnakoski *et al.*, 2018).

There are three classes of BGCs, namely non-ribosomal peptides (NRPs) and polyketides (PKs), gene clusters being the most abundant, while also hybrid NRP-PK classes, prenyltransferases (DMATSs), terpene cyclases (TCs) are commonly found in fungi (Bergmann *et al.*, 2007; Khaldi *et al.*, 2010; Linnakoski *et al.*, 2018). The NRPs are distinguished by three main domains: the adenylation (A) domain responsible for amino acid recognition, the activation-responsible peptidyl carrier protein (PCP), and the bond formation-responsible condensation (C) domain (Alvin *et al.*, 2014). Typical PKs also consists of three domains, namely acyltransferase (AT), ketosynthase (KS), and thioesterase (TE). Acetyltransferase acts as a gatekeeper for the specificity of the substrate, selecting and activating the monomers, the intermediate acyl chain. Ketosynthase (KS) catalyzes the formation of the C-C bond through the Claisen condensation in the elongation of the polyketide chain. Finally, the TE domain is responsible for the formation of the C-C bond. These BGCs offer a potential source for new scaffolds for novel drug discovery (Tyurin *et al.*, 2018). Fungal secondary metabolites not produced by the synthases or synthetases discussed above include ustiloxin ribosomal-derived peptide, fatty acid-derived oxylipins and the recently discovered isocyanide xanthocillin, which requires isocyanide synthase (Pettit, 2011; Lim and Keller, 2014).

Genome mining opens new avenues for exploring the genetic basis of secondary metabolite biosynthesis and devising ways to activate inactive metabolic pathways (Bergmann *et al.*, 2007; Ochi and Hosaka, 2013). Fungal biosynthetic gene clusters are known to be multimodal in terms of enzymatic domains and can exceed 100 kb in size. This trait presents significant

drawback in the utilization of molecular biology techniques to capture their activities (Reen *et al.*, 2015). The expression of these gene clusters is frequently controlled by epigenetic regulation mediated by chromatin structure, resulting in the tight regulation of these BGCs (Pettit, 2011; Strauss and Reyes-Dominguez, 2011). Conversely, the advent of genome sequencing platforms indicated a statistically substantial mismatch between the number of genes involved in secondary metabolism and the known secondary metabolites in fungi (Clevenger *et al.*, 2017). This is because most of the genes in fungi encoding secondary metabolites are not accessible, and they remain silent or are produced in low concentrations under standard laboratory settings (Nützmann *et al.*, 2013). To awaken these BGCs and identify their cryptic products, various innovative cultivation and gene manipulation approaches have been established (Larsen *et al.*, 2005; VanderMolen *et al.*, 2013).

2.4. Strategies for inducing silent biosynthetic gene cluster in endophytic fungi

A fundamental understanding of the regulatory circuits that control secondary metabolites gene clusters is required to activate silent BGCs successfully. It is not yet known what triggers the expression of the silent or transiently expressed BGCs in natural environments (Reen *et al.*, 2015). Several strategies have been discovered to awaken the 'silent' genes and alter the profile of secondary metabolites (Larsen *et al.*, 2005; VanderMolen *et al.*, 2013; Hewage *et al.*, 2014). **Figure 2.4** indicates two classes of strategies, culture-based approaches and genetic modification.

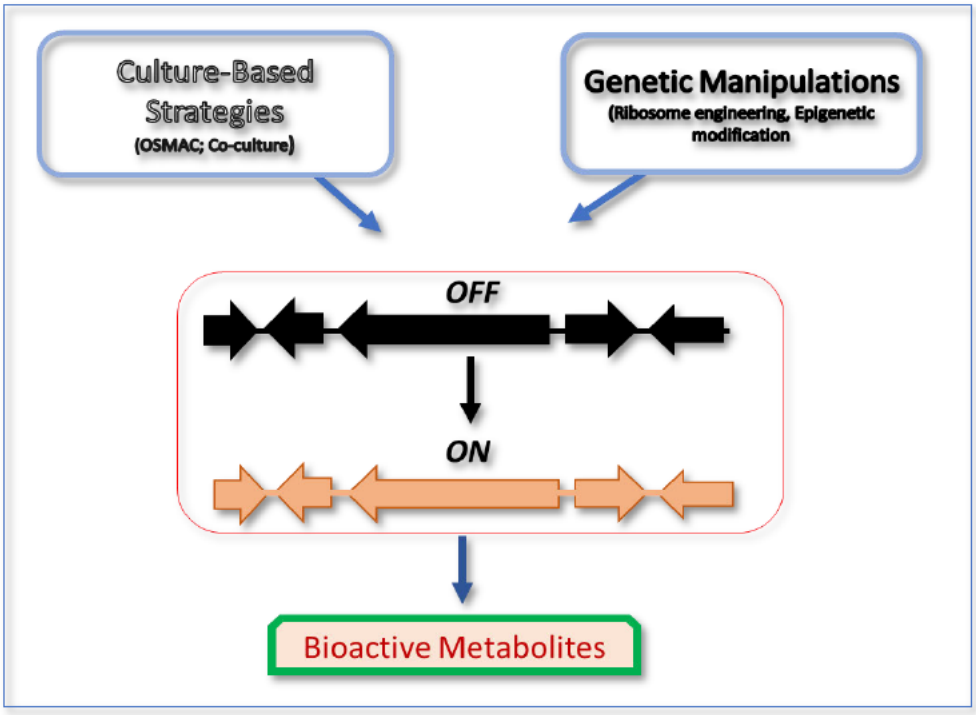

Figure 2.4: Strategies for activation of silent biosynthetic gene clusters. The culture-based strategy includes OSMAC, co-culture, and genetic manipulation, including ribosome engineering, epigenetic modifications (Adapted and modified from Okada and Seyedsayamdost. (2017) (Okada and Seyedsayamdost, 2017).

2.4.1. Culture-based strategies

The culture-based strategies include the systematic modification of growth conditions, known as one strain many compounds (OSMAC) approach (Pan *et al.*, 2019). The key concept behind this technique is that since each microbial strain has the ability to synthesize a variety of compounds, only a subset of these compounds is produced under specified growth conditions (Bode *et al.*, 2002; Romano *et al.*, 2018). Therefore, discrepancies in cultivation parameters can elicit the production and lead to the discovery of new secondary metabolites by modifying cultivation parameters such as temperature, salinity, aeration, and even the shape of the flasks. Bode *et al.* (2002) illustrated that the fungus *Aspergillus ochraceus*, which was thought only to yield the metabolite aspinonene, was able to yield 15 additional metabolites under altered culturing conditions (Bode *et al.*, 2002). More studies have exploited this technique by varying several cultivation parameters to yield jadomycin isolated from *Streptomyces venezuelae*, validomycin from *Streptomyces hygroscopicus*, and cerulenin from *Dimorphosporicola tragani* (González-Menéndez *et al.*, 2019; Doull *et al.*, 1994; Liao *et al.*, 2009).

The second culture-based approach comprises co-culturing with microorganisms such as bacteria, where interaction between the co-cultivated organisms induces the production of

metabolites encoded by the silent BGCs (Rutledge and Challis, 2015; Bertrand *et al.*, 2014). The co-culture strategy is modelled on the complex communities of fungi and bacteria in the natural environment, allowing them to survive on the shared resources and against their niches' environmental adversities (Strobel *et al.*, 2004). In addition, the organisms in these niches, compete with each other for survival which may require the respective organisms to produce unique defence mechanisms. Hence, mimicking these environments in a laboratory can enforce competition for survival, leading to the activation of silent biosynthetic pathways and the subsequent isolation of new bioactive secondary metabolites (Netzker *et al.*, 2018). One of the examples is the discovery of penicillin G, produced by *Penicillium notanum* cultured on a petri dish with *Staphylococcus aureus* (Okada and Seyedsayamdost, 2017; Radetsky, 1996). Since this discovery, researchers have reported more compounds produced from co-cultivation of different species, including Emericellamides A and B from *Emericella sp* and oosponol and oospoglyco produced from *Heterobasidion annosum* (Oh *et al.*, 2007; Sonnenbichler *et al.*, 1994). However, co-culture approaches are, laborious, and require scientists to conduct multiple experiments under different conditions, with the outcome sometimes being unknown (Romano *et al.*, 2018).

Chemical elicitors are small molecules that has been shown to differentially induce silent BGCs at subinhibitory concentrations. This induction often results in the overexpression and production of natural products since the control of BGC expression is complex, the addition of chemical elicitors is an efficient technique to induce the expression of silent BGCs. the process of determining which small molecule elicitor will influence the synthesis of a particular secondary metabolite is based on randomization. However a new method high-throughput elicitor screening (HiTES) has been proposed, the advantage of HiTES is that it allows insights into both the product(s) and regulation of a silent BGC (Mao *et al.*, 2018). The HITES approach consists of insertion of a reporter gene into the BGC of interest to provide a rapid read-out for its expression. Subsequently, small molecule libraries are screened to identify candidate elicitors (Seyedsayamdost, 2014). Etoposide is an example of a successful compound that was used in a study employing the HiTES technique. Etoposide induced the production of acyl-surugamide A by activating the sur BGC in *Streptomyces albus* J1074 (Xu *et al.*, 2017).

2.4.2. Genetic modification strategies

Gene modification is yet another strategy that can be utilized to activate the silent gene clusters in endophytic fungi to yield secondary metabolites. Several genetic manipulation tools have been developed to awaken fungi's silent biosynthetic gene clusters (Jiang *et al.*, 2021). Engineering of transcription and translation machinery by introducing mutational variants to RNA polymerase and proteins is one technique for increasing BGC expression in this strategy (Rutledge and Challis, 2015; Pfannenstiel and Keller, 2019). Another genetic approach is the manipulation of global regulators to activate multiple pathways for the discovery of multiple metabolites; this event entails manipulating pathway-specific regulators, using reporter guides to select mutants and refactoring using inducible or constitutive promoters and heterologous hosts (Rutledge and Challis, 2015; Baral *et al.*, 2018). Successful genetic modification efforts has been shown in the production of lovastatin, clavulanic acid, natamycin, and pacidamycin D (Askenazi *et al.*, 2003; Xiang *et al.*, 2009; Wang *et al.*, 2016; Casini *et al.*, 2018).

The limitation of manipulating pathway-specific regulators is that prior knowledge of genes encoding putative pathway-specific transcription factors to increase expression of BGCs is required (Rutledge and Challis, 2015). The reporter-guided mutant selection approach facilitates the detection of mutants expressing target BGCs (Rutledge and Challis, 2015). Refactoring by substituting natural promoters with constitutive or readily inducible promoters allows both direct capture of BGCs and simultaneous cloning of multiple DNA fragments (Rutledge and Challis, 2015; Fan *et al.*, 2017; Baral *et al.*, 2018). Following the identification of the BGC of interest, the genes are activated and expressed in a heterologous host. The heterologous expression approach improves workflows for identifying metabolites. In comparison, conventional strategies for capturing target BGCs used to involve screening of cosmid gene libraries, which had a slow and inefficient cloning process and failed to clone the desired DNA fragment accurately. However, recent advances in direct cloning and DNA assembly tools have led to more viable alternatives (Rutledge and Challis, 2015; Fan *et al.*, 2017). Despite their success in numerous fungal species, these genetic approaches have been restricted in their application in fungi due to complex fungal physiology and a lack of molecular tools to manipulate fungal genomes (Rutledge and Challis, 2015; Jiang *et al.*, 2021).

Advantages of culture-based over genetic manipulation approaches

The use of culture-based approaches seems feasible, particularly for the discovery of non-model environmental fungal isolates. As opposed to genetic manipulation, these approaches do not require prior knowledge of BGC types and the regulatory processes that govern their expression. Furthermore, these approaches can be applied to microbes that are less amenable to genetic manipulation and are ideal tools for unlocking genetically refractory marine microorganisms (Reen *et al.*, 2015; Romano *et al.*, 2018). One successful approach is adding epigenetic modifiers belonging to the class of DNA methyltransferase (DNMT) and histone deacetylase (HDAC) inhibitors (Okada and Seyedsayamdost, 2017). It induced secondary metabolite pathways in a subset of diverse endophytic fungi that would not otherwise occur under standard culture conditions (González-Menéndez *et al.*, 2016).

2.4.3. Epigenetic tools to induce silent BGCs via chromatin remodeling

Fungal DNA, like that of any other higher eukaryote, is coiled around histones (H2A, H2B, H3, and H4) to form nucleosomes which results into chromatin (Cutter and Hayes, 2015). The chromatin have been implicated in the inaccessibility of DNA by transcription factors. In fungi, the diversity of genomes varies between 8970 kb and 177570 kb (Aghcheh and Kubicek, 2015; Sun and Guo, 2012). Chromatin exists in two states: a "*closed*" state called heterochromatin, which is densely packed and therefore transcriptionally silent, while an "*open*" state called euchromatin is more loosely packed and transcriptionally active (**Figure 2.5**) (Brosch *et al.*, 2008; Gacek and Strauss, 2012). Post-translational modifications can alter histone proteins' interactions with DNA and other proteins found in the eukaryotic nucleus. Several post-translational modifications of histones, including acetylation, methylation, and phosphorylation, have been shown to influence chromatin arrangements that facilitate DNA accessibility by transcription factors (Bannister and Kouzarides, 2011; Aghcheh and Kubicek, 2015). This has been observed in filamentous fungi, where histone acetylases, methyltransferases, and histone-binding proteins have proven to be significant for the expression of secondary-metabolite biosynthesis gene clusters (Reyes-Dominguez *et al.*, 2010).

One of the mechanisms involved in regulating fungal secondary metabolite biosynthesis has been identified as chromatin-level control of gene silencing or activation (Keller, 2019; Brakhage, 2013). Small chemical molecules known as epigenetic modifiers have been shown to alleviate such regulatory hurdles in fungi (Akone, 2016; Baral *et al.*, 2018). It has been

discovered that chemical targeting of histone and DNA post-translation modifications can increase the expression of fungal secondary metabolites by activating or inhibiting gene clusters that encode secondary metabolites (Cichewicz, 2010; Gakuubi *et al.*, 2021). **Figure 2.5** illustrates the mechanism of epigenetic modifiers to induce silent BGCs (Pillay *et al.*, 2022). Target strains are cultured in the presence of epigenetic modifying compounds, notably HDAC inhibitors and DNA methyltransferase (DNMT) inhibitors during epigenetic modification (Yang *et al.*, 2014). Magotra *et al.* (2017) found that adding such compounds in growth cultures inhibited or activated the related enzymes, resulting in secondary metabolite biosynthesis pathways re-engineering (Magotra *et al.*, 2017).

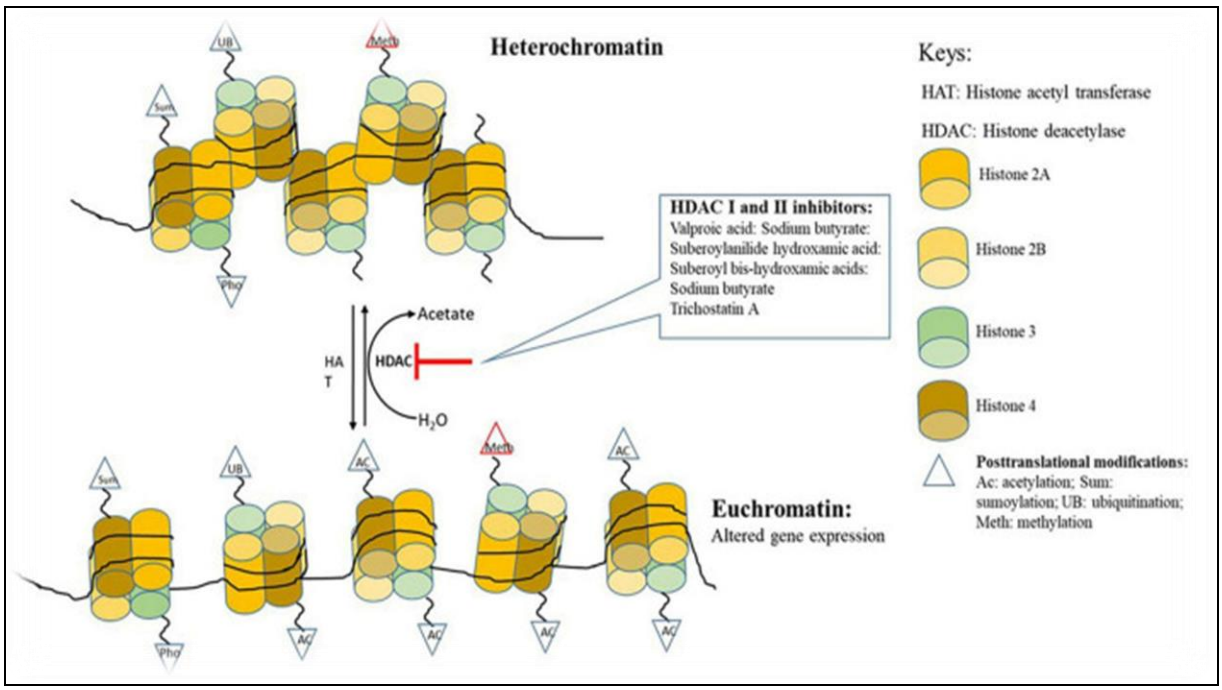

Figure 2.5: Epigenetic alterations: Histone acetyltransferase (HAT) mediated acetylation or Histone deacetylase (HDAC) mediated deacetylation of Histones. These tags are associated with chromatin modulation required for the expression of cryptic genes (adapted from Pillay *et al.* 2022) (Pillay *et al.*, 2022).

The first evidence that chromatin contributes to secondary metabolite gene cluster regulation was obtained from the deletion of the HDAC *hdaA* in *A. nidulans*, leading to the activation of two secondary gene clusters producing sterigmatocystin and penicillin (Shwab *et al.*, 2007). The same study discovered that application of the HDAC inhibitor trichostatin A to *Alternaria alternata* and *Penicillium expansum* induced the production of several unknown compounds signifying that both genetic and chemical manipulation of chromatin modifications can lead to the activation of secondary gene clusters (Shwab *et al.*, 2007). Two histone post-translational

modifications involved in secondary metabolites gene expression have been extensively studied: acetylation and methylation (Strauss and Reyes-Dominguez, 2011).

The growth of the endophytic fungus *Aspergillus fumigatus*, isolated from *Grewia asiatica* in potato dextrose broth (PDB containing 500 µM of valproic acid as an HDAC inhibitor, resulted in a reduction in the quantity of several compounds identified in the control culture (Magotra *et al.*, 2017; Krämer *et al.*, 2003). In contrast, fumiquinazoline C concentrations in cultures of strains grown with the HDAC inhibitor increased ten-fold (Magotra *et al.*, 2017; Krämer *et al.*, 2003).. Other than the ones mentioned above, there are numerous reports on the use of epigenetic modifiers to activate silent BGCs for secondary metabolite production **(Table 2.2)** (Henrikson *et al.*, 2009; Smith and Edlind, 2002; Shwab *et al.*, 2007; Sun and Guo, 2012; Vrba *et al.*, 2011). In conclusion, these findings imply that small molecules capable of altering epigenetic factors at sub-inhibitory concentrations may activate silent gene clusters or increase the production of fungal metabolites that are naturally present in fungi.

Table 2.2: Epigenetic modifiers are used to induce fungal biosynthetic gene clusters encoding secondary metabolites.

Epigenetic modifier	Class of inhibitor	Fungal species	Plant species	Induced compound	Concentration	Reference
Valproic Acid	HDAC inhibitor	*Aspergillus fumigatus*	*Grew asiatica*	Fumiguinazdine C	500 µM	(Magotra *et al.*, 2017; Krämer *et al.*, 2003)
SAHA/ 5-azacytidine	HDAC inhibitor	*Chaetomium sp*	Unidentified	Isosulochrin	Unidentified	(Henrikson *et al.*, 2009)
Sodium Butyrate	HDAC inhibitor	*Leucotoma personii*	Unidentified	Cytosporone B, C, and E	Varied concentrations	(Vrba *et al.*, 2011)
5-azacytidine	DNMT inhibitor	*Alternaria sp*	*Datura stramonium*	Mycotoxin alternariol	250 µM	(Sun and Guo, 2012)
Nicotinamide	NAD+ dependant HDAC inhibitor	*Eupenicillium sp*	*Xanthium sibiricum*	Eupenicinicol C and D; Eujavanicol A and eupenicinola A	Unidentified	(Li *et al.*, 2014)
Trichostatin A	HDAC inhibitor	*Alternaria alternata and Penicillium expansum*	Unidentified	sterigmatocystin and penicillin	Unidentified	(Smith and Edlind, 2002; Shwab *et al.*, 2007).

2.5. Rationale for exploring endophytic fungi from *Albizia adianthifolia*.

The plant, *adianthifolia* (Flat crown), is a medium to a large tree from the family *Fabaceae,* subfamily *Mimosoideae*. The species belongs to *Albizia durazz*., a genus valued worldwide for its ecological, economic, and medicinal properties (Louppe *et al.*, 2008). The *Albizia* species have historically been used in traditional medicine for treating coughs, indigestion, constipation, insomnia, irritability, rheumatism, stomach ache, tuberculosis, and wounds (Singab *et al.*, 2015).

Phytochemical studies of *Albizia* species have led to the isolation of secondary metabolites, including saponins, terpenes, alkaloids, and flavonoids (Singab *et al.*, 2015; Lacaille-Dubois *et al.*, 2011). There is evidence that saponin compounds isolated from the genus *Albizia* possess cancer-related properties, including analgesic, anthelmintic, antidysenteric, antihistaminic, anti-inflammatory, antimicrobial, antimutagenic, antiseptic, antispermatogenic, antitumor, anxiolytic, cytotoxic, immunomodulatory, nootropic, and apoptosis-inducing properties (Lacaille-Dubois *et al.*, 2011). An example of bioactive saponin is adanthifoliosides A, B, and D, which have induced apoptosis in Jurkat cells at 5 µM and 1 µM, respectively (Haddad *et al.*, 2004). Based on fungal endosymbiosis with plants, this discovery may further suggest that endophytic fungi isolated from *A.adianthifolia* could produce similar compounds (Louppe *et al.*, 2008). However, there are least or no reports on endophytic fungi isolation from *A.adianthifolia*. Thus, isolation and identification of fungi from *A.adianthifolia* will be relevant.

2.6. Endophytic fungi as a source of anti-HIV-1 compounds.

To combat antiviral resistance, there is an urgency for the development of new anti-HIV drugs since approved ARVs are associated with various shortfalls such as toxicity and rapid emergence of the resistant strain. These ARV's limitations make anti-HIV discovery an intriguing area of research. The bioactive metabolites from endophytic fungi stand a chance as suitable candidates for synthesizing anti-HIV agents. In search of anti-HIV inhibitors from fungal endophytes, Sch 210971 and Sch 210972 isolated from *Chaetomium globosum* compound inhibited CCR-5 inhibitory activity at 1.2 and 79 µg/mL (Shafer *et al.*, 2007; Martinez *et al.*, 2015). More anti-HIV agents discovered from endophytic fungi, which can be further developed, are presented in **Table 2.3**. Bioprospecting of endophytic fungi for anti-HIV agents could be a promising field. However, there has been little documentation on their exploration thus far (Rajamanikyam *et al.*, 2017). The obstacles to the discovery of antiviral compounds include highly specialized and incompatible screening measures or the lack of

innovative antiviral screening strategies in the more significant part of secondary metabolite compound discovery programs (Kaul *et al.*, 2012).

Table 2.3: Anti-HIV-1 compounds isolated from endophytic fungi and their mechanisms of action

HIV-1 Replication targeted stages	HIV-1 Agent/compound	Endophytic fungi	Class of compounds	Mechanism of action	Active concentration	reference
Fusion or entry	Isochromophilones I and II	*Penicillium nicillium*	Entry inhibitor	Inhibited the gp120–CD4 binding	6.6 and 3.9 µM	(Omura *et al.*, 1993)
	Variecolin	*Emericella aurantiobrumea*	Entry inhibitor	Competed with macrophage inflammatory protein (MIP)-1 for binding to CCR5	9 µM	(Yoganathan *et al.*, 2004).
	Sch 210971 and Sch 210972	*Chaetomium globosum*	Entry inhibitor	Strongly inhibited CCR-5 inhibitory activity	1.2 µM and 79 nM	(Shafer *et al.*, 2007; Martinez *et al.*, 2015).
Reverse Transcription	Phenylspirodrimanes	*Stachybotrys chartarum*	Reverse transcriptase inhibitor	Inhibited the RT RNA-dependent DNA polymerase activity in a dose-dependent manner	8.4 µM	(Ma *et al.*, 2013)
	Helotialins A and Helotialins B	*Helotialean Ascomycete*	Reverse transcriptase inhibitor	Unidentified	8.01 and 27.9 nM	(Zou *et al.*, 2009)
	Altertoxins	*Alternaria tenuissima*	Reverse transcriptase inhibitor	Unidentified	≤ 2.20 µM	(Bashyal *et al.*, 2014)
Integration	Xanthoviridicatins E and F	*Penicillium. chrysogenum*	Integrase Inhibitor	Inhibited the cleavage reaction of HIV-1 integrase.	6 and 5 µM	(Singh *et al.*, 2003)
	Aquastatin A	*Fusarium aquaeductuum*	Integrase inhibitor	Moderately inhibited the strand transfer reaction of HIV-1 integrase	50 µM	(Hamano *et al.*, 1993)

	Atrovenetinone methyl acetal	*Penicillium sp. FKI-1463*	Integrase inhibitor	Unidentified	19 µM	(Shiomi *et al.*, 2005)
Translation or budding	Hinnuliquinone	unidentified fungus	Protease inhibitor	Unidentified	2.5 µM.	(O'Leary and Hanson, 1982)
	Colossolactones	*Ganoderma colossum*	Protease Inhibitor	Inhibited HIV-1 protease in a dose-dependent manner	4,1-4,4 µM	(Herath *et al.*, 2004; Singh *et al.*, 2000)
	Mulberroside C	Unidentified fungus	Protease Inhibitor	Inhibited HIV-1 protease in a dose-dependent manner	<7.8 µg/mL	(Vora *et al.*, 2021)

Efforts have been made to target enzymes and proteins responsible for the HIV-1 life cycle. The interaction of the HIV glycoprotein gp120 envelope protein with a $CD4^+$ T cell on the surface of most susceptible cells promotes HIV entry into the host cell. It is well established that the chemokine receptors CCR5 and CXCR4 are required as coreceptors for binding gp120 and $CD4^+$ T cells (Henrich and Kuritzkes, 2013). Inhibition of such binding can prevent viral entry into the host cell and prevent replication, and this is a highly effective alternative target for HIV therapy. Several anti-HIV-1 compounds derived from endophytic fungi targeting the gp120–CD4 binding have been discovered, including isochromophilones I and II obtained from *Penicillium nicillium*, which have been shown to disrupt gp120–CD4 interaction at a concentration of 6.6 and 3.9 µM, respectively, and prevent HIV-1 entry into the host cell (**Table 2.3**) (Omura *et al.*, 1993). Other fungal endophytes explored as a source of HIV-1 entry inhibitors include *Emericella aurantiobrumea* and *Chaetomium globosum*, presented in **Table 2.3**. (Yoganathan *et al.*, 2004; Shafer *et al.*, 2007). However, clinical trials on these compounds would offer more insight into their mechanisms and potential for drug development.

Endophytic fungi have also been explored as a source of reverse transcriptase inhibitors. In a study by Bashyal *et al.* (2014), Altertoxins extracted from *Alternaria tenuissima* were found to inhibit the reverse transcriptase enzyme from transcribing HIV-1 RNA into DNA (Bashyal *et al.*, 2014). Ma *et al.* (2013) discovered phenylspirodrimanes from *Stachybotrys chartarum* to be a reverse transcriptase-RNA-dependent DNA polymerase inhibitor that suppressed DNA polymerase activity in a dose-dependent manner (Ma *et al.*, 2013). A Helotialean ascomycete has also been looked at as a reverse transcriptase inhibitor (**Table 2.3**) (Zou *et al.*, 2009). These

reports prove that endophytic fungi could be the source of reverse transcriptase inhibitors. However, additional research is needed to corroborate this assertion.

In 2004, Singh *et al.* (2003) showed that xanthoviridicatins E and F at the concentration of 6 and 5 µM are potent inhibitors of HIV-1 integrase enzyme derived from *Penicillium chrysogenum*. Both compounds inhibited the cleavage process of HIV-1 integrase (Singh *et al.*, 2003). In addition, Atrovenetinone methyl acetal and Erabulenol B were also discovered to be integrase inhibitors (**Table 2.3**) (Shiomi *et al.*, 2005).

Human Immunodeficiency Virus-1 has developed resistance to approved protease inhibitors primarily due to amino acid mutations within or proximal to the drug's catalytic binding site (Zdanowicz, 2006). Fungal compounds that have been found to inhibit protease enzymes include colossolactones isolated from *Ganoderma colossum*. Colossolactones inhibited the HIV-1 protease enzyme in a dose-dependent manner at concentrations ranging from 4, 1-4, 4 µM (Herath *et al.*, 2004). The discovery of Hinnuliquinone from an unidentified fungus by O'Leary and Hanson. (1982) added to the protease inhibitors literature; however, the mechanism of action of this compound is unknown (O'Leary and Hanson, 1982; Singh *et al.*, 2004).

While promising and successful efforts have been made to develop anti-HIV-1 drugs, fungi have been less extensively studied for their antiviral activity. Secondary metabolites isolated from endophytic fungi have revealed the potential to inhibit HIV-1 at distinct stages of its life cycle. However, there are few reports regarding the mechanisms of these compounds during HIV-1 replication. more research is necessary to identify the mechanisms of these beneficial compounds isolated from endophytic fungi with low cytotoxicity.

Summary

The preceding review demonstrates the importance of employing small epigenetic molecules of fungal chromatin to induce silent BGCs for the secretion of novel secondary metabolites for application in the quest for anti-HIV drugs. Furthermore, the potential of endophytic fungi to produce pharmacologically active secondary metabolites with profiles similar to their hosts' plants is an intriguing concept that warrants further investigation, particularly in anticipation of discovering new drugs that can address the current anti-HIV resistance, which is a global challenge. Thus, the present study focused on assessing the secondary metabolite profiles of small molecule modifier-treated endophytic fungi isolated from *A.adianthifolia*. To achieve

this, selected small-molecule inhibitors with the potential to modify the fungi' chromatin were used to treat the isolated endophytic fungi.

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CHAPTER 3

MANUSCRIPT

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Epigenetic induction of secondary metabolites production in endophytic fungi *Penicillium chrysogenum* and GC-MS analysis of crude metabolites with anti-HIV-1 activity.

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3.1. Abstract

The continuous burden of Human Immunodeficiency Virus-1 in Sub-Saharan Africa, coupled with the inability of antiretroviral agents to eradicate HIV-1 from viral reservoirs, the potential risks of drug resistance development, and the development of adverse effects, emphasizes the need to develop a new class of HIV-1 inhibitors. Here, we cultivated five endophytic fungi isolated from *Albizia adianthifolia* with the addition of small epigenetic modifiers, sodium butyrate and valproic acid, to induce the expression of biosynthetic gene clusters encoding active secondary metabolites with probable anti-HIV activities. We identified a non-toxic crude extract of the endophytic fungus *Penicillium chrysogenum* treated with sodium butyrate to possess significantly greater anti-HIV activity than the untreated extracts. Single-round fractionated extracts of treated *P.chrysogenum* showed potent anti-HIV activity with an IC_{50} of 5.90 µg/mL and a 5-fold increase compared to the untreated fraction. The active fractionated extracts were subjected to gas chromatography-mass spectrometry (GCMS), and more bioactive compounds were detected in treated *P.chrysogenum* fractions than in untreated fractions. These results indicate that treatment of endophytic fungi with small epigenetic modifiers enhances the secretion of secondary metabolites with anti-HIV-1 properties, acknowledging the feasibility of epigenetic modification as an innovative approach for the discovery of cryptic fungal metabolites as therapeutic compounds.

Keywords: Human Immunodeficiency Virus; endophytic fungi; epigenetic modifiers; biosynthetic gene clusters; secondary metabolites

3.2. Introduction

Highly active antiretroviral therapy (HAART) has significantly reduced viral load and improved life expectancy patient survival (De Clercq, 2010). However, this therapy has not been able to eradicate the virus from the resting CD4+ T cells. Reportedly, HAART is associated with various side effects, including drug-resistance HIV strains and toxicity (Mayer and Venkatesh, 2010; Broder, 2010). On this account, it is essential to discover innovative and inexpensive anti-HIV agents from natural sources that may have fewer or no adverse effects with novel mechanisms (Kurapati *et al.*, 2016).

Several bioactive compounds from endophytic fungi have shown to have the potential to inhibit HIV-1 enzymes and proteins involved in the HIV-1 life cycle (Roy, 2017). Some fungal compounds explored as anti-HIV agents include altertoxins from *Alternaria tenuissima*, and phenylspirodrimanes from *Stachybotrys chartarum* (Bashyal *et al.*, 2014; Ma *et al.*, 2013).

These compounds inhibit a reverse transcriptase enzyme responsible for constructing a complementary deoxyribonucleic acid (DNA) from a ribonucleic acid (RNA) template (Bashyal *et al.*, 2014; Hu and Hughes, 2012). The integrase enzyme, which is responsible for the insertion of HIV DNA into the host genome during the DNA recombination mechanism, is yet another HIV-1 target inhibited by fungal compounds (Reeves and Piefer, 2005). Singh *et al.* (2003) showed that xanthoviridicatins E and F derived from *Penicillium chrysogenum* are potent inhibitors of the HIV-1 integrase enzyme. Both compounds inhibited the cleavage process of HIV-1 integrase (Singh *et al.*, 2003).

Apart from the aforementioned HIV-1 targets, Roy. (2017) has reported on protease and glycoprotein 120 as potential HIV-1 targets, as well as active fungal compounds that have been identified against them (Henrich and Kuritzkes, 2013; Kuritzkes, 2009; Monini *et al.*, 2003; Roy, 2017; Flexner, 1998). These studies indicate feasible support that endophytic fungi could be the source of anti-HIV compounds. However, there are few reports regarding the mechanisms of these compounds during HIV-1 replication (Linnakoski *et al.*, 2018). One of the limitations is that fungal compounds rarely enter clinical trials, and this is due to their high toxicity and uncertain mechanisms.

Advances in genomics and innovative culturing methods have made it possible to revisit microbial-derived natural products after they were abandoned due to the increasing rediscovery of already known molecules (Rutledge and Challis, 2015). The availability of high throughput fungal genome sequences has revealed that many fungal biosynthetic gene clusters (BGCs) are silent or transiently expressed when fungi are cultivated under laboratory conditions, in addition the regulatory circuits and environmental stimulus responsible for their expression in the natural environment have not been explained (Reen *et al.*, 2015; Ibrahim *et al.*, 2016). Several innovative culturing methods like spiking fungal cultures with small molecular epigenetic modifiers such as histone deacetylase (HDAC) and DNA methyltransferase (DNMT) have shown remarkable success in eliciting the expression of these silent BGCs, yielding a plethora of bioactive compounds that are not produced under normal growth conditions (Magotra *et al.*, 2017; Yang *et al.*, 2014).

Epigenetic modifiers have been shown to alter gene expression in fungi without altering the DNA sequence (Xiao *et al.*, 2013). *Aspergillus fumigatus* treated with the HDAC inhibitor valproic acid resulted in a 10-fold increase in fumiquinazoline C isolation, demonstrating the

efficacy of epigenetic modifications using chemical inhibitors (more specifically, HDAC or DNMT inhibitors) or inducers in stimulating the transcription of attenuated or silenced BGCs (Cichewicz, 2010; Magotra *et al.*, 2017). Three HDAC-like genes were found in *Streptomyces coelicolor* after an analysis for the presence of HDAC genes. After exposure to sodium butyrate, the BGC for actinorhodin production, which was inactive in *S.coelicolor*, was substantially expressed (Moore *et al.*, 2012; Zhu *et al.*, 2014). These discoveries indicate that epigenetic modifiers, particularly sodium butyrate and valproic acid, may be employed to induce silent BGCs in fungi. More compounds were also induced when fungi were cultured in the presence of epigenetic modifiers (Henrikson *et al.*, 2009; Krämer *et al.*, 2003; Liu *et al.*, 2014; Shwab *et al.*, 2007; Smith and Edlind, 2002; Vrba *et al.*, 2011).

In this study, we have assessed the effects of epigenetic modifiers in activating silent BGCs in endophytic fungi *P.chyrysogenum* isolated from *A.adianthifolia* following an antiviral screening assay for profiling the produced secondary metabolites as anti-HIV-1 compounds. Further, we performed GC-MS analysis on fractionated *P.chrysogenum* crude extracts. Sodium butyrate treated extracts revealed increased anti-HIV activity and increased production of secondary metabolites compared to untreated extracts. These findings show a promise toward epigenetic modification as a powerful strategy to induce silent BGCs of endophytic fungi to produce secondary metabolites with anti-HIV properties.

3.3. Materials and methods

3.3.1. Endophytic fungi isolation

Leaves and bark of *Albizia adianthifolia* (flat crown) were harvested from the South Coast area of the eThekwini Municipality, KwaZulu-Natal. The plant samples were submitted for taxonomic identification and registration (voucher number 18232) at the University of KwaZulu-Natal (UKZN) School of Life Science Herbarium. A method outlined by Arivudainambi *et al.*, (2011) and Petrini. (1986) was followed to isolate five endophytic fungi from the leaves and bark of *A.adianthifolia* (Arivudainambi *et al.*, 2011; Petrini, 1986). In summary, the plant samples (leaves and barks) were properly washed with distilled water to eliminate epiphytes. The surfaces were then sterilized in 70% (v/v) ethanol for one minute, then after dipped in sodium hypochlorite % (v/v) (Novachem South Africa) for three minutes before dipping them once more in 70% (v/v) ethanol for one min. The last wash was performed three times in distilled water and air-dried. The leaves and barks were cut into small pieces (5-7 mm), and aseptically added onto potato dextrose agar (PDA) and malt extract agar (MEA)

plates supplemented with 100 µg/mL ampicillin to inhibit bacterial growth. The cultures were placed in the dark at 25ºC for 14 days, followed by subsequent sub-culturing until single fungal cultures were retained and preserved in 25% glycerol at -80ºC for future purposes.

3.3.2. Treatment of endophytic fungi with small epigenetic modifiers

Small-molecule modifiers, Sodium butyrate and valproic acid (Sigma-Aldrich, South Africa) were diluted in distilled water and added to 10 mL of malt extract (ME) broth at a final concentration of 25 µM inoculated with three small pieces (1 cm) of fungal mycelia (Gonzalez-Menendez *et al.*, 2016). The fungal isolates treated and untreated with small-molecule modifier-treated cultures were placed in the dark at 25ºC for 14 days. Ten milliliters of fungi-free PD broth (control) were treated with small-molecule modifiers added to final concentrations of 25 µM of valproic acid and sodium butyrate. The culture controls were treated the same as the experimental controls to determine if the small-molecule modifiers and solvent altered the observed activity in the absence of fungal extract/culture. The assays were performed in duplicates.

3.3.3. Crude extraction of fungal secondary metabolites

After ten days of cultivation, an equal volume (10 mL) of 100% methanol (Lichro chemicals, South Africa) was added to the cultures to extract secondary metabolites. The cultures were placed on an orbital shaker (BUCHI) for one night to shake at 150 rpm at 25ºC. The following day, gauze was used to isolate the extracts from the mycelia. The extracts were dried at 40ºC to minimize the degradation of secondary metabolites constituents while mycelia were discarded. The dried extracts were stored at 4ºC and diluted in distilled water to a final concentration of 300 µg/mL prior antiviral activity screening.

3.3.4. Molecular Identification of endophytic fungi of active fungal extracts

3.3.4.1. Cultivation and DNA isolation of endophytic fungi

The fungal isolate (PO3MB2) was cultured in the dark on solid malt extract agar (MEA) for 14 days at 25ºC. For genomic DNA extraction, 100 mg of mycelia from the fresh fungal hyphae was collected. The Norgen Plant/Fungi DNA isolation kit (25240, Norgen Biotek, Thorold, ON, Canada) was used to extract genomic DNA per the manufacturer's instructions. The extracted DNA was evaluated for its quality with regard to purity and concentration using 1% agarose and a NanoDrop 2000c Spectrophotometer (ThermoScientific, South Africa), respectively.

3.3.4.2. Amplification of fungal DNA

The internal transcribed spacer (ITS) sequence region of the extracted fungal DNA was amplified using primers, forward primer, ITS1 (5′-TCCGTAGGTGAACCTGCGG-3′) and reverse primer, ITS4 (5′-TCCTCCGCTTATTGATATGC-3′). The polymerase chain reaction (PCR) master mix made up of Phusion hot start II high fidelity polymerase (BioLabs, New England, USA) (1X), ITS1 primer (0.5 µM), ITS4 primer (0.5 µM), 50 ng DNA, and nuclease-free water (9 µl). The PCR was carried out under the subsequent conditions: initial denaturation at 98ºC for the 30s, denaturation at 30 cycles of 98 ºC for 10s, annealing at 45 ºC for 15s, extension at 72 ºC for 15s, and final extension at 72 ºC for 2 mins. The PCR products were evaluated on a 1% agarose gel and purified using the PureLink® PCR Purification kit, Invitrogen (Thermo Scientific, Carlsbad, CA, USA), as per the manufacturer's instructions. The purified amplicons for each fungal isolate were measured using a NanoDrop 2000c (Thermo Scientific, South Africa).

3.3.4.3. Identification of endophytic fungi by ITS sequencing

The amplicons were sent to Central Analytical Facility (CAF) at Stellenbosch University (Stellenbosch, South Africa) for ITS region sequencing. The quality assessment of the ITS sequences was performed using Snap gene Viewer version 6.0.6. Subsequently, the quality of ITS sequences was compared using Basic Local Alignment Tool (BLAST) application of the nucleotide database of the National Centre for Biotechnology Information (NCBI) with gene databases (Accession number, ON989875). The sequences with the highest hits were chosen to develop phylogenetic tree using the maximum likelihood algorithm in MEGA X (Kumar *et al.*, 2018; Tamura and Nei, 1993). Evolutionary distances were computed using the Tamura-Nei model (Tamura and Nei, 1993) was used to determine evolutionary distances. The percentage of replicate trees and trees robustness was evaluated using a bootstrap test (1000 replicates) (Felsenstein, 1985).

3.3.5. Cell cultures

The TZM-bl and HEK 293T cell lines were obtained from the NIH AIDS research and reference reagents program. TZM-bl and HEK 293T cells were cultured in high-glucose Dulbecco's Modified Eagle's medium (D-MEM) (Lonza Walkersville, Inc. MD) containing 25 mM HEPES buffer, 10% FBS, and 50 µg/mL Gentamycin. Both cell lines were incubated at 37°C with 5% CO_2.

3.3.5.1. Transformation of XL1 Blue super competent cells and construction of viral plasmids

For transformation, one µl of plasmid DNA (pNL4.3 Luc, VSV-g, and CMV) were added to 50 µl of XL-gold competent cells to a pre-chilled Luria-Bertani (LB) broth (Thermo Fisher, South Africa). The transformation reaction was gently mixed and incubated on ice for 30 mins. Afterwards, the reactions were placed on heat pulse for 45 s at 42°C, followed by another incubation on ice for 2 mins. Pre-heated super optimal broth with catabolite repression (S.O.C) medium (Thermo Fischer, South Africa) at 42°C was added to the transformation solution and incubated at 37°C for 1 hour, shaking at 230 rpm. Transformation reactions were plated on nutrient agar plates supplemented with 100 µg/mL ampicillin, then incubated at 37°C for 16 hours. After incubation, the colonies were transferred to the master plate using pipette tips. Afterward, the tips were dislodged into 15 ml of pre-heated LB broth supplemented with 100 µg/mL ampicillin and placed in a shaking incubator at 37°C at 230 rpm.

3.3.5.2. Plasmid DNA purification

The GeneJET plasmid miniprep kit (Thermo Scientific, Lithuania, EU) was used to purify plasmid DNAs, following the manufacturer's instructions. The DNA quality was assessed using NanoDrop™ 2000 software (Thermo Scientific, South Africa), followed by 1% Agarose gel electrophoresis.

3.3.5.3. Generation of pseudoviruses by transfection

HIV-1 NL4.3 stocks were generated by transfection HEK 293T cells. Briefly, 3×10^6, 293T cells/mL were seeded in a T-75 culture flask with 5 ml of supplemented D-MEM one day before transfection to achieve 50-80% confluency of monolayers. The transfection solution containing 12 µL of Env plasmid DNA pCMV-VSV-g and 12 µL of backbone plasmid DNA pNL4.3 GFP-VSV-g in DMEM was transfected using 45 µL of XtremeGene (Promega, UK) according to the manufacturer's instructions. After 48 hours of incubation, the transfection medium was replaced with a fresh medium. The supernatant was harvested and filtered after 48 hours, and aliquots were stored at −80°C until use.

3.3.5.4. Titration of virus (TCID assay)

The $TCID_{50}$ of the generated HIV-1NL4.3 stocks was determined. Briefly, TZM-bl cells prepared at a density of 1×10^5 cells/well in DMEM (supplemented with 25 mM HEPES buffer, 10% FBS, and 50 µg/mL Gentamycin) containing 37.5 mg/mL DEAE dextran (Thermo Scientific, South Africa) were cultured in a 96-well plate. TZM-bl cells were infected with a serial dilution of the virus stock in duplicate, starting with a 1/10 dilution. After 48 hours of incubation, the culture medium (100 µL) was replaced with fresh DMEM media, and 100 µL Bright-Glo luciferase reagent (Promega, Madison, WI, USA) was added to each well under low light conditions. Complete cell lysis was achieved following incubation of the plate at room temperature for two minutes. All the contents were transferred to a corresponding 96-well bottom flat black plate (Costar, Corning Incorporated, USA). The relative luminescence was measured immediately using a Victor 2 luminometer plate reader (Perkin-Elmer Life Sciences, Shelton, CT) at 540 nm. The $TCID_{50}$ was determined by the virus dilution that was able to elicit 50000 relative light unit (RLU). A standard amount of virus (50000 RLU; Multiple of infection (MOI) =0.05) was used in the replication and drug susceptibility assays and the level of viral replication was expressed as a percentage of the RLUs.

3.3.6. Evaluation of cytotoxicity of fungal crude extracts

MTT (3-[4, 5-dimethylthiazol-2-yl]-2, 5-diphenyltetrazolium bromide) cell viability assay kit (Thermo Scientific, South Africa) was used to evaluate the cytotoxicity of fungal crude extracts and compounds used in this study according to the manufacturer's instructions. Briefly, 10 µL of 300 µg/mL treated and untreated methanol fungal crude extract with epigenetic modifiers and standard drug Azidothymidine (AZT) at 300 µg/mL (NHI AIDS research and reagent program) were 3-fold diluted to each well containing 100 µL of D-MEM in duplicate. Wells without any drug/crude extract were used as a negative control. TZM-bl cell suspension prepared at a density of 1×10^5 cells/mL in DMEM containing 37.5 µg/mL diethyl aminoethyl (DEAE)-dextran were seeded in the 96-well plate and incubated at 37°C, 5% CO_2 for 48 hours. After incubation, 12 mM of MTT stock solution (10 µL) was added to each well and incubated for 4 hours. Afterward, replaced the media with a fresh D-MEM medium. Formazan crystals were solubilized by adding 50 µL of diluted DMSO (0.2%) and incubated for 10 mins. The absorbance was read at 540 nm using an ELISA plate reader (PerkinElmer, USA). The percentage cell viability was calculated using the formula:

$$(\%)\text{Cell Viability} = [(\text{Sample Absorbance} - \text{Cell free sample blank})/(\text{Mean media Control Absorbance})] \times 100 \quad (1)$$

The 50% cytotoxic concentration (CC_{50}) causing visible morphological changes in 50% of TZM-bl cells, with respect to cell control, were determined using GraphPad Prism Software (v.5) (Hsiang, 2001).

3.3.7. Luciferase-based antiviral assay

A luciferase-based antiviral assay was performed using TZM-bl cell lines (Modi *et al.*, 2013). In this experiment, 10 µL of each treated and untreated crude methanol extract was 3-fold diluted in DMEM in 96-well flat-bottom plate. Fifty microliters of diluted HIV-1 NL4.3 (MOI = 0.05) virus were dispensed to all the wells except cell control wells and incubated for 1 hour at 37°C, 5% CO_2. TZM-bl cells prepared at a density of 1×10^5 cells/mL in a growth media containing 37.5 mg/mL DEAE dextran were seeded in a 96-well plate and incubated at 37°C 5% CO_2 for 72 hours. AZT at 300 µg/mL was used as a positive control, while the uninfected and untreated cells were negative controls. After incubation, the culture medium (100 µL) was replaced with a new DMEM media, and 100 µL Bright Glo luciferase reagent (Promega, Madison, WI, USA) was added to each well under low light conditions. Complete cell lysis was achieved following incubation of the plate at room temperature to a corresponding 96-well bottom flat black plate (Costar, Corning Incorporated, USA). The relative luminescence unit was measured immediately using a Victor 2 luminometer plate reader (Perkin-Elmer Life Sciences, Shelton, CT) at 540 nm. The percentage of viral inhibition was expressed as following this equation:

$$(\%)\text{HIV inhibition} = [(\text{Average Sample-Average Control})/(1-(\text{Average Viral Control-Average Control})] \times 100 \quad (2)$$

The half-maximal inhibitory concentration (IC_{50}) causing morphological changes in the HIV inhibition dose-response curve by 50% was calculated using GraphPad Prism Software (v.5).

3.3.8. Bioassay-guided isolation and chemical characterization of secondary metabolites

3.3.8.1. Extraction of secondary metabolites through large-scale Fermentation

Endophytic fungi that exhibited bioactivity in preliminary screening following MTT and luciferase-based assays were cultured (6 plugs) in 500 mL Erlenmeyer flasks containing 150 mL of malt extract broth at 25°C for 14 days, with shaking on a rotary evaporator at 150 rpm. After incubation, an equal volume of absolute methanol was added to the fungal culture and incubated overnight, shaking on a rotary evaporator at 150 rpm. The culture broth was filtered through gauze into a pre-weighed Erlenmeyer flask, and mycelium was discarded. Solvents were evaporated at 40°C to dry the extracts.

3.3.8.2. Solid Phase Extraction (SPE) of fungal crude extracts

Active crude extracts were fractionated as described by Stoszko *et al.* (2020) with some modifications (Stoszko *et al.*, 2020). Crude extracts were dried at 40°C, followed by a sequential fractionation step. Lipids were removed by hexane/water extraction 50/50 (v/v), 4-mL total volume], and an aqueous phase was collected and dried. The dried material was reconstituted in 50% methanol (MeOH), and 1 mL of such solution was spiked with 20 µL of phosphoric acid and loaded onto HLB, 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%) organic solvent (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 and filtered with a 0.2 µm filter before use. HLB was based on N-vinylpyrrolidone–divinylbenzene copolymer. MCX was a cation-exchange sorbent that represents the HLB material modified with SO_3H^- groups. MAX was an anion-exchange cartridge. After each round of fractionation, all samples were tested for anti-HIV-1 activity using the luciferase-based antiviral assay described above.

3.3.8.3. Gas chromatography-mass spectrometry analysis

A gas chromatography-mass spectrometer (GC-MS) analysis was performed to identify secondary metabolites from the partially purified crude fungal extracts. GC-MS was performed in the chemistry department at the University of KwaZulu-Natal (PMB). Briefly, GC-MS analysis was performed by splitless injection (spilt 20:80-8-200-5M-8-260-10M10-280-HP5-ETOH) of 1.0 µl of the sample in methanol on a Hewlett Packard 6890 (USA) gas chromatograph. The Agilent 19091S - 433 column (30m x 250µm x 0.25µm) was used to separate the samples. The starting column temperature was 35°C with a hold time of three minute. The temperature was set to increase at an 8°C/min, with a maximum temperature of 280°C. One microliter of the sample was injected into the port, subsequently vaporized, and transported down the column utilizing helium as the carrier gas at a flow rate of 1 ml/min. At 70 eV, the MS Spectrum was captured. Following the separation in the column, the components were identified and evaluated using a Flame Ionization Detector (FID). Compounds were identified by comparing the spectrum of unknown compounds to the spectrum of known compounds in the National Institute of Standards and Technology (NIST MS 2.0) structural library to determine their names, molecular weight, and structure (Mishra and Patnaik, 2020).

3.4. Results

3.4.1. Isolation and molecular identification of endophytic fungi

Five sterilized samples (barks and leaf) from *A.adiathifolia* cultured on MEA and PDA revealed fungal mycelia after 14 days of incubation at 25 °C. Fungal mycelia was then carefully transferred onto new plates for final preservation. Following the internal transcribed spacer (ITS) sequencing, one of five endophytic fungal isolates, P03MB2, was identified as *P.chrysogenum* following its antiviral activity in preliminary screening while other fungal isolates where not considered for identification as they did not show any inhibitory effects against HIV. The basic local alignment search tool (BLAST) analysis revealed that the P03MB2 strain belonged to *Penicillium spp. P.chrysogenum* was the most frequent isolate in 62 of 99 *Penicillium* isolates (66,67%). Sequence analysis of the ITS region of the encoded DNA displayed a substantial alignment of 99-100% in *P.chrysogenum* with 100% (550/550) identities. Following the data analysis from NCBI, a phylogenetic tree of the endophytic fungi *P.chrysogenum* was constructed and has revealed 100% relatedness *to Penicillium* species (**Figure 3.1**).

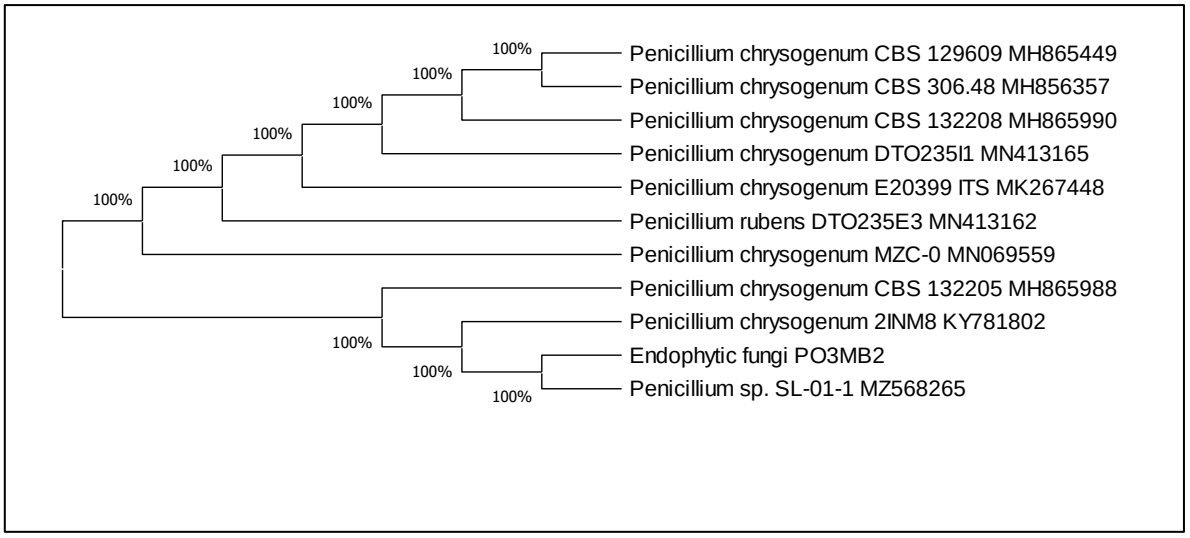


Figure 3.1. The maximum-likelihood tree based on ITS gene sequences of P03MB2, with closely related strains accessed from GenBank using the BLASTN tool. The sequences were aligned using ClustalW. Bootstrap values included 1000 replicates using MEGA software (version 11.06) and are displayed on the tree branches.

3.4.2. Cytotoxicity effects of crude methanol extracts

To determine the concentration at which fungal extracts are not toxic, the crude extracts were standardized to 300 µg/mL for cytotoxicity testing against TZM-bl cell lines. The percentage cell viabilities of TZM-bl cells against sodium butyrate-treated and untreated fungal methanol extracts are shown in **Figure 3.2**. The results revealed that all endophytic fungal extracts showed no cytotoxicity on TZM-bl cells with >80% cell viability and CC_{50} >1000 µg/mL. The positive reference, AZT at 300 µg/mL, did not show toxicity on TZM-bl cells with average cell viability of 106% and CC_{50} of 21.4 µg/ml. **Figure 3.3** indicates dose response curves representing cytotoxic effects of extract with their cell viability at 50%.

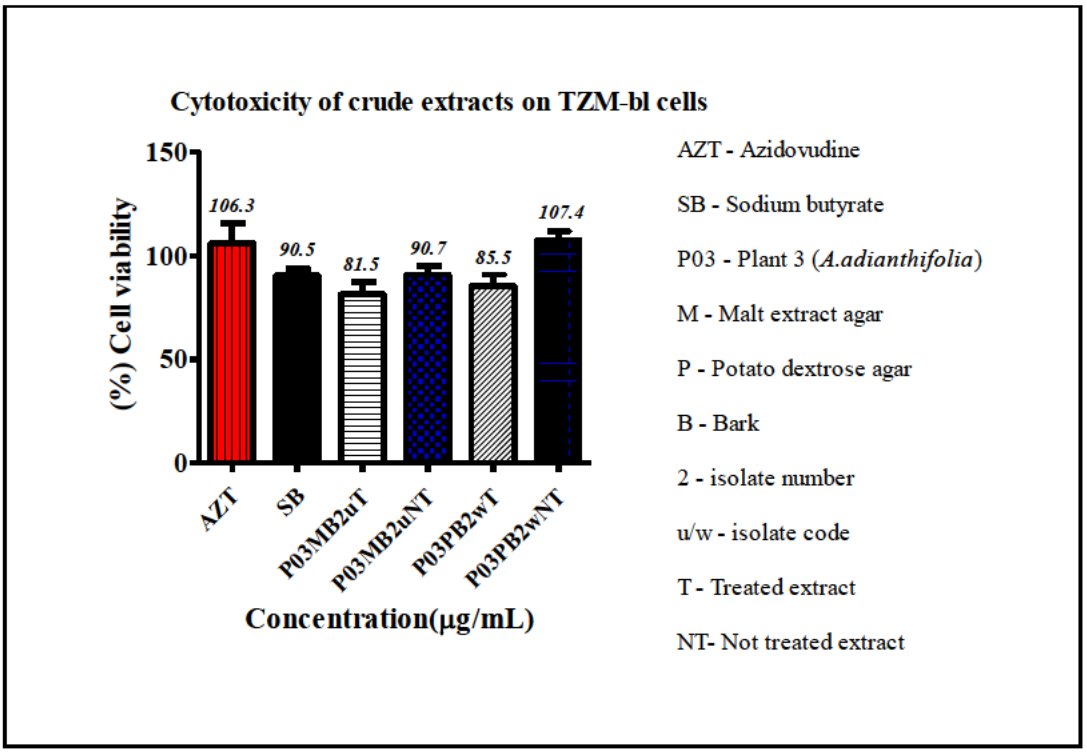


Figure 3.2: Percentage of cell viability of TZM-bl cell lines against methanol crude fungal extracts treated and untreated with Sodium butyrate. Results were obtained from two independent experiments; data are mean SEM.

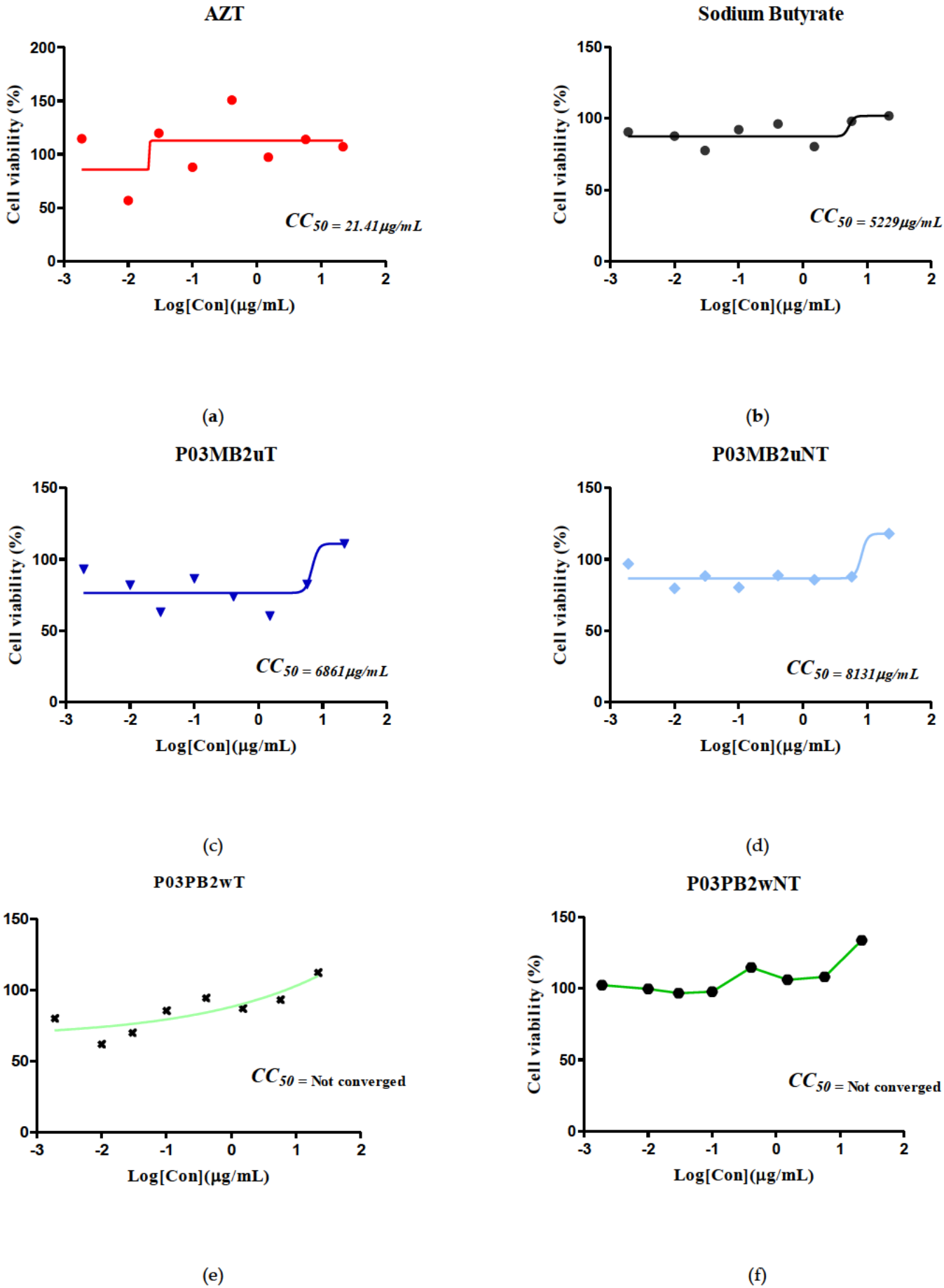


Figure 3.3: Dose-response curves showing cytotoxic effects of six crude extracts against TZM-bl cells of cartridges (a: AZT, b: Sodium butyrate, c: PO3MB2uT, d: P03MB2uNT, e:

P03PB2wT, and f: P03PB2wNT). Cytotoxicity concentration at 50% cell viability (µg/mL) were calculated.

3.4.3. Luciferase-based antiviral activity assay on crude extracts

Antiviral effects of the crude extract were measured by calculating the percentage of virus inhibition of extracts at varying 3-fold dilutions from the initial 300 µg/mL concentration. **Figure 3.4** showed that among all treated and untreated extracts, only treated *P.chrysogenum* strain P03MB2 extract showed anti-HIV activity with a half-maximal inhibitory concentration IC_{50} of 9.30 µg/mL with a fold change of 1.01 (**Figure 3.4**). The positive reference, AZT, indicated a positive dose-dependent curve with IC_{50} of 33.3 µg/ml.

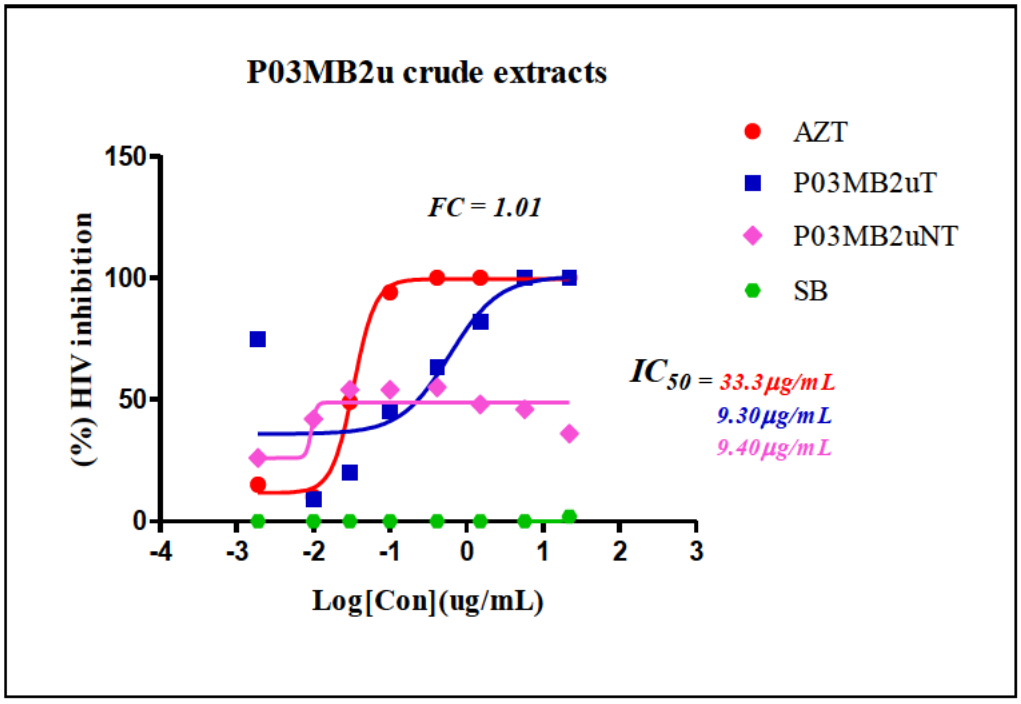
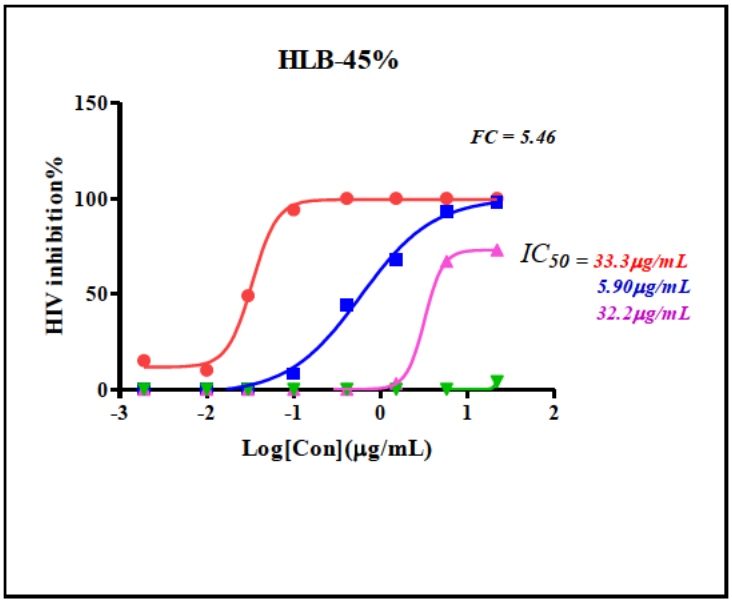


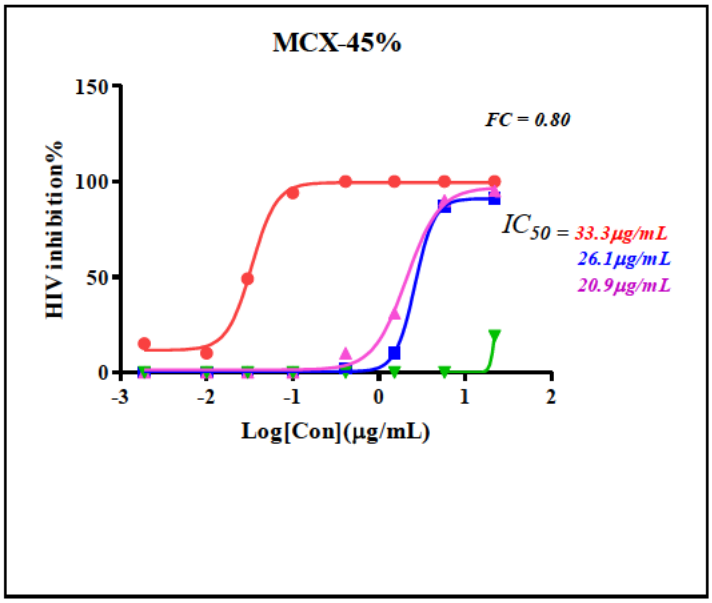
Figure 3.4: Dose-response curve showing anti-HIV-1 activity of sodium butyrate-treated and untreated crude extracts from *P.chrysogenum* tested in TZM-bl cell lines. Sodium butyrate (SB) extracts without fungal culture were used as a negative control, while AZT was used as a positive control. Inhibitory concentration at 50% inhibition (µg/mL) and fold change (FC) were calculated.

3.4.4. Bio-assay guided approach to the anti-HIV activity of secondary metabolites fractions (Solid-Phase extraction)

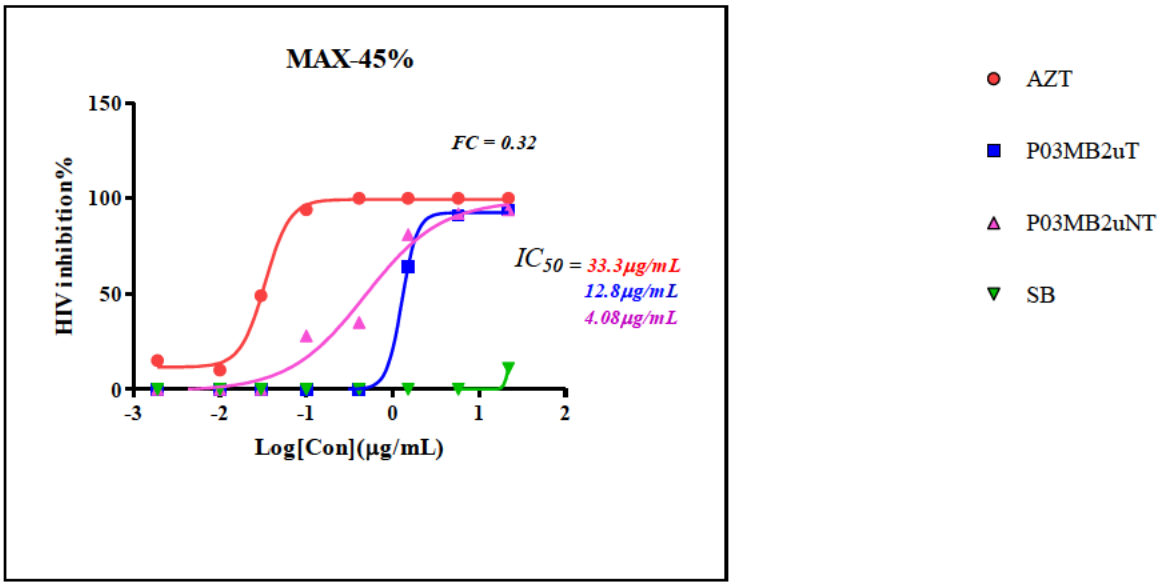
Large-scale crude extracts were standardized to 100 µg/mL with 50% MeOH. From a single round of fractionation, six fractions of treated and untreated *P.chrysogenum* P03MB2 isolates, and three sodium butyrate fractions were screened for anti-HIV activity at varying 3-fold dilutions from the initial concentration of 100 µg/mL. Sodium butyrate fractions were used as negative controls, and they did not reveal any anti-HIV activity when eluted with 5, 45, and 95% MeOH from all cartridges. *P.chrysogenum* fractions eluted with 45% MeOH from all three cartridges (HLB, MCX, and MAX) revealed anti-HIV activity, while fractions eluted with 5 and 95% did not indicate potent anti-HIV activity (**Figure 3.5**). Both cartridges produced active secondary metabolites fractions for both treated and untreated, with fold changes of 0.34 for fractions from MCX and 3.32 from MAX. HLB cartridge produced the best anti-HIV activity for treated *P.chrysogenum* fraction, with IC_{50} of 5.09 µg/mL compared to 32.3 µg/mL with a fold-change of 5.46.



(a)



(b)

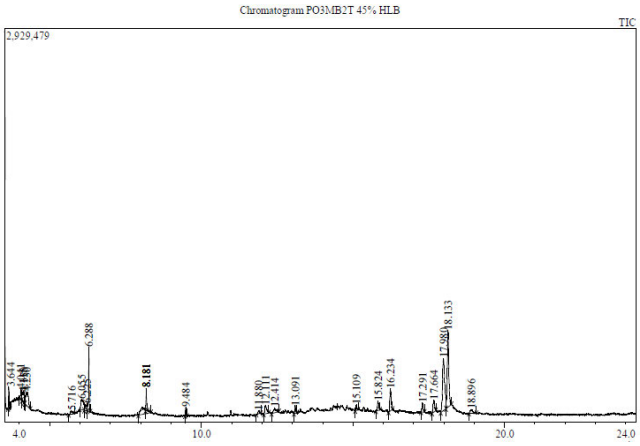


(c)

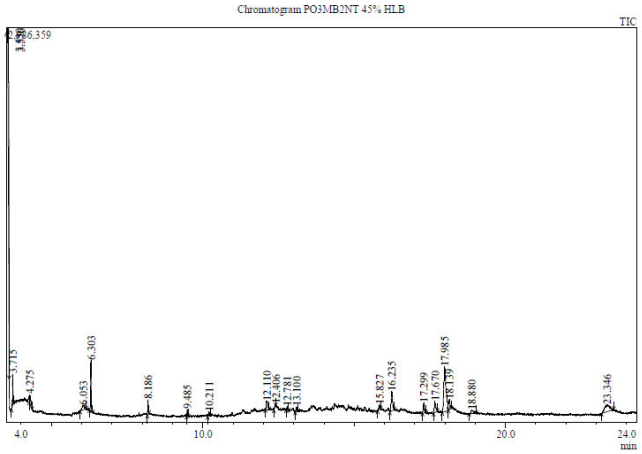
Figure 3.5: Dose-response curves of three pre concentration cartridges (a: HLB, b: MCX, and c: MAX) were combined with variable content of extracting solvent (5% MeOH, FT, flowthrough). The anti-HIV activity of fractionated secondary fungal metabolites was tested via the treatment of TZM-bl cells. Inhibitory concentration at 50% inhibition (µg/mL) and fold change (FC) were calculated.

3.4.5. GC-MS analysis

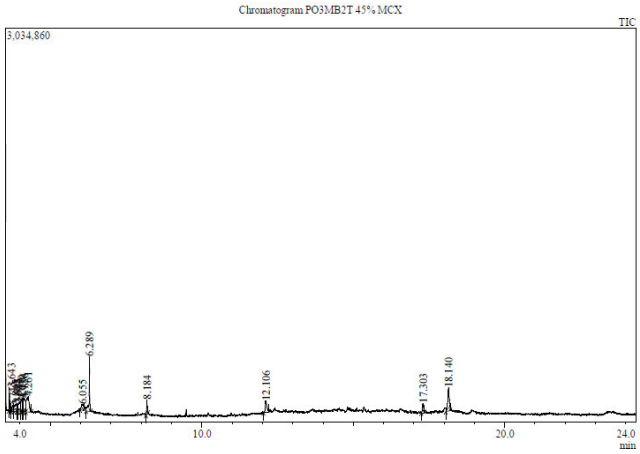
GC-MS analysis of methanolic (45%) extracts from *P.chrysogenum* isolate revealed the presence of different compounds as mapped from the NIST library. The gas chromatogram of acetonitrile fractions of treated and untreated *P.chrysogenum* indicated various peaks with different retention times per cartridge; the results are shown in **Figure 3.6**. A total of 110 compounds were detected from *P.chrysogenum* treated and untreated fractions produced from HLB, MCX, and MAX cartridges (**Table 3.1**, **Table 3.2**, and **Table 3.3**). The most prominent compounds include Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro (13,64%), Cyclotrisiloxane, hexamethyl (8,18%), Cyclotetrasiloxane, octamethyl (7,23%), Cyclopentasiloxane, decamethyl (6,36%), Quinoline, 1,2-dihydro-2,24-trimethyl (5,45%), Propanenitrile (4,55%), Deca-6,9-diene (4,55%), Dibutyl phthalate (4,55%) and Silane,[1,1-dimethyl-2-propenyl)oxy]dimethyl (2,73%). Compounds detected from sodium butyrate fractions were excluded from *P.chrysogenum* fractions as it was used as a negative control. **Table 3.4**



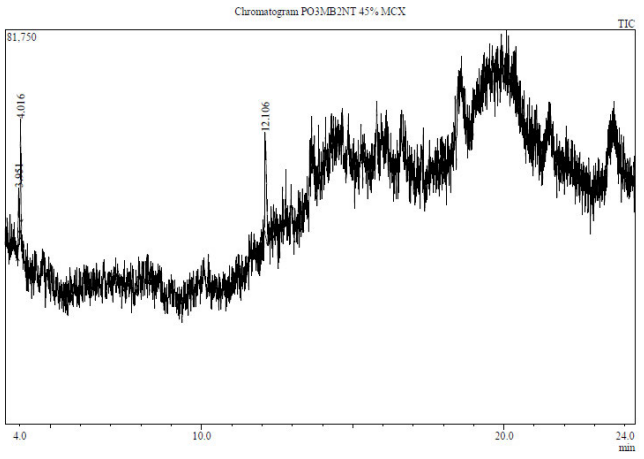
(**a**)



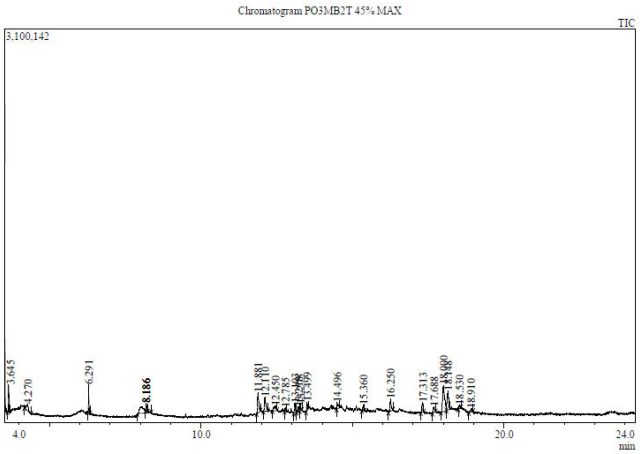
(**b**)



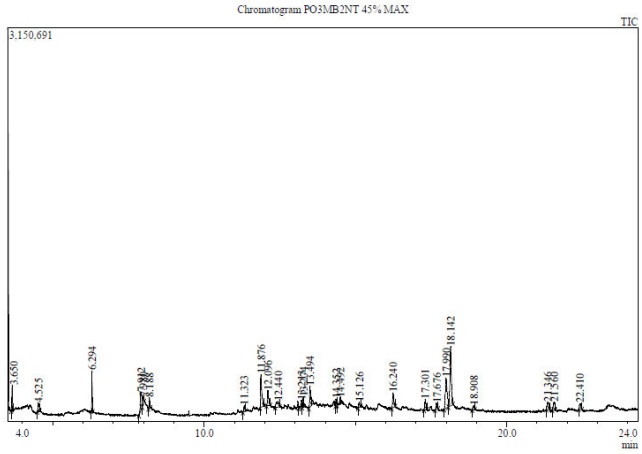
(**c**)



(**d**)



(**e**)



(**f**)

Figure 3.6: GC-MS chromatographs of methanolic fractions (a to f); (a - b) indicate chromatographs of treated (a), and untreated (b) fractions produced by the HLB cartride. (c - d) chromatographs of treated (c), and untreated (d) fractions produced by MCX cartridge, and lastly (e - f) indicate chromatographs of treated (e), and untreated (f), fractions produced by MAX cartridge.

Table 3.1: Bioactive compounds identified from *P.chrysogenum* treated and untreated fractions produced from HLB cartridge and their retention time, peak area, and biological properties.

RT (min)	Peak Area (%)	Treated Fraction. HLB Name of compound	Activity	RT (min)	Peak Area (%)	Untreated Fraction HLB Name of compound	Activity
4.041	2.00	Trimethylsilyl fluoride	Nr	3.576	27.26	1,2-Butadiene	Nr
4.115	2.35	Nickel, bis(N,N,N'-trimethyl-o-phenylenediamine-	Nr	9.485	0.84	3,5-Dibutoxy-1,1,1,7,7,7-hexamethyl-3,5-bis(tri	Nr
5.716	1.29	Oxime-, methoxy-phenyl-	Nr	12.406	0.58	Acetamide, N-(2-piperidin-4-ylethyl)-	Nr
6.225	2.51	1,1,3,3,5,5,7,7-Octamethyl-7-(2-methylpropoxy	Nr	12.781	0.60	5-Fluoro-3-trifluoromethylbenzoic acid, undecyl	Active
12.414	1.84	E-11,13-Tetradecadien-1-ol	Active	13.100	1.17	Adipic acid, di(2-tert-butyl phenyl) ester	Active
13.091	1.17	Methyl 4-O-acetyl-2,3,6-tri-O-ethyl-.alpha.-d-mannopyranoside	Active	15.827	1.31	Acetic acid, 1-cyano-1-(1-cyclopenten-3-one-1-yl	Nr
15.109	0.98	Trichloroacetic acid, dodecyl ester	Active	18.880	1.57	Triethylene glycol monododecyl ether	Active
15.824	2.04	1-Nitroso-2,4-dimethylamino-benzene	Nr				
18.896	1.65	1-Propanol, 3-(octadecyloxy)-	Nr				

Active = Other biologically active; Nr = Not reported; Active*** = HIV-1 activity.

*source; Dr. Duke's Phytochemical and Ethnobotanical Databases (Duke, 2004).

Table 3.2: Bioactive compounds identified from *P.chrysogenum* treated and untreated fractions produced from MCX cartridge and their retention time, peak area, and biological properties.

RT (min)	Peak Area (%)	Treated Fraction MCX Name of compound	Activity	RT (min)	Peak Area (%)	Untreated Fraction MCX Name of compound	Activity
3.745	2.58	Pyridine, 1,2,3,6-tetrahydro-1-methyl-4-[4-chlorophenyl	Active	4.016	23.89	Pentanoic acid, 2-hydroxy-4-methyl-, (S)-	Active
3.845	5.28	Pyridine, 1-acetyl-1,2,3,4-tetrahydro-5-(2-piperidinyl	Active				
3.905	2.33	1-Methyl-3-phenylindole	Active				
3.945	7.98	[1,2,4]Triazolo[1,5-a]pyrimidine-6-carboxylic acid	Nr				
4.040	5.04	Trimethylsilyl fluoride	Nr				

Active = Other biological activity; Nr = Not reported; Active*** = HIV-1 activity.

*source: Dr. Duke's Phytochemical and Ethnobotanical Databases (Duke, 2004).

Table 3.3: Bioactive compounds identified from *P.chrysogenum* treated and untreated fraction produced from MAX cartridge and their retention time, peak area, and biological properties.

RT (min)	Peak Area (%)	Treated Fraction. MAX; Name of compound	Activity	RT (min)	Peak Area (%)	Untreated Fraction MAX; Name of compound	Activity
12.450	3.48	2-Dimethylsilyloxytetradecane	Nr	7.912	6.67	Benzaldehyde, 3-methyl-	Active
13.103	1.17	2-Fluoro-5-trifluoromethylbenzoic acid, 3-Hexa	Active	7.980	2.25	Bicyclo[4.2.0]octa-1,3,5-trien-7-ol	Nr
13.218	3.13	l-Methionine, N-(2-methoxyethoxycarbonyl)-, hexyl	Active	12.440	3.17	3,6-Dimethyl-1,4-di[(2-phenyl-1,3-oxazol-4-yl)	Nr
13.499	1.44	Sym-tetramethyl(diisopropyl)disiloxane	Active	13.274	1.83	trans-1-(p-Ethoxyphenyl)-1-dodecen-3-one	Active***
13.496	2.60	Coumarin, 3,4-dihydro-4,5,7-trimethyl-	Active***	13.494	6.01	1-Trimethylsilyloxy-2-methyl-4-methoxybutane	Active
14.496	2.63	i-Propyl 9-tetradecenoate	Active***	13.352	1.39	Pyrazol-5(4H)-one, 3-(4-methoxyphenyl)-	Nr
15.360	1.63	Imidazo[4,5-d]imidazole-2,5-(1H,3H)dione, tetrahydro	Inactive	14.492	1.65	trans-2-Hexadecenoic acid	Active***
18.530	1.21	.beta.-Thionaphthol tetraacetyl-.alpha.-d-galactoside	Active	15.126	1.37	2-Hexadecene, 3,7,11,15-tetramethyl-, [R-[R*,R*-(	Nr
18.910	1.15	Chloroacetic acid, 2-pentadecyl ester	Active	18.908	1.57	Oxirane, [(hexadecyloxy)methyl]-	Nr
				21.346	2.47	Methyl 10-trans,12-cis-Octadecadienoate	Active***
				12.560	2.75	6-Octadecenoic acid, methyl ester.	Active
				22.410	1.98	Heptadecanoic acid, 16-methyl-, methyl ester	Active

*Active = Other biological activity; Nr = Not reported; Active*** = HIV-1 activity.

*source; Dr. Duke's Phytochemical and Ethnobotanical Databases (Duke, 2004).

Table 3.4: Bioactive compounds detected from sodium butyrate fractions produced from HLB, MCX, and Max cartridges.

RT (min)	Peak Area (%)	SB Fraction HLB Name of compound	RT (min)	Peak Area (%)	SB Fraction MCX Name of compound	RT (min)	Peak Area (%)	SB Fraction MAX Name of compound
3.550	48.39	1,2-Cyclobutanedicarbonitrile, cis-	3.660	5.72	Propanenitrile	3.515	9.94	1-Buten-3-yne
3.698	3.25	Propanenitrile	4.230	10.76	Cyclotrisiloxane, hexamethyl-	3.545	38.97	1,2-Cyclobutanedicarbonitrile, cis-
4.279	4.17	Cyclotrisiloxane, hexamethyl-	6.293	7.53	Cyclotetrasiloxane, octamethyl-	3.683	1.86	Propanenitrile
6.043	2.35	Cyclotetrasiloxane, octamethyl-	12.111	12.92	Quinoline, 1,2-dihydro-2,2,4-trimethyl-	4.251	8.67	Cyclotrisiloxane, hexamethyl-
6.299	3.69	Cyclotetrasiloxane, octamethyl-	14.790	3.72	Ethanol, 2-(eicosyloxy)-	6.299	2.54	Cyclotetrasiloxane, octamethyl-
11.745	3.76	2,5-Piperazinedione, 3,6-bis(2-methylpropyl)-	15.351	3.16	Indane-4-carbonitrile, 2,2,5,7-tetramethyl-1-oxo-	8.189	0.49	Cyclopentasiloxane, decamethyl-
12.105	5.09	Quinoline, 1,2-dihydro-2,2,4-trimethyl-	15.935	3.44	1,1,3,3-Tetramethyl-1,3-bis[(3Z)-non-3-en-1-yloxy]dis	12.110	3.92	Quinoline, 1,2-dihydro-2,2,4-trimethyl-

12.385	2.89	1-Heptafluorobutyryloxydecane	16.072	3.63	Eicosanoic acid, 2,3-bis[(trimethylsilyl)oxy]propyl ester	12.411	0.94	Trichloroacetic acid, decyl ester
14.796	1.92	Ethanol, 2-(tetradecyloxy)-	16.256	4.83	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-	14.525	0.75	Decane, 4-ethyl-
15.360	1.20	2,8-Dibenzofurandiamine	17.304	5.73	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene	16.246	2.98	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(
16.259	2.34	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(	18.006	16.72	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(	17.303	1.74	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene-2,
17.295	2.33	7,9-Di-tert-butyl-1-oxaspiro(4,5)deca-6,9-diene	18.135	12.13	Dibutyl phthalate	17.675	1.45	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-
17.999	7.30	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-(2-	18.895	9.72	Triethylene glycol monododecyl ether	17.998	10.54	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-
18.140	3.46	Dibutyl phthalate				18.135	3.01	1,2-Benzenedicarboxylic acid, bis(2-methylpropyl) ester
18.904	2.96	Diethylene glycol monododecyl ether				18.919	3.49	Diethylene glycol monododecyl ether
23.310	2.47	1H-Imidazole-4,5-dicarboxamide, 1-benzyl-				23.339	8.69	Pyrrolo[1,2-a]pyrazine-1,4-dione, hexahydro-3-
23.384	2.42	N-(2,6-Dichlorophenyl)-3-methylbenzamide						

3.5. Discussion

The medicinal properties of *A.adianthifolia* are extensively established, and the various parts of the plant are known to have distinct biological activities. Pharmacological studies on *A.adianthifolia* extracts and compounds have shown potent pharmacological activities (Beppe *et al.*, 2014; Chukwuma *et al.*, 2015; Pujol *et al.*, 1990; Tamokou *et al.*, 2012; Watt and Breyer-Brandwijk, 1962). Apart from these potent pharmacological activities, the plant has not yet been evaluated for the innovation of biologically active compounds from its endophytic microbiota, notably for anti-HIV activities. In our ongoing research to activate silent BGCs from endophytic fungi, we investigated the effects of epigenetic modifiers in activating silent BGCs of *P.chrysogenum* P03MB2 isolated from *A.adianthifolia* for profiling the produced secondary metabolites as anti-HIV compounds.

In the current study, the identified *P.chrysogenum* P03MB2 (**Figure 3.1**) by ITS sequencing was isolated from the bark of *A.adianthifolia* upon surface sterilization using ethanol and sodium hypochlorite and bicarbonate to eliminate epiphytes. This sterilization method has shown significant efficacy in previous studies (Petrini, 1986; Saini *et al.*, 2016; Arivudainambi *et al.*, 2011). The efficiency of surface sterilization of *A.adianthifolia* bark was validated by the absence of microbiological growth after five days. This indicates that the surface sterility approach inhibited the growth of epiphytic bacteria and fungus. As a result, the isolates from

the subsequence may be deemed actual endophytic fungi. To the best of our knowledge, this is the first report of *P.chrysogenum* isolation from *A.adianthifolia*.

The toxicity examination of the active extract was conducted on TZM-bl cells to eliminate possible non-specific antiviral activities and toxic concentrations of extracts. All extracts were non-toxic, with cell viability of ≥80% (**Figure 3.3**). The cell cytotoxicity at 50% was analyzed in accordance to study described by Zhou *et al.,* (2013) (Toxic < 10 µg/mL; Moderate toxicity ≥10 but <100 µg/mL; Less/No toxicity ≥100 µg/mL) (Zhou *et al.*, 2013). It was critical to evaluate the extracts' cytotoxicity since the inhibitory activity of any compounds or extracts against HIV infection is often attributable to their toxicity, which might lead to an inaccurate conclusion (Wellensiek *et al.*, 2013).

During our preliminary screening, we discovered one non-toxic crude extract of *P.chrysogenum* treated with sodium butyrate (**Figure 3.2**) that inhibited HIV-1 replication in TZM-bl cells **(Figure 3.4)**. *P.chrysogenum* is well investigated for production of a wide range of bioactivities, including antibacterial and antifungal properties, but there have been few reports on its anti-HIV activities (Khalil *et al.*, 2019; Puškárová *et al.*, 2017; Rodino *et al.*, 2014). To cite a few compounds that have been described as anti-HIV compounds, Singh *et al.* (2003) discovered xanthoviricandins E and F, indicating that *P.chrysogenum* might be a potential source of anti-HIV inhibitors (Singh *et al.*, 2003).

Five bioactive compounds that exhibit anti-HIV properties were detected from treated and untreated *P.chrysogenum* P03MB2 fractions produced from MAX cartridges during the current study (**Table 3.6**). These compounds include coumarin and i-propyl-9-tetradecenoate from treated fraction and trans-1-(-p-Ethoxyriphenyl)-1-dodecen-3-one, trans-2-Hexadecenoic Acid, and methyl-10-trans-12-Cis-octadecadienoate from an untreated fraction of MAX (**Table 3.3**) reported in Dr. Duke's Phytochemical and Ethnobotanical Database (Duke, 2004). Active crude extracts were semi-purified using a mixed-mode SPE approach that employed three distinct cartridges (HLB, MCX, and MAX) to target neutral, acidic, and basic compounds. The use of three cartridges was adopted from a study published by Stoszcko *et al.* (2020) reporting on the discovery of gliotoxin. This latency reversal agent resulted in a more than 20-fold increase in HIV-1 expression after purification (Stoszko *et al.*, 2020). To our knowledge, no reports have been published on the isolation of any of the metabolites mentioned above as anti-HIV inhibitors from *P.chrysogenum*. In summary, our findings are in good agreement

with previous studies describing *Penicillium sp* as a producer of antiviral compounds against HIV-1. However, further research is required to fractionate crude extracts using a bioassay-guided approach and uncover the mechanisms of the compound among the five to establish which compound is accountable for the reported anti-HIV activity in this study.

Various epigenetic modifications involving chemical inhibitors or inducers, such as Histone deacetylase (HDAC) inhibitors or DNA methyltransferase (DNMT) inhibitors, have been found to stimulate the transcription of attenuated gene clusters successfully, subsequently producing a variety of novel secondary metabolites or enhancing their production (Okada and Seyedsayamdost, 2017; Yang *et al.*, 2014). In this study, *P.chrysogenum* isolate was treated with sodium butyrate to enhance the secretion of secondary metabolites from silent BGCs. The treated fungal fractions revealed a 5-fold increase in anti-HIV activity from the HLB cartridge. In contrast, MCX and MAX did not show any variation between treated and untreated (Stoszko *et al.*, 2020) fractions (**Figures 3.5b and 3.5c**). This finding indicates that only hydrophilic compounds were induced from the *P.chrysogenum* gene, while acidic and basic compounds were unaffected by sodium butyrate (**Figure 3.5a**). Comparative profiling with GC-MS spectra showed an increased production of secondary metabolites from the *P.chrysogenum* treated fraction than the untreated fraction produced from the HLB cartridge (**Figure 3.6**). A similar phenomenon was observed when valproic acid was added to the growth media of *Aspergillus fumigatus* (Magotra *et al.*, 2017). The addition of valproic acid overexpressed genes encoding fumiquinazoline C and improved its production by 10-fold (Magotra *et al.*, 2017; Gupta *et al.*, 2020). In conclusion, our study results suggest possible activation of genes in *P.chrysogenum* by the addition of sodium butyrate due to increased anti-HIV activity and increased bioactive compound from spectra. However, more rounds of purification of the extracts and deep genome analysis of fungal species are recommended.

3.6. Conclusion

In this study, the effects of epigenetic modifiers in activating silent BGCs of endophytic fungi *P.chrysogenum* for increased production of secondary metabolites as anti-HIV-1 inhibitors were investigated for the first time. Our results showed that *A.adinthifolia* harbored abundant fungal endophytes representing a high-level taxonomic diversity. One of five treated endophytic fungal methanol extracts exhibited inhibitory activities against the HIV-1 strain without associated toxicity. Fractionated *P.chrysogenum* extract revealed an increased anti-HIV activity. Expressly, fractions of treated fungal isolates produced from the HLB cartridge indicated a 5-fold increase in anti-HIV activity compared to untreated fungal fractions. Furthermore, GC-MS analysis revealed more production of secondary metabolites from the treated fungal fraction than from the untreated fraction. Five secondary metabolites exhibiting anti-HIV properties were detected from treated and untreated fungal fractions. This finding describes *P.chrysogenum* as a promising source of anti-HIV inhibitors. Overall, the cultivation of *P.chrysogenum* with the addition of sodium butyrate enhances the biosynthetic pathways for the secretion of secondary metabolites exhibiting anti-HIV activity. It further presents epigenetic modification as a powerful tool in screening bio-control agents and the discovery of new bioactive compounds.

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CHAPTER 4

SYNTHESIS

4.1. General discussion

The current study demonstrated that epigenetic modifiers have the potential to induce silent biosynthetic gene clusters (BGCs) in endophytic fungi. Our results further showed that sodium butyrate-treated fungal extracts isolated from the bark of *Albizia adianthifolia* have an increased anti-HIV compared to untreated fungal extracts. Our finding is in line with a report from Magotra *et al.,* (2017), where they observed a 10-fold increase in the production of Fumiquinazoline C by *Aspergillus fumigatus* when it was cultivated in the presence of valproic acid (Magotra *et al.*, 2017). This implies that there may be specific BGCs induction by the addition of sodium butyrate, which led to the increased anti-HIV activity observed in this study.

The use of small molecular compounds such as valproic acid or sodium butyrate provides a simple approach for inducing silent biosynthetic pathways even in non-model environmental species with no known genetic information (Chiang *et al.*, 2011). However, to facilitate genome mining of the BGCs encoding for bioactive compounds of these non-model fungal isolates will require the knowledge of the whole genome. Differential expression due to epigenetic modifiers can be further probed using transcriptomics (Pfannenstiel and Keller, 2019). The transcriptional analysis of *Aspergillus nidulans* has indicated that the deletion of *hdaA* from the secondary metabolite gene cluster using histone deacetylase inhibitors (HDACs) leads to the activation of two BGCs of sterigmatocystin and penicillin (Reyes-Dominguez *et al.*, 2010; Shwab *et al.*, 2007).

Various other advanced tools have been developed to facilitate the linking of biosynthetic gene clusters to their bioactive compounds (Kjærbølling *et al.*, 2019). The clustered regularly interspaced short palindromic repeats associated protein (CRISPR cas9) system has been successfully established in a variety of fungal species, including *Neurospora crassa*, *Trichodema reesei, Pyricularia oryzae,* and *Candida albicans* (Vyas *et al.*, 2015; Matsu-Ura *et al.*, 2015; Liu *et al.*, 2015; Arazoe *et al.*, 2015). While heterologous expression has shown its success in *Talaromyces stipitatus*, *Penicillium patulum*, and *Aspergillus oryzae* (Davison *et al.*, 2012; Kealey *et al.*, 1998; Watanabe *et al.*, 2000). These tools can also facilitate the development of the hit compounds identified in this study and their genome-mediated optimization.

Molecular identification of endophytic fungi exhibiting anti-HIV properties was conducted following internal transcribed spacer (ITS) region sequencing. The isolated fungi in this study was identified as *Penicillium chrysogenum.* As the most sequenced region of fungi, ITS sequencing serves as an excellent barcode for systematics, phylogenetics, and identification (Bellemain *et al.*, 2010; Begerow *et al.*, 2010). The ITS sequencing method has been employed to identify various fungal isolates, including *P.chrysogenum* and *Aspergillus fumigatus* (Stoszko *et al.*, 2020; Ding *et al.*, 2020). The polymerase chain reaction (PCR) based approach is beneficial not only for identifying cultured species but can also be used to identify uncultured species from the natural environment (Aslam *et al.*, 2017). The use of molecular tools that complement morphological tools is auspicious for species identification, and they can also be used for rapid and reliable evaluation of biological diversity (White *et al.*, 1990).

A luciferase-based assay was used to investigate viral inhibition of *P.chyrosgenum* extracts, it is well explained by relative light units (RLUs), which are directly proportional to virus particles in TZM-bl cells (Modi *et al.*, 2013). The rationale behind the use of this assay, it provides a rapid, highly sensitive, and direct method for monitoring HIV infection and cell viability (Mitsuki *et al.*, 2016). There has been increasing attention toward *P.chrysogenum* since its discovery by Flemming (1929) as a potential source of antibacterial compounds (Fleming, 1929; Tan and Tatsumura, 2015). Some recent antibacterial compounds discovered from *P.chrysogenum* include roquerfortine E and F and meleagrin (Ding *et al.*, 2020). However there have been few reports on *P.chrysogenum*'s anti-HIV activities (Linnakoski *et al.*, 2018; Roy, 2017). Examples of compounds that have been found to inhibit HIV-1 include discovered xanthoviricandins E and F and sorbicillactone A from *P.chrysogenum* (Singh *et al.*, 2003; Bringmann *et al.*, 2003).

The bioassay-guided approach to isolate and purify active anti-HIV compounds was followed. The single-round fractionated active crude extracts revealed potent anti-HIV with a 5-fold increase compared to non-fractionated crude extracts. According to research, fold change (FC) which is calculated as a ratio of averages from control and test sample values, is used to measure gene regulation, levels of change, or cutoffs (e.g., 0.5 for down- and 2 for up-regulated) (Schena *et al.*, 1995; Lockhart *et al.*, 1996). Comparative profiling with gas chromatography-mass spectrometry (GC-MS) spectra showed an increased production of secondary metabolites from the *P.chrysogenum* treated fraction than the untreated fraction. The GC-MS results corroborate the increased anti-HIV activity observed in this study by the increased presence of compounds in treated fungal fractions.

Moreover, five compounds (coumarin, i-propyl-9-tetradecenoate, trans-1-(-p-Ethoxyriphenyl)-1-dodecen-3-one, trans-2-Hexadecenoic Acid, and methyl-10-trans-12-Cis-octadecadienoate) have been detected in this study. A coumarin isolated from Alternaria species has been shown to inhibit HIV-1 reverse transcriptase (Govindappa *et al.*, 2015). However, none of the above compounds have been isolated from *P.chrysogenum* and reported to exhibit antiviral properties. *Penicillium species* are known to have the potential to produce a wide range of volatile and non-volatile compounds with therapeutic properties (Kermasha *et al.*, 2002; Gillot *et al.*, 2017). Therefore the cultivation of *P.chrysogenum* with addition of sodium butyrate might have resulted in the increased secretion of non-volatile compounds. There is a significant possibility that the observed anti-HIV activity in this study is also attributable to undetectable non-volatile compounds, due to the fact that GC-MS detects mainly volatile compounds (Maurer *et al.*, 1997). In this case liquid chromatography-mass spectrometry (LC-MS) coupled with nuclear magnetic resonance (NMR) would be significant in detecting both the volatile and non-volatile compounds.

Penicillium chrysogenum isolated from *A.adianthifolia* may be a potential source of anti-HIV compounds; however, for future studies, a machine-learning model where these candidate compounds with potential as anti-HIV compounds can be probed in-silico against HIV target site to see which of these compounds have high binding affinity and can potentially be developed further. Overall, sodium butyrate has enhanced secondary metabolites production in *P.chrysogenum*, highlighting the potential of epigenetic modification as a suitable strategy for producing cryptic fungal metabolites as therapeutic compounds. This is the first report on the use of epigenetic modifiers to induce silent BGCs in endophytic fungi, *P.chrysogenum*, for increased production of bioactive compounds for anti-HIV agents.

4.2. Conclusion

The aim of the study was successfully achieved as the effects of epigenetic modifiers on endophytic fungi revealed potent bioactivity in treated fungal fractions compared to untreated fractions suggesting possible activation of silent BGCs. In conclusion, the results from the study demonstrate epigenetic modification as a potential strategy for silent BGC induction and endophytic fungi as a potential source of anti-HIV compounds.

- Endophytic fungal isolate *P.chrysogenum* treated with sodium butyrate, methanol extracts exhibited inhibitory activities against the HIV-1 strain without associated toxicity.

- Fractionated *P.chrysogenum* extract revealed an increased anti-HIV activity; expressly, fractions of treated fungal isolates indicated a 5-fold increase in anti-HIV activity compared to untreated fungal fractions.
- Gas chromatography mass-spectrometry analysis revealed that the treated fungal fraction produced a more significant number of secondary metabolites than the untreated fraction. Five compounds have previously been reported to exhibit anti-HIV properties among the secondary metabolites detected from both treated and untreated fungal fractions.

The findings of this study serve as a blueprint and contribute to the scientific literature in the pursuit of potential strategies to explore microbial compounds as sources of anti-HIV activity without cytotoxic side effects in drug discovery and development.

4.3. Limitations and Recommendations

Despite the significant anti-HIV of fungal extracts observed in this study, the effects of extracts or fractions on HIV-1 replication remain unknown as luciferase-based assay only affirms the antiviral potential of bioactive compounds and does not reveal any information regarding their mechanism of action. Therefore, more detailed knowledge of the actual molecular targets and laboratory assays targeting various steps along virus infection are needed to understand the mechanisms of action in the future. Binding assays, computational simulation (Molecular docking), time of addition (TOA), and HIV-1 reverse transcriptase , integrase, protease activity assays are some methods that could be employed in elucidating the mechanisms of action of fungal compounds detected in this study.

Several compounds were detected from semi-purified fungal fractions with various biological activities reported. The setback is that our study cannot report which compound amongst the identified compounds is responsible for the observed anti-HIV activity in this study. As a result, future studies can individually evaluate the secondary metabolite identified in this study to derive one responsible for the observed anti-HIV activity. The other setback is using GC-MS to analyze active fractions and identify compounds, mainly because the technique only identifies volatile compounds. Therefore, to improve the study, we recommend liquid chromatography-mass spectrometry (LC-MS) coupled with nuclear magnetic resonance (NMR) to accommodate the non-volatile compounds since they also might be responsible for the observed anti-HIV activity even though they were not detected.

Compared to untreated fractions, the improved anti-HIV activity of sodium butyrate treated fractions indicate possible induction of silent fungal BGCs. To affirm silent BGCs induction, we recommend a secondary metabolite profile and transcriptional analysis of fungal isolates. We believe that the study's findings will be an inspiration and helpful resource for future research in the fungal metabolite-based antiviral drug discovery.

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APPENDICES

Appendix 1: Ethical clearance for research study

07 October 2021

Mr John Phuti Makhwitine (220016491)
School of Laboratory Medicine & Medical Science
Medical School

Dear Mr Makhwitine,

Protocol reference number: BREC/00001519/2020
Project title: Profiling of secondary metabolites secreted by endophytic fungi isolated from S.birrea for potential use as drug leads against multi drug resistant clinical pathogens
Degree Purposes: Masters
NEW TITLE: Activation of silent BGCs and profiling of secondary metabolites secreted by endophytic fungi for potential use as antiviral agents.

We wish to advise you that your application for amendments to change the title to then new title above received on 20 September 2021 for the above study has been noted and approved by a subcommittee of the Biomedical Research Ethics Committee.

The committee will be advised of the above at its meeting to be held on 09 November 2021.

Yours sincerely

[redacted]

Ms A Marimuthu
(for) Prof D Wassenaar
Chair: Biomedical Research Ethics Committee
cc: sibongilew@nicd.ac.za