ANTI-HIV, ANTICANCER AND ANTIOXIDANT ACTIVITY OF *FOCKEA MULTIFLORA* EXTRACTS

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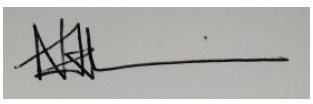
SIGNATURE 

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SUMMARY

ANTI-HIV, ANTICANCER AND ANTIOXIDANT ACTIVITY OF *FOCKEA MULTIFLORA* EXTRACTS

By

Nomthandazo Khuboni

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Background: Over the past four decades, the Human immunodeficiency virus (HIV) has been a great problem for public health globally. The immunosuppressive ability of HIV is associated with life-threatening opportunistic infections like the human papillomavirus (HPV) that causes cervical cancer. The development of highly active antiretroviral therapy (HAART) has transformed HIV infection into a manageable chronic disease and has improved life expectancy by providing steady viral suppression. However, HAART comes with unpleasant side effects that complicates adherence to treatment, leading to the increase in plasma viral load, the drug-resistant HIV strains development and treatment failure. To achieve the goal of eradicating HIV completely, it is imperative to develop novel antiviral agents with tolerable levels of toxicity, fewer resistance profiles and that are cost-effective. Because of the existence of plant derived natural products that have antiviral and anticancer properties, this study investigated the bioactivity of *Fockea multiflora* extracts with the aim of identifying anti-HIV and associated activities. The plant belongs to Apocynaceae, a family that has shown to be a vital source of beneficial bioactive compounds contained in the isolated constituents which include flavonoids, coumarins, and saponins.

Methods: The chloroform, water and diethyl ether extracts of the root and leaf of *F. multiflora* were investigated for their cytotoxicity profile using MTT [3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenyltetrazolium bromide] and Neutral Red Uptake (NRU) of peripheral blood mononuclear cells (PBMCs) and TZM-bl cells. This study also investigated the inhibitory effects of *F. multiflora* on two of the crucial enzymes of the HIV-1 life cycle; reverse

transcriptase (RT) and protease (PR). The anticancer profile of *F. multiflora* was then monitored using MTT in a cervical cancer cell line-(HeLa) and liver cancer cell line-(HepG2). Real-time cell analysis (RTCA) was used to investigate the effects of the extracts in cancer cell proliferation in real-time. Lastly, the radical scavenging ability of *F. multiflora* was investigated by measuring the nitric oxide (NO) produced by HeLa and HepG2 cells.

Results: Evaluating the cytotoxicity of plant extracts is important for general safety of extracts as well as for determining the treatment ranges for further investigation. Results obtained from MTT assay revealed that some *F. multiflora* extracts were cytotoxic towards PBMCs; extract DL (diethyl ether of leaf) gave a CC_{50} of 10.39 ± 0.26 µg/mL and CL (chloroform of leaf) with a CC_{50} of 12.14 ± 0.12 µg/mL. Natural products possess reducing agents which tend to interfere with MTT indicators and this may lead to false results unless these agents are eliminated. The NRU assay was then employed, as it involves the washing of cells which eliminates the interference. The cell viability results obtained from NRU using the TZM-bl cell line gave a dose-dependent curve for most of the extracts except for WR (water of root) and WL (water of leaf), where cells were highly viable for all concentrations with predicted CC_{50} values way above the tested concentrations. This confirmed the safety of the polar solvent (water) used in the extraction of these two extracts at all tested concentrations (4 – 250 µg/mL). The NRU assay revealed that none of the extracts was cytotoxic towards TZM-bl cells. The inhibition activity of *Fockea multiflora* extracts on HIV-1 RT resulted in a percentage inhibition of 73% at 100 µg/mL for CL, with an estimated IC_{50} of <10 µg/mL. All *F. multiflora* leaf extracts exhibited relatively high inhibition (>50%) and extract CR (chloroform of root) and DR (diethyl ether of root) were statistically significant (p = 0.0085 and 0.0406, respectively). A known inhibitor for HIV-1 RT, doxorubicin exhibited a percentage inhibition of 80%. The inhibitory activity of extracts on HIV-1 protease was investigated and a percentage inhibition of 70% at 100 µg/ml for CR was obtained with an estimated IC_{50} of 10 µg/mL (p = 0.0250). It was noted that all root extracts exhibited relatively high inhibition (>50%) and a known inhibitor for HIV-1 PR, acetyl pepstatin (AP) exhibited a percentage inhibition of 85%. The anticancer ability of *F. multiflora* was assessed in HeLa cells through MTT and none of the extracts exhibited inhibition activity below 50%. However, RTCA revealed lower IC_{50} values than those obtained in the MTT assay; extract WR for HeLa cells gave an IC_{50} of 35.53 µg/mL and all extract activities were found to be statistically significant (p-values ranging from 0.0001 to 0.0187). The radical scavenging ability of all *F. multiflora* extracts on the production of NO was observed to be higher in HepG2 cells to almost undetectable levels, with IC_{50} ranging from

0.001 ± 1.21 to 2.02 ± 0.09 µg/mL and all extracts were found to be statistically significant (p = 0.0001).

Conclusion: This study showed that *F. multiflora* extracts have the potential to inhibit both HIV-1 RT and PR, with the highest percentage inhibition being close to that obtained for known inhibitors. Results also demonstrated that *F. multiflora* extracts are rich in antioxidants, with the greatest radical scavenging ability observed in liver cancer cell lines. These findings provide the first report on the anti-HIV-1 activity of *F. multiflora* extracts and supports the isolation and purification of active compounds for further studies.

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LIST OF ABBREVIATION

AIDS – Acquired Immunodeficiency Syndrome

cART – Combination of Antiretroviral Therapy

CDC – Canters for Disease Control and Prevention, USA

ER – Endoplasmic Reticulum

ETC – Electron Transport Chain

FDA – Food and Drug Administration

HAART – Highly Active Antiretroviral Therapy

HBV – Hepatitis B Virus

HCC – Hepatocellular Carcinoma

HIV – Human Immunodeficiency Virus

IARC – International Agency for Research on Cancer

MTT – 3-(4,5-dimethylthiazol-2-yl)-2,5-diphenyltetrazolium bromide

IN – Integrase

NCI – National Cancer Institute, USA

NO – Nitric Oxide

NNRTIs – Non-Nucleoside Reverse Transcriptase Inhibitors

NRU – Neutral Red Uptake

NRTIs – Nucleoside Reverse Transcriptase Inhibitors

OIs – Opportunistic Infections

PBMCs – Peripheral Blood Mononuclear Cells

PIs – Protease Inhibitors

PR - Protease

ROS – Reactive Oxygen Species

RNS – Reactive Nitrogen Species

RT – Reverse Transcriptase

RTCA – Real Time Cell Analyses

SD – Standard Deviation

SEM – Standard Error of the Mean

SSA – Sub-Saharan Africa

1. INTRODUCTION

Since its discovery in 1981, Human Immunodeficiency Virus type 1 (HIV-1) has caused serious health problems globally. Studying the pathogenesis of HIV for drug development purposes has been a crucial focus for many researchers (Tough and McLaren, 2019). HIV causes a chronic life-long infection known as acquired immunodeficiency syndrome (AIDS) which is kept at bay through the effect of antiretrovirals. The combination of antiretroviral therapy (cART), otherwise known as highly active antiretroviral therapy (HAART) has been highly effective in suppressing HIV-1 viral load, impeding disease progression and partially preserving or partially restoring immune competence (Pau and George, 2014) (Balasubramaniam, 2019). Life expectancy has drastically increased for persons living with HIV due to HAART, however, the burdensome side effects lead to drug toxicity (Cheney *et al*., 2021), the emergence of multidrug resistance caused by patient non-compliance (Salehi *et al*., 2018) and opportunistic infections like tuberculosis and the Human Papilloma virus (HPV) which leads to cervical cancer (Yusmazura *et al*., 2017).

The overproduction of free radicals with the insufficient release of antioxidants to scavenge the produced radicals also plays a role in making HAART ineffective. As a result, people living with HIV become subjected to oxidative stress which affect vital body organs, leading to neurodegenerative diseases and cancer (Boora *et al*., 2014). According to a review conducted in 2016 by Ivanov and colleagues, it was pointed out that HIV infection triggers pronounced oxidative stress and deregulates the antioxidant defence system. It has been noted that people living with HIV exhibit enhanced ROS production. Moreover, HIV-1 tends to compromise the antioxidant defence system, leading to more oxidative stress that increases the chance of developing cancer (Ivanov *et al*., 2016). The incidence of cervical cancer is higher among HIV-positive women, with sub-Saharan Africa accounting for 85% of cervical cancer cases attributed to HIV (Stelzle et al., 2021). Therefore, this study aimed at evaluating the inhibitory effect of *F. multiflora* extracts on the production of nitric oxide (NO) as a marker of oxidative stress in cervical cancer cells, HeLa.

The non-AIDS defining malignancies have now become the major cause of mortality among people living with HIV, this can be attributed to the ageing of this population, the rise in prevalence of cancer-causing coinfections and immunologic susceptibility to cancers (Makinson *et al*., 2021). Cervical cancer remains one of the major cancer burdens worldwide, especially in under-developed and developing regions like sub-Saharan Africa. Apart from the

physical impact caused by cervical cancer, women are also subjected to social and sexual impacts in their communities (Yusmazura *et al*., 2017). Despite the significant development and improvement of HIV treatment, the unacceptable adverse effects limit the efficacy of current therapies in controlling HIV-infection and preventing the onset and progression of cervical cancer (Cary and Peterlin, 2018).

Scientific literature exists of natural products being utilised as treatments for preventing and managing a vast array of diseases including HIV/AIDS and cancer (Shabani, 2016) (Mensah *et al*., 2019). Since ancient times, the human race has made use of natural products that have been derived from plants and marine organisms to formulate medicines with the aim of reducing and treating different illnesses (Yuan *et al*., 2016). Low-income countries like Africa and Asia do not have adequate and consistent access to medicines, especially in rural areas, due to inflated medicine prices as well as the reduced buying strength of the community. Therefore, traditional medicine have served as the most accessible form of treatment for ailments in these communities (Santos *et al*., 2019). The natural products comprise of diverse chemical entities that have found multiple essential functions as vital constituents of traditional medicine (Katz and Baltz, 2016). The use of traditional medicine is a regular practice in all rural communities in Africa, as it caters to approximately 80 – 90% of the inhabitants (Mensah *et al*., 2019).

In South Africa, there is a growing trend among people living with HIV to opt for remedies derived from plants as alternative medicine. This is mainly due to cultural beliefs, the extended distance to health care centres, lengthy waiting periods when clinic dispensary run out of stock and the stigma associated with being seen interacting with community and home-based caregivers associated with HIV/AIDS treatment or education. The use of water to extract various plant materials by traditional health practitioners in preparing herbal mixtures has proven to be easily attainable and cheaper for people living with HIV in various African societies (Matotoka *et al*., 2019). South Africa is not the only country that follows the practice of traditional medicine over conventional treatments, other countries have also been documented to follow the same practice and these include; India (Eram *et al*., 2018), Brazil (Santos *et al*., 2019) and China (Yuan *et al*., 2016). The two latter countries have made major progress in the research of natural products possessing anti-HIV activity, as most systems for traditional medicine in India encompass some herbs, medicinal plant and/or natural plant products (Eram *et al*., 2018). This is also noted with traditional Chinese medicine whose clinical studies reveal affluent data that is constantly being developed (Yuan *et al*., 2016).

Unfortunately, the African continent has fallen behind on this progress as Africa only represents 16% of research efforts on anti-HIV-1 natural products while China represents 41%. Meanwhile, about 70% of HIV infection occurs in sub-Saharan Africa and the prevalence rate in China is less than 0.1% among adults. Africa cannot always be looking to the outside world for anti-HIV remedies when our continent is endowed with abundant medicinal plant resources (Chinsembu, 2019). This study therefore aimed at contributing to the African research efforts already conducted on anti-HIV-1 natural products by investigating the anti-HIV-1 activity, antioxidant capacity and anticancer profile of *Fockea multiflora* extracts. Nowadays more studies are directed towards elucidating active compounds, however, crude plant extracts form bases of these research studies. Crude plant extracts are still deemed worthy of being evaluated due to a notion that the overall activity is retained in crude extracts as mixtures of constituents working synergistically or antagonistically, whereas the activity is somehow lost or reduced through fractionation (Caesar and Cech, 2019). As an approach to elucidate the anti-HIV-1 profile of *F. multiflora*, the investigation had to first be conducted on crude plant extracts.

2. BACKGROUND AND LITERATURE REVIEW

2.1 The origins of HIV

The human immunodeficiency virus emerged in the 1980s as a public health threat, which compelled the attention of clinical and scientific research communities to understand the pathogenesis of this virus (Das and Arnold, 2013). It was discovered that the progressive waning of the immune system due to HIV infection leads to the onset of AIDS which ultimately leads to death (Balasubramaniam, 2019) if left untreated. HIV belongs to the Retroviridae family under the subfamily of Orthoretrovirinae and within the Lentivirus genus. According to the phylogenetic analyses and epidemiologic studies HIV was initially transmitted to human species around 1920 to 1940 (Seitz, 2016) which began from independent zoonotic events of dissemination between primates and humans (Tough and McLaren, 2019).

There are two categories of HIV, namely type 1 (HIV-1) and type 2 (HIV-2). HIV-1 is responsible for the ongoing HIV/AIDS pandemic, accounting for over 95% of infections globally (Balasubramaniam, 2019). HIV-1 is thought to have originated from non-human primate immunodeficiency viruses (SIVcpz) from chimpanzees in Central Africa (Seitz, 2016). The strains of HIV-1 have been categorised into four groups: M, O, N and P (Subramaniam *et al.*, 2019). Group M is the cause of disease and includes nine viral subfamilies, namely A, B, C, D, F, G, H, J and K. Subtypes A and D are regarded as the oldest subtypes and subtypes B and D are closely associated (Seitz, 2016). Subtype C is responsible for the cause of HIV-1 infection that is reported worldwide and particularly in India and sub-Saharan Africa, where HIV-1 accounts for more than 80% of the global infections (Subramaniam *et al.*, 2019). According to phylogenetic analyses, several chimpanzee viruses can be distributed between group N and O, while SIV of gorillas (SIVgor) is allocated in group P. It is inconclusive whether the group P chimpanzee virus was directly transmitted to humans from chimpanzees or gorillas (Seitz, 2016).

The HIV-2 was first reported in 1985, it is relatively less pathogenic compared to HIV-1 (Bhatti *et al*., 2016) (Balasubramaniam, 2019) and it evolved from West African sooty mangabeys (SIVsm) (Seitz, 2016). Type 2 of HIV bears a resemblance to simian virus that infects macaques in captivity. The simian virus that naturally infect African primates are suspected to have been disseminated to humans through transmissions of multiple cross-species leading to the spread of HIV-1 and HIV-2 (Bhatti *et al*., 2016). Despite decades of intense research, no effective preventive vaccine or cure for HIV has been developed and this results in millions of new HIV infections every year (Balasubramaniam, 2019).

In 2016, the United Nations General Assembly's Political Declaration on Ending AIDS established (United Nations Programme on AIDS) UNAIDS' 90-90-90 target strategy, aimed at bringing HIV testing and treatment to most of the global people living with HIV by the end of 2020. The purpose of the strategy was to diminish the viral load to subtle levels, to augment the immune response, prolong life, and to curb the virus from spreading (UNAIDS, 2020).

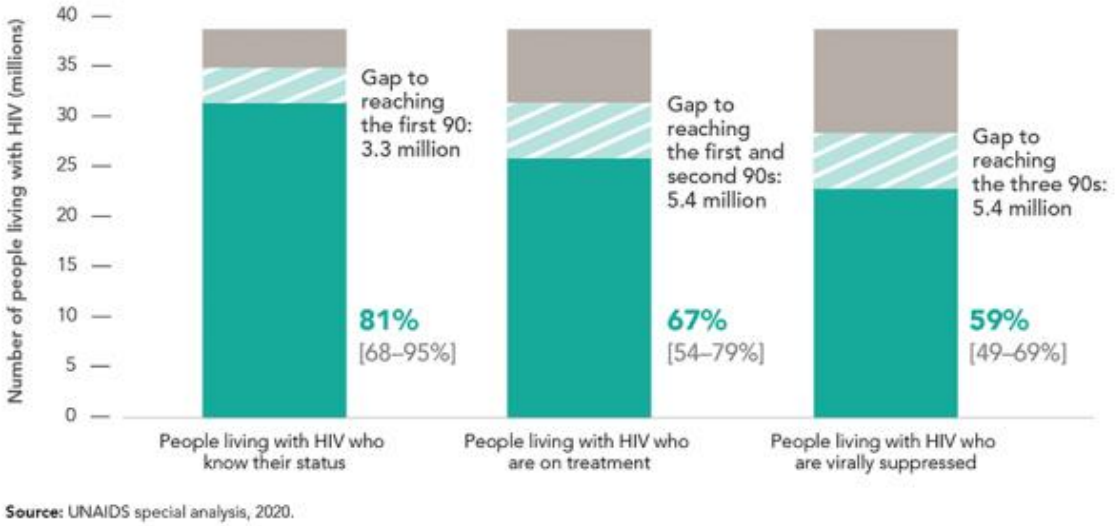


Figure 1| Global HIV testing and treatment cascade for 2019, showing the progress to UNAIDS 90-90-90 target. Bar graphs (from left to right) represent 81% of people living with HIV who got tested, 67% of people living with HIV who are on treatment and 59% of people living with HIV who have their viral load suppressed (UNAIDS, 2020).

The strategy proved to be effective in a sense that 81% of people living with HIV knew their status, 67% were on antiretroviral therapy and 59% had suppressed viral loads globally by 2019 as shown in **Figure 1**. However, the UNAIDS 90-90-90 target was not met by the end of 2020 (UNAIDS, 2020).

2.1.1 HIV Statistics

The recorded statistic on HIV emphasizes the devastating impact HIV has had on the lives of people living with HIV. Since the discovery of HIV, about 75.7 million people have tested positive for HIV and about 32.7 million people have perished from AIDS-related diseases. These figures could be way more as not all persons know their HIV status. In 2019, people living with HIV were estimated at 38 million and 1.7 million people were recorded as new HIV infections globally. Although the new infections marked a 23% decrease compared to year 2010, however, this is way more than the 500 000 HIV infections which was aimed for by the year 2020 (Palshetkar *et al*., 2020) as shown in **Figure 2**.

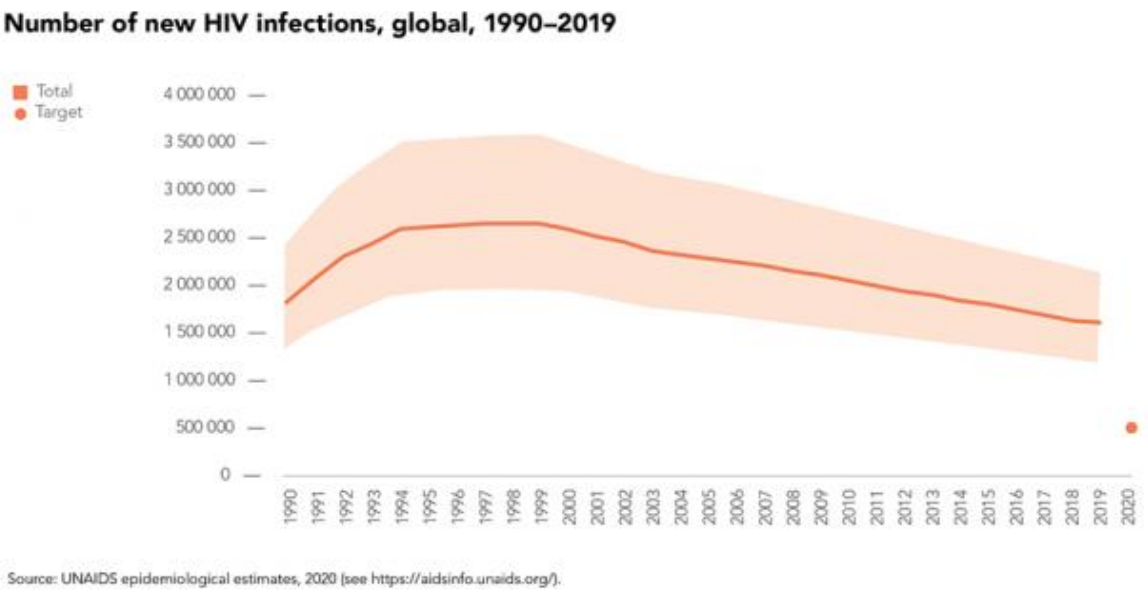


Figure 2| Global cases of HIV infection from 1990 to 2019. The HIV infection appreciated from 1990, reached a stationary phase between 1994 and 1999 then started depreciating until 2019. The dot at 2020 represents the UNAIDS 90-90-90 target that was aimed to be at 500 000 cases of new HIV infection, which was not met as new infections were estimated at 1.7 million by 2019.

The regions contributing the highest proportion to HIV infections are eastern and southern Africa, where out of 730, 000 new HIV infections in 2019, 30% were young women (UNAIDS, 2020). A devastating issue for women infected with HIV is the high risk of development of cervical cancer and exposure to an array of opportunistic infections.

2.1.2 HIV pathogenesis

Several factors dictate the aftermath of HIV-1 infection as well as the progression to AIDS, these factors include the viral phenotype, the viral entry into the host cell and the immune response of the host towards the virus (Naif, 2013) (Fanales-Belasio *et al*., 2010). HIV thrives in the bloodstream or lymphatic tissue and its transmission depends on the biological properties of the virus isolate, the concentration of the virus in the fluid of the infected person, and the susceptibility of the host to infection (Fanales-Belasio *et al*., 2010). HIV target the T-helper cells or CD4 cells which are responsible for fighting off infection and diseases. During infection, the CD4 cells are utilised as the machinery for the reproduction of new virions (Pau and George, 2014), subsequently disabling the immune system from protecting the host against the invasion of pathogens.

The consequence of HIV infection vary greatly from one individual to another, but often disease progression and clinical symptoms advance slowly (Naif, 2013). In the first few days

and weeks starting from the time of exposure to HIV, people show flu-like symptoms or mononucleosis-like illness, like fever, oral ulcers, maculopapular rash, arthralgia, lymphadenopathy, pharyngitis, myalgia, malaise, and weight loss (Fanales-Belasio *et al*., 2010). The acute phase shown in **Figure 3** lasts for three months after infection, until the detection of HIV-specific antibodies in the blood serum of infected people (Naif, 2013).

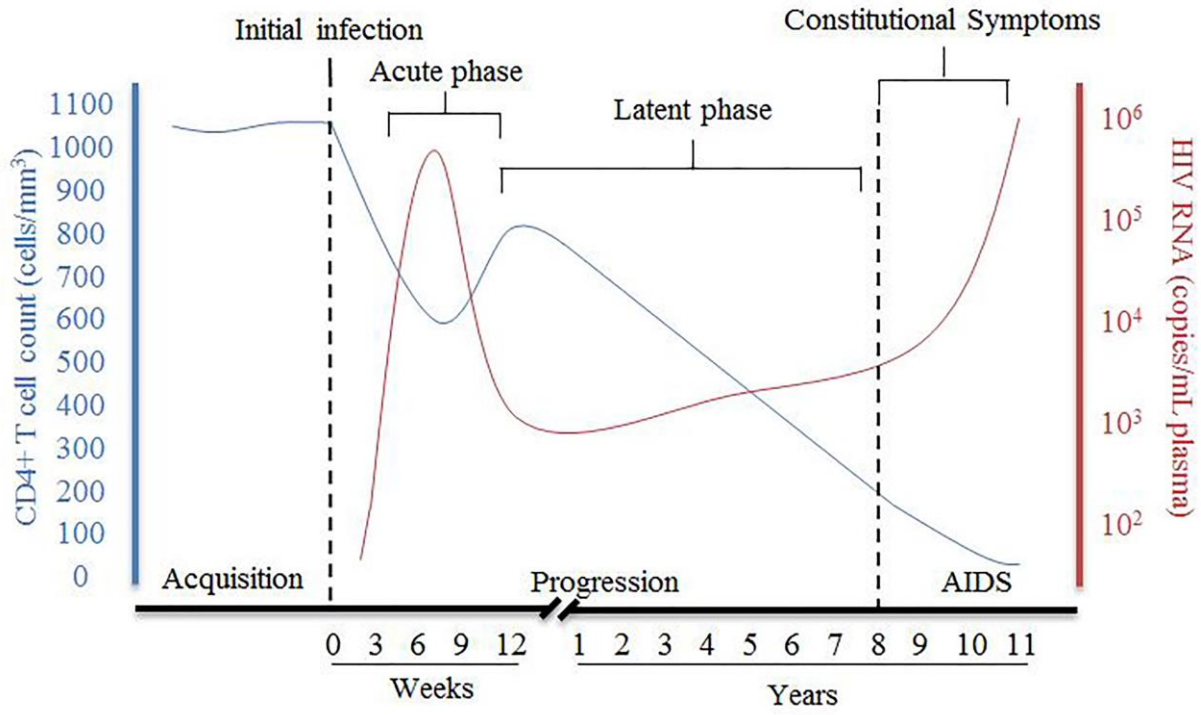


Figure 3| The HIV-1 infection phases. The progression of the HIV replication phase is depicted by the CD4 cell count (marked in blue) and HIV viral RNA (marked in red) and their relationship starting from infection to the phase where clinical symptoms are observed (Tough and McLaren, 2019).

People depicting more severe symptoms during acute phase tend to progress more quickly to AIDS and the CD4 cell count declines drastically in relation with high levels of viral RNA (Fanales-Belasio *et al*., 2010). Post this phase, a brief recovery stage ensues where CD4 T cells increase and the viral RNA decreases. This stage is the beginning of the latent phase of the infection where a person might not show any symptoms of infection, however, a slow progressive decline of CD4 T cells with an increase in the viral RNA still occurs (Tough and McLaren, 2019). This occurs because the viral replication still occurs in the lymph nodes and blood stream of the infected host. Consequently, the immune system becomes exhausted by the continuous exposure to virus that results in a continuous immune response (Naif, 2013). The impairment of the lymphoid system progresses and CD4 cell numbers further drop to levels less than 200 mm^3 (Fanales-Belasio *et al*., 2010) where the infected individuals are said to have

developed AIDS (Naif, 2013). The late latent phase that is also known as the symptomatic stage makes individuals susceptible to opportunistic infections (OIs) by bacteria, fungi, viruses, parasites, and tumours due to the immune system deteriorating. The most prevalent OIs are caused by Candida albicans, tuberculosis, Microcystis carinii, Cytomegalovirus, Herpes zoster or enteropathic parasites (Giardia species, Cryptosporidium and Isospora belli), each of these may cause life-threatening diseases (Fanales-Belasio *et al*., 2010) (Zumla *et al*., 2015). Considerable attention has been dedicated to studying the replication process of HIV-1 and related immunological responses. However, a persistent obstacle is the development of antiviral therapies utilising novel compounds with less resistance profile and reduced toxicity (Kurapati *et al*., 2016).

2.1.3 HIV-1 Life Cycle

The HIV infects T cells with high levels of CD4, particularly memory T cells. However, molecules of CD4 seem insufficient to grant viral entry into other types of cells, like monocytes and dendritic cells. This issue led to the discovery of a second gateway the virion uses to infect other types of cells, which is the chemokine receptor or coreceptors (Swanstrom and Coffin, 2012) (Naif, 2013). About 17 chemokine ligands that function as HIV coreceptors have been discovered and are categorized according to their ligand structure. The current nomenclature uses CXC, CC, CX3C or C followed by R (receptor) and a number (*e.g.*, CXCR1-5, CCR1-10, CX3CR1, etc). The most commonly used coreceptors are CXCR4 and CCR5, however, CCR5 is the most desired coreceptors by almost all primary HIV-1 isolates regardless of viral genetic subtype (Fanales-Belasio *et al*., 2010). The exposed coreceptors on the lipid membranes of the target cells contribute to initiating the replication of HIV.

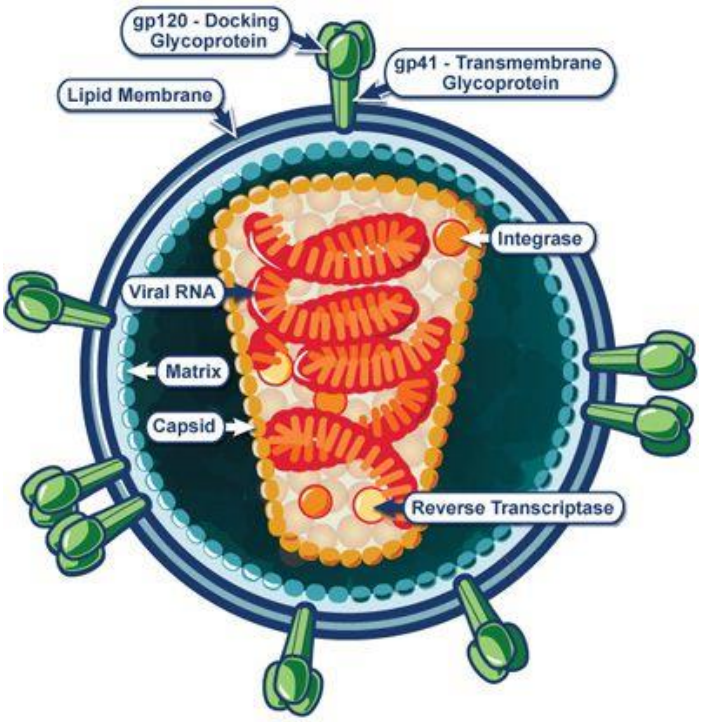


Figure 4| HIV structure with finger-like projections glycoproteins gp120 and gp41 which assist the virion to attach onto the host cell. Inside the lipid bilayer of the virion is a viral capsid that harbours the single-stranded viral RNA and viral enzymes: reverse transcriptase (RT), integrase (IN), and protease (PR). (https://studylib.net/doc/6821292/hiv-life-cycle-diagram Accessed: 16 September 2021).

The HIV is an icosahedral structure of nearly 100 nm with 72 external spikes generated by glycoproteins envelope of gp41 and gp120**,** which are implanted on the membrane as shown in **Figure 4** above. The lipid bilayer of the virion is studded with host-cell proteins which play an integral part during the budding process. Inside the virus is a conical nucleocapsid of single-stranded positive polarity RNA genome copies which are identical. The nucleocapsid also contains viral enzymes namely, reverse transcriptase (RT), integrase (IN), and protease (PR). The machinery of the host cell is exploited by the virion to complete its life cycle (Ramdas *et al.*, 2020) (Rankovic *et al*., 2017). The virion also encodes Tat and Rev genes which are responsible for activating the transcription of the viral genes and for late mRNAs transportation from the nucleus to the cytoplasm. In addition to regulatory genes encoded by the virion, there is also the Nef gene, which is responsible for decreasing CD4 cells and Vif which plays a major role in enhancing the viral infectivity. Genes such as Vpr and Vpu, play an essential role in the transportation of the viral core from the cytoplasm to the nucleus and also enhance the release of the virion from the cell (Lata *et al*., 2015). Although the features of virion play different roles, as can be seen in **Figure 5**, they sequentially conspire to bring about successful infectivity.

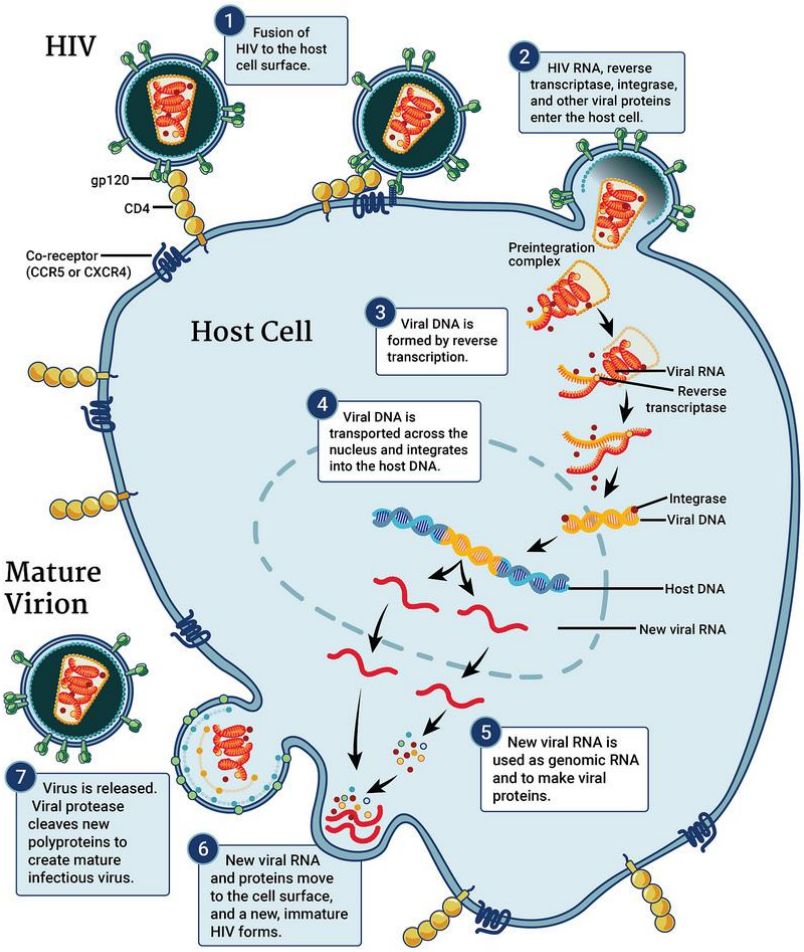


Figure 5 | The HIV-1 replication cycle, which commences when HIV attaches to the CD4 cells. This is followed by the fusing of the virion and the host cell surface (step 1). A viral capsid empties its content into the cytoplasm (step 2), leading to the disintegration of the capsid shell. HIV RT transcribes the viral RNA into complementary DNA (step 3), which is then carried across the nucleus (step 4), where the HIV IN integrates the HIV DNA into the host's DNA. The host's machinery then transcribes the HIV DNA into multiple copies of new HIV RNA and some of these RNA become the genome of a new virus, while other RNA copies are utilised by the cell to make new HIV proteins (step 5). The newly generated viral RNA and HIV proteins assemble toward the cell surface (step 6), where a new non-infectious and immature HIV is created. The host cell then releases the virus and the last viral enzyme, HIV PR cleaves newly synthesized polyproteins to generate an infectious virus (step 7) (https://www.niaid.nih.gov/diseases-conditions/hiv-replication-cycle Accessed: 16 September 2021).

From the time of HIV-1 infection to the time of observable immunodeficiency, the viral population undergoes significant changes. The viral population in a person infected with HIV is usually initiated by a single CD4 T cell from the mucosal tissue proximal to the site of exposure to HIV (Swanstrom and Coffin, 2012). The entry of HIV-1 is characterised by complex protein-protein interactions where the envelope glycoprotein gp120 contacts the surface-expressed CD4 via the C4-domain of gp120 and this permits for a more stable two-pronged attachment of the virus. This interaction prompts a change in the structures of CD4

and gp120, leading to an extra site to be created for gp120 for the coreceptors to attach onto the surface of the cell, as shown in step 1 of **Figure 5** (Ramdas *et al.*, 2020) (Seitz, 2016). The virus interacts with a legion of host glycoproteins expressed on the plasma membrane such as CD43 and P-selectin glycoprotein ligand-1 (PSGL-1) which regulate the attachment of HIV by being merged with the virions (Ramdas *et al*., 2020). The PSGL-1 has been discovered to be upregulated during inflammation and this interferes with the infectivity of the virion. The HIV-1 overcomes this hindrance by downregulating PSGL-1 to remove it from the virions to guarantee the effective attachment to the host cell membrane. This is aided by the HIV-1 encoded Vpu together with co-clustered Gag at the virion membrane (Fu *et al.*, 2020) (Ramdas *et al.*, 2020). The next phase is the fusion of N-terminus of gp41 into the plasma membrane of the host cell and this action is made easy by the hydrophobicity of gp41, as shown in step 2 of **Figure 5** (Fanales-Belasio *et al*., 2010) (Seitz, 2016). Past fusion, the capsid of the virion is translocated into the cytoplasm of the host cell where it is taken up by an endosome. The release of the capsid contents into the cytoplasm is induced by the change of pH in the phagosome before nuclear import through the process known as uncoating. The strand of HIV-1 RNA is then degraded by RNase H, followed by the second enzymatic activity that is facilitated by the DNA-dependent DNA polymerase activity of HIV-1 reverse transcriptase (HIV-1 RT) to convert a single-stranded complementary DNA (cDNA) into double-stranded DNA (proviral DNA), shown in step 3 of **Figure 5**. The newly converted proviral DNA is then transported into the nucleus of the host cell through nucleopores in the form of a cluster consisting of the HIV-1 integrase (HIV-1 IN) and linear or circular proviral DNA. The IN enzyme integrates the proviral genome into the host cell genome, as shown in step 4 of **Figure 5** (Seitz, 2016) (Rankovic *et al*., 2017).

HIV-1 exploits the host cell mechanism to generate copies of its genomic material and messenger RNA (mRNA) strands, as shown in step 5 of **Figure 5**. These short mRNA strands are used to generate long chains of HIV-1 proteins, then moved from the nucleus to the surface of the cytoplasm and assembled as immature non-infectious virion, as shown in step 6 of **Figure 5** (Yang *et al*., 2012) (Lv *et al*., 2015). After assembly, the immature virions bud out of the target cell and this sets in motion HIV-1 protease (HIV-1 PR), a homodimeric aspartyl protease that cleaves polyproteins of Gag and Gag-Pol at twelve cleavage sites to generate mature active proteins that are encoded by the HIV-1 virus genome. The HIV-1 PR first cleaves off Pol from Gag-Pol polyprotein, Pol is responsible for producing active viral enzymes (RT, PR, and IN) and viral structural proteins (capsid and nucleocapsid) which are required by the

newly produced virions, as shown in step 7 of **Figure 5** (Yang *et al*., 2012) (Lv *et al*., 2015) (Lockbaum *et al.*, 2019).

The duration of the attachment of HIV onto a CD4 cell needs about 30 minutes to 2 hours, while the process of transcription takes roughly 6 hours and the integration process requires another 6 hours. The initial virus particles produced after integration phase can be detected and destroyed by the immune system after about 12 hours, which is 24 hours after infection. The virus overcomes the action of host immune system by generating a form of variability which serves as a shield not only from the immune response but also from the effects of therapeutics. HIV-1 carries out its variability in three unique ways: (1) the RT facilitates an "error-prone" mechanism of action where one substitution is introduced in a genome in a single round of replication, on average; (2) initiating a quick viral replication that produces a large quantity of virions per day, which are estimated around 10^{10} and (3) the recombination of two or more different HIV viruses occurring within the same person (Fanales-Belasio *et al*., 2010). Over time, a wide spectrum of various population of mutants develops in infected individuals and escape the immune response to infect more cells and maintain HIV replication. At this point the epitopes of antigens that are essential for neutralising antibodies are already affected by the mutation process, therefore, the HIV replication exacerbates uncontrollably (Seitz, 2016).

2.1.4 HIV-1 treatment strategies

The Food and Drug Administration (FDA) has approved over 30 antiretroviral drugs to be used in the treatment of HIV infection (Subramaniam *et al.*, 2019). The FDA approved antiretrovirals can be classified as HIV-1 entry, nucleoside reverse transcriptase, non-nucleoside reverse transcriptase, integrase, and protease inhibitors (Henes *et al*., 2019). HIV-1 is now a manageable infection and the plasma viral suppression is achievable with the help of HAART that attacks several phases of the HIV-1 cycle. The treatment includes two nucleoside reverse transcriptase inhibitors (NRTIs), a non-nucleoside reverse transcriptase inhibitor (NNRTI), and a protease inhibitor (PI) or an integrase inhibitor (INSTI). The improvement with longevity and quality of life among people living with HIV has been greatly attributed to the development of HAART, however, no effective cure or vaccine for HIV exist to date (Gavegnano *et al.,* 2019) (Tough and McLaren, 2019) (Zhuang *et al.,* 2020). Antiretrovirals must be administered daily and if defaulted, the plasma viral load increases. Moreover, HAART has proven to be an expensive treatment for underdeveloped and developing countries, where HIV infected patients still struggle to access antiretrovirals (Subramaniam *et al.*, 2019)

(Palshetkar *et al.*, 2020), this challenge has greatly hampered treatment strategies such as UNAIDS 90-90-90 target that was put in place as a strategy for ending the AIDS epidemic.

Research reveals that most HIV drug development strategies have been directed towards the two crucial viral enzymes namely: HIV-1 RT and PR (Henes *et al*., 2019). This study also looked at the inhibitory ability of plant extracts against these enzymes.

2.1.4.1 HIV-1 Reverse Transcriptase inhibition

Reverse transcriptase is a molecular tool for HIV-1 that is regarded as a primary drug target by several researchers. There are two types of HIV-1 RT inhibitors, first one being nucleoside RT inhibitors (NRTIs) which binds to the active site on the RT enzyme and the second one being non-nucleoside RT inhibitors (NNRTIs) which bind to an allosteric site located about 10 A° away from the DNA polymerase active site (Das and Arnold., 2013) (Lai *et al.*, 2018) (Zhuang *et al*., 2020). Most NRTIs are base analogues that act as DNA polymerization chain terminators catalysed by RT. The NRTI carries out its inhibitory action either by lacking a 3′-hydroxyl group which prevents the formation of a 3'-5'-phosphodiester bond required for DNA chain expansion or by containing an altered sugar moiety that rejects the inclusion of the next nucleotide (Das and Arnold., 2013) (Lai *et al.*, 2018). The nucleoside analogue, zidovudine (AZT) was the first drug to be approved for NRTI. Clinical studies conducted on AZT revealed that treating HIV with a drug that targets only a single phase of HIV replication cycle was ineffective. It was therefore noted that AZT alone could not achieve maximum suppression of the viral load, moreover, drug-resistant strains caused AZT to lose its sensitivity (Das and Arnold., 2013). The NRTI drug resistance occurs because of mutations that prevents the NRTI to be featured into the newly forming DNA chain. Another form of NRTI drug resistance occurs through RT mutations that escalate the rate of hydrolytic exclusion of NRTI and enable the synthesis of DNA to continue (Shafer *et al*., 2012) (Das and Arnold., 2013).

The ineffectiveness of zidovudine led to the approval of NNRTIs which became a more prominent class of drugs (Chinsembu, 2019). The NNRTIs are regarded as the crucial component in HAART because of the antiviral property with low toxicity profile and high specificity (Zhuang *et al*., 2020). Over 50 chemotypes of NNRTIs have been identified, however, FDA approved only six of them, namely: delavirdine (DLV), nevirapine (NVP), etravirine (ETR), efavirenz (EFV), doravirine and rilpivirine (RPV) (Zhuang *et al*., 2020). Although doravirine was shown to suppress mutations associated with NNRTI in vitro *(*Lai *et al*., 2018) however, clinical trials depicted that the effectiveness of NNRTIs was being

compromised by adverse reactions, drug-resistant variants of HIV-1 RT and weak drug-drug interactions (Chinsembu, 2019). The development of resistance to HAART is greatly attributed to the promiscuity of RT in two ways: directly to RT inhibitors at molecular level and/or indirectly as a primary source for producing genetic variations which include mutant HIV-1 proteins and mutant RTs (Das and Arnold, 2013).

The promiscuity of HIV-1 is observed with several mutations occurring during replication while the virus retains its activity, moreover, these strains of mutants are able to produce new variants (Das and Arnold., 2013). A typical HIV-1 drug is formulated to target the wild-type virus, however, the least inhibited mutants emerge and adapt as the predominant variants to the host environment (Das and Arnold, 2013). Two classes of mutants have been identified as immunological-escape mutants and drug-resistant mutants (Abram *et al*., 2014) and this variation in mutation arise during the HIV-1 viral life cycle. This ability of the virus to diverge abruptly serves an integral part in escaping the immune system and antiretrovirals thereby becoming resistant (Hu and Hughes, 2012).

2.1.4.2 HIV-1 Protease inhibition

HIV-1 protease is an aspartyl protease of a homodimer with 99-amino acids. The protease cleaves 12 peculiar cleavage sites on Gag and Gag-Pol polyproteins to produce individual viral proteins which are necessary for the viral maturation and replication (Lockbaum *et al*., 2019). The inhibition of protease is considered as the most reliable path of treating HIV/AIDS, as PR inhibitors prohibit the conversion of virions into infectious state and because of that, HIV-1 PR is regarded as a prime focus for the development of antiretrovirals (Filho *et al*., 2010). However, the capability of the virus to be resistant towards antiretrovirals illustrates a great obstacle in the significant developments made with HIV therapy to date. The first approved HIV-1 PI was saquinavir (SQV) in 1995 by FDA, it was a peptide that was able to bind tightly to the activation site of PR (Shi *et al*., 2020). However, SQV together with amprenavir, indinavir, nelfinavir and ritonavir have become insignificant due to production of resistance mutation and toxicity, with the exception of ritonavir which is still being used as a PI booster (Doktorgrades, 2020). The subsequent generations of PIs namely: atazanavir, darunavir (DRV), lopinavir and tipranavir had an improved efficacy, selectivity and had a lower toxicity profile compared to the first generation of PIs (Doktorgrades, 2020). Darunavir, has shown great binding affinity that was proven to be effective against strains which had developed resistance towards other inhibitors. Despite the effectiveness observed with darunavir, great levels of resistance are observed with the accumulation of legions of mutations. The mutations

that are far from the catalytic site of HIV-1 protease tend to accumulate in great numbers compared to mutations which are proximal to the catalytic site and this makes the binding of inhibitors to be less effective (Henes *et al*., 2019) (Whitfield *et al*., 2020). The mutations that are found proximal to the activation site are considered as the ones conferring drug resistance, while mutations distal from the activation site are thought to weaken inhibitor binding and they also compensate and restore catalytic functions (Henes *et al*., 2019).

Patient noncompliance due to adverse reactions as well as high pill load have greatly contributed to the rising of mutations, and a combination of complex multiple mutations is caused by variants from patients who failed therapy. HIV-1 protease can tolerate many mutations, with 45 out of 99 amino acids with over 20 substitutions known to cause drug resistance (Henes *et al*., 2019). Grasping how mutations thwart the binding of inhibitors while carrying out their enzymatic activity is the most important factor to comprehend the mechanism of drug resistance in HIV-1 protease. Substrate recognition has been identified as the major core of drug resistance in HIV-1 PR, as structural studies reveal that PIs contend with natural substrate for binding in the catalytic site and this impairs the effectiveness of inhibitors. The improvements made with subsequent generation of PIs promote them to fit perfectly within the substrate envelope. However, most inhibitors still fall short as they exhibit shallow resistance profile as resistance mutations accumulate (Doktorgrades, 2020) within and outside of the catalytic site. This clearly indicates that drug design strategies need to be more advance not only to combat the replication of the virus but also to tackle the resistance mutations which renders the current existing antiretrovirals ineffective.

Looking at the improvement brought by combining drugs with HAART, drug design strategies with PIs also gravitated towards combining different PIs for more efficacy and to overcome PI-resistance mutations. The PIs atazanavir/ritonavir (ATV/r) and lopinavir/ritonavir (LPV/r) at one point became available and the generic was cheaper but for regions like sub-Saharan Africa it was still expensive (Boender *et al*., 2016). The combination of darunavir and ritonavir (darunavir-ritonavir or DRV/r) proved to be more efficacious and was supported by Lancet laboratories not just as a second line of PI treatment for patients who are resistant to the first line of treatment but also for people living with HIV at any stage of treatment (Clotet *et al*., 2007). The DRV/r has shown a broad range of efficacy and is well tolerable among people living with HIV, especially by treatment-naïve individuals, early treatment-experienced as well as the highly treatment-experienced individuals. The findings of clinical trials conducted on DRV/r revealed the necessity of using this second-line treatment on patients with AIDS and

those who have experienced extensive antiretroviral therapy failure (Arasteh *et al*., 2009). The escalating number of people that are infected with HIV who are experiencing treatment failure due to PI-resistance mutations require a second line of PI-based antiretrovirals. However, the high costs of DRV/r make it inaccessible to low income countries like sub-Saharan Africa (Boender *et al*., 2016). The devastating issue with the lack of second line of antiretroviral therapy in regions that are mostly affected by the plight of HIV/AIDS is that resistance mutation strains get to be transmitted among individuals. This is a setback to all the advancements that have been made with HIV therapies.

2.1.4.3 Limitations and setbacks associated with HIV therapy strategies

Although HAART has considerably reduced the rate of mortality among people living with HIV, however, it is not without side effects which are not well tolerated and this cause patients to default on treatment, thereby making them prone to developing multidrug resistant infections (Palshetkar *et al*., 2020). The burdensome side effects include prevalence of cardiovascular disease (CVD), malignancy, metabolic, liver, bone and renal diseases and nephrotoxicity, technically occurring due to aging in the affected population (Kapewangolo *et al*., 2013) (Chinsembu, 2019). One of the limiting factors on the significant progress made with HIV therapies points to the rising incidences of HIV drug-resistance strains, which is said to be 5.6% in countries like Nigeria, Zimbabwe, Kenya, Uganda, South Africa, and Zambia. Another setback is brought about by the ingesting of tenofovir and indinavir which leads to severe liver toxicity. Both toxicity and HIV resistance takes away from the adherence and efficacy of HAART (Chinsembu, 2019).

2.1.4.3.1 Effects of COVID-19 pandemic on the management of HIV

This study was conducted during the emergence of the COVID-19 pandemic, there has never been a global pandemic before COVID-19 that has converged with the HIV pandemic, so it is crucial to look at the interplay between the two infections (Brown *et al*., 2021) as well as the effect of COVID-19 on the progress to eradicate HIV/AIDS. In late 2019 and early 2020 COVID-19 surfaced as a major health threat on a global scale. The COVID-19 is caused by a novel severe acute respiratory syndrome coronavirus 2 (SARS-CoV-2) (Barbera *et al*., 2021). As of July 2020, 12 102 328 people were infected with COVID-19 and about 551 046 deaths were related to COVID-19 worldwide. The factors contributing to COVID-19 mortality included old age, chronic disease co-morbidities, chronic kidney disease, chronic lung disease, asthma, obesity, cardiovascular conditions, diabetes, chronic liver disease, and

immunocompromised individuals, like people who are infected with HIV (Mirzaei *et al*., 2021).

Analyses conducted in the Western Cape province, South Africa reveal that HIV elevated the risks of mortality during COVID-19 pandemic, as about 8% of COVID-19 related deaths were among people living with HIV. Those with high viral load were found to be at even greater risks of dying from COVID-19 compared to people whose viral load was suppressed (Barbera *et al*., 2021). This evidence increased the vulnerability of people living with HIV, making it even more urgent for this population to have access to antiretrovirals (WHO, 2020). However, the COVID-19 restrictions which were formulated with the aim to curb the virus from spreading ended up disrupting the health care services for HIV therapies, as some countries reported a 20% reduction in medicine collection due to lockdowns. There were also reports of multiple incidences of people living with HIV going for two months without antiretrovirals and others running out of food thereby abandoning HIV treatment (UNAIDS, 2020). Such disruptions in HIV treatment strategies may have contributed to more AIDS-related deaths, as was estimated that the COVID-19 pandemic could result in over 500,000 additional AIDS-related deaths in sub-Saharan Africa between 2020 – 2021. Even a little disruption of 20% was still estimated to result in about 110 000 cases of mortality (UNAIDS, 2020) (WHO, 2020). The outbreak of COVID-19 pandemic added onto already existing limitations and setbacks in HIV therapy strategies.

Prior to the development of COVID-19 vaccines there was a desperation to urgently find remedies for the treatment and prevention of COVID-19, which led to countries like China, South Africa, and Madagascar to resort to medicinal plants like *Artemisia annua*. The anecdotal use of these plant extracts was overly exaggerated with no scientific proof of efficacy and safety (Kapepula *et al*., 2020) (Attah *et al*., 2021). The widespread use of medicinal plants during COVID-19 pandemic showed how most people still rely on medical plants as remedies and indicated that plants are the next available resources within our ecosystem. Screening of medicinal plants to discover their antiviral profile towards HIV, COVID-19 as well as other opportunistic infections which affect mankind on a global scale is of vital importance.

2.2 Opportunistic infections

HIV infected people are vulnerable to a multitude of fungal, bacterial, protozoal, and viral infections which include *Mycobacterium tuberculosis*, *influenza, Pneumocystis jirovecii, Pneumococcal pneumonia*, Cytomegalovirus (CMV), *Cryptococcus neoformans,* Herpes virus infections, meningococcal diseases, *Mycobacterium avium*, *Toxoplasma gondii*, and

Histoplasma capsulatum (Naif, 2013) (Seitz, 2016) (Barbera *et al*., 2021). Additionally, individuals who are infected with HIV have shown to develop various cancers, resulting in what is known as AIDS defining cancers (ADC), which include invasive cervical cancer, Kaposi sarcoma and non-Hodgkin lymphoma (Stelzle *et al*., 2021). Research conducted in high-income countries has shown a decline in ADC incidence and a rise in non-AIDS defining cancers (NADC), such as skin, lung and liver cancer (Dhokotera *et al*., 2019) (Sasco *et al*., 2010). However, the contrary has been observed in sub-Saharan African regions among people living with HIV (Stelzle *et al*., 2021) (Nkwonta *et al*., 2022). The high proportion of immunosuppressed individuals with a high risk in developing ADC in the era of HAART in low-income countries is possibly due to the aging population of people living with HIV, delayed HIV diagnosis, poor access to treatment, insufficient suppression of viral load and recent exposure to high viral load (Sun *et al*., 2021) (Nkwonta *et al*., 2022).

This study only focused on Human papillomavirus (HPV) and Hepatitis B or C virus (HBV or HCV) as infections associated with cancer.

2.2.1 Human papillomavirus-related cancer

The infection of HPV is the core agent for the development of cervical cancer, which is the malignancy of the cervix (Domenico *et al*., 2012) (Park *et al*., 2021). Apart from the HPV infection contributing to the development of cervical cancers, it also plays a part in the development of a larger group of other cancers such as that of anus, oropharynx, vagina, penis, and vulva (Lekoane *et al.*, 2020). The abnormal growth and uncontrollable proliferation of cancerous cells lead to life-threatening complications, which have posed a major health burden globally (Alshehri, 2020). Cervical cancer ranks number four as both the most diagnosed cancer as well as a major cause of death among women, with new cases that are estimated at 604 000 and 342 000 deaths globally in year 2020. This AIDS defining cancer is the most diagnosed in 23 countries and the leading cause of cancer death in 36 countries, with some of these countries being sub-Saharan Africa, South America, Melanesia, and South-Eastern Asia. Globally, Malawi has the highest recorded cases of cervical cancer incidence and mortality, while Eastern Africa, Southern Africa, and Middle Africa have the highest incidences regionally (Sung *et al*., 2021) (WHO, 2018).

The association between cervical cancer and HPV infection was mapped out by Harold zur Hausen in the early '70s. The HPVs can be classified into two categories, namely the low-risk HPV and high-risk HPV depending on the susceptibility of lesions associated with HPV to progress to malignant stage (Domenico *et al*., 2012). The HPV is further categorised into

different types with type 16 being the most prevalent high-risk HPV type, followed by HPV 18. The clinical symptoms of low-risk HPVs are observed through the generation of genital warts and moderate dysplasia, while symptoms of high-risk HPVs are linked with cervical intraepithelial neoplasia or cervical dysplasia. The immune response can effectively resolve most HPV infections, however, when the infection is not eliminated, it progresses to cervical cancer (Domenico *et al*., 2012). The evidence of correlation between HIV and HPV-associated cervical cancer has been well documented in the scoping review done on nine studies conducted in sub-Saharan Africa, where it was intensively demonstrated that HIV-1 infected women are at greater risks of developing cervical cancer compared to the general population (Lekoane *et al.*, 2020). Due to the interplay of HIV and HPV the International Agency for Research on Cancer (IARC) has categorised HPV as a direct carcinogen and HIV-1 as an indirect carcinogen because of the ability of the infection to suppress the host immune response (Lekoane *et al.*, 2020) (Sung *et al*., 2021).

2.2.2 Hepatitis C or B virus-related cancer

The HBV or HCV infection is a major element for the development of hepatocellular carcinoma (HCC). A study conducted in 2014 illustrated that over half a million people globally were diagnosed with HCC each year (Díaz-Sánchez *et al*., 2014). The HCC emanate as the final severe stage of liver diseases, which commences with sustained inflammation that progresses to liver cirrhosis due to HBV and/or HCV infections, biliary diseases, alcoholic liver disease, drugs, non-alcoholic fatty liver disease, metabolic disorders, toxins, 1-antitrypsin deficiency and genetic conditions such as hereditary hemochromatosis (Yu *et al*., 2018). Liver cancer ranks sixth as the cancer that is often diagnosed and ranks third as the leading cause of mortality worldwide. As of 2020, new cases of liver cancer were estimated at 906 000 with roughly 830 000 fatalities. Liver cancer is prevalent among men, with rates of both incidence and mortality ranging between 2 to 3 times higher compared to women (Sung *et al*., 2021).

A vast number of researchers have explicitly demonstrated the rising incidence of HCC among individuals who are infected with HIV. Moreover, the association between HCC and the long term use of HAART has also been brought to light (Díaz-Sánchez, 2014) (Thornton *et al*., 2017) (Sun *et al*., 2021). About 75 - 85% of HCC cases are predominantly found in China, sub-Saharan Africa, and the Republic of Korea. People who are infected with HIV have a greater burden of HCC compared to people who are not infected. Over 90% of HCC cases arise as a consequent of hepatic injury that activates inflammation. Persistent inflammation generates

reactive oxygen species (ROS) and reactive nitrogen species (NOS) (Yu *et al.*, 2018) (Sun *et al*., 2021), which leads to more oxidative stress and this further deteriorates the immune system that is already compromised.

Reports from cohort studies reveal that most people who are living with HIV are no longer dying from HIV/AIDS-related causes but more cases of mortalities are attributed to liver-related diseases (Díaz-Sánchez, 2014) (Thornton *et al*., 2017). Individuals living with HIV who are coinfected with HBV and/ HCV progress quicker to the severe stage of liver disease than the population with only hepatitis infection (Thornton *et al*., 2017). People who are at great risks of developing and progressing to HCC are those with unsuppressed HIV plasma loads, those with extremely low CD4 cell counts, and people who inject themselves with drugs (Sun *et al*., 2021). Sorafenib was approved by the FDA as treatment for HCC, however, the aggressive nature of the treatment further strains the liver that is already compromised. This often leads to more side effects with moderate improvements and improbable chances of survival (Yu *et al*., 2018). Other drugs such as, regorafenib, nivolumab, lenvatinib, pembrolizumab and cabozantinib were also approved for the treatment of HCC, however, some limitations were experienced in trials. There are currently ongoing clinical trials, which will contribute in the HCC drug development strategies (Luo *et al*., 2021). Improved cancer therapies devoid of unbearable adverse reactions and toxicity are of vital importance to reduce the death rates not only among people living with HIV but for the entire human population.

2.3 Cancer therapies

The high prevalence of cancer shows medical needs that are not being met due to low rate of success on the development of anticancer therapies. About 10% of cancer drug agents in clinical trial stages get to be approved (Yan *et al*., 2011). Numerous cytotoxic agents or conventional chemotherapy were invented as the initial agents to treat cancer. These agents include alkylating agents (temozolomide, cyclophosphamide, oxaliplatin, cisplatin), anti-metabolites (capecitabine, cytarabine, methotrexate, fluorouracil), antitumor antibiotics (doxorubicin, bleomycin, epirubicin), microtubule stabilizers (docetaxel, paclitaxel) and topoisomerase inhibitors (etoposide, irinotecan) (Palumbo *et al*., 2013). The disadvantage about cytotoxic drugs is that they act on all rapidly dividing cells; consequently, killing normal cells as well. The reoccurrence of tumours due to dispersion of drug-resistant cells and low specificity of the drug remains an inescapable impediment to attaining the effectiveness of anticancer therapy. The high cancer related mortality rate is more attributed to the side effects of anticancer therapies than it is in cancer itself. Chemotherapy is the commonly preferred

cancer treatment; however, it is aggressive in that it further weakens the patient's immune system (Padmaharish and Lakshmi, 2017) (Lichota and Gwozdzinski, 2018) (Alshehri, 2020). Another shortfall with chemotherapy is the limited response to different types of cancer, like renal cell carcinoma, melanoma, gastrointestinal stromal tumours (GIST) and hepatocellular carcinoma (Palumbo *et al*., 2013). Cancers treated with chemotherapy make people infected with HIV to be more susceptible to OIs because of the impaired immune system coupled with the complications caused by the HIV infection. Some studies have demonstrated that people living with HIV might experience a further decrease in CD4 cell count after being treated with chemotherapy or radiotherapy (Makinson *et al*., 2021). This shows the need to develop anti-HIV agents that could also be effective against cancers or the development of cancers.

Targeted cancer agents were invented to resolve the non-specificity of conventional chemotherapy. The categories of targeted therapy are monoclonal antibodies and signal transduction inhibitors (Bansode, 2017) (Conde-Estévez, 2017). Targeted cancer therapies function to impede the proliferation of cancer by interfering with specific molecules or molecular targets that are involved in the growth, progression, and spread of cancer. Targeted therapies are designed to interact with specific molecular targets that are associated with cancer, this is made possible using a patient's information of genes and proteins to prevent, diagnose, and treat cancer. This specificity has gained targeted therapies names such as precision medicines or molecularly targeted drugs. What set targeted therapies apart from conventional chemotherapies is that they are cytostatic, which make them the focus of anticancer drug development (Bansode, 2017).

As much as targeted therapies are specific and have become a fundament of anticancer drug development, several limitations are also experienced with them. Human tumours bear various mutation events that lead to the activation of multiple signalling pathways. The repetition in the signalling systems at the disposal of the cancer cells often render targeted agents inactive in treating most cancers. In order to overcome this limitation, the use of combinations of targeted therapies has been implemented as a potentially effective approach to simultaneously inhibiting multiple pathways, suppressing feedback reactivation of the compensatory signalling network, thereby preventing recurrence (Yan *et al*., 2011). While this solution may have been effective for those not simultaneously dealing with HIV/AIDS, it bears devastating outcomes for people living with HIV who are taking HAART. With multiple drugs being taken orally, drug–drug interactions (DDI) concerning gastrointestinal absorption may occur, thereby leading to adverse drug reactions or loss of efficacy. Fatal events associated with DDI are

estimated to account for approximately 4% among cancer patients and incidences of DDI arise when absorption, distribution, metabolism, or elimination of drugs are changed, leading to alterations in the amount and duration of drug availability at receptor sites (Conde-Estévez, 2017).

Just like conventional therapies, targeted cancer therapies are also not without adverse reactions. Side effects include hypertension, an increase in arterial thromboembolic events, thyroid dysfunction, cutaneous toxicity, cardiotoxicity, hepatotoxicity, skeletal muscle wasting, hypertension, bowel perforation, arterial thromboembolic events, impaired wound healing, bleeding events, venous thromboembolic events, and electrolyte abnormalities (Palumbo *et al*., 2013). Although targeted cancer therapies have had significant improvement in the lives of cancer patients, they still pose unbearable side effects. Therefore, there is still a great need for the development of new therapeutic drugs that are more efficient than existing drugs or that can synergize with them.

2.4 Oxidative stress

A major factor associated with disease development and progression is the emergence of oxidative stress. Free radicals are continuously generated endogenously as byproducts of biochemical and physiological processes of all aerobic human cells, and are also encountered exogenously (smoke, pesticides, air pollutants and ultraviolet light) (Williams *et al*., 2017) (Aslantürk and Çelik, 2013) (Skouta *et al*., 2018). Free radicals are categorised into two classes namely, reactive oxygen species (ROS) and reactive nitrogen species (RNS) (Gupta, 2015). The reactive oxygen species include free radicals such as superoxide anion ($O2^{\cdot -}$), hydroxyl radical ($OH^{\cdot}$), hydroperoxyl ($HO_2^{\cdot}$), peroxyl ($ROO^{\cdot}$), alkoxyl ($RO^{\cdot}$) and non-radicals hydrogen peroxide (H_2O_2), hypochlorous acid (HOCl), ozone (O_3) and singlet oxygen ($^1O_2^{\cdot}$). Similarly, reactive nitrogen species (RNS) include nitric oxide ($NO^{\cdot}$), peroxy nitrite ($ONOO^{\cdot}$), nitrogen dioxide (NO_2^{-}) and dinitrogen trioxide (N_2O_3) (AsokKumar *et al*., 2009) (Domenico et al., 2012). Radical species have highly unstable, unpaired electrons with a short half-life and this nature makes free radicals to be highly reactive (Williams *et al*., 2017). The production of free radicals occurs in conjunction with adequate antioxidant compounds. Low levels of free radicals are essential for the maintenance of normal physiological function and act as defence mechanism for the immune system, for example neutrophils generate ROS towards invading pathogens (Williams *et al*., 2017). However, high levels of free radicals result in oxidative stress, which damages cells, tissues and macromolecules such as proteins, DNA and lipids (Domenico et al., 2012). Therefore, the balance between oxidants and antioxidants determines

the health of a person and guarantees adequate antioxidants to scavenge free radicals, chelate metal ions and also act as electron donors (AsokKumar *et al*., 2009) (Kedare and Singh, 2011). Antioxidants such as, glutathione (GSH), vitamins C and E, play a major role in quenching free radicals (Boora *et al*., 2014).

The initiation of lipid peroxidation that leads to atherosclerosis, cardiovascular diseases, inflammatory diseases and ageing points to the overproduction of reactive species and oxidative stress (Boora *et al*., 2014). The association has also been made between elevated levels of radical species and the development of neurodegenerative diseases like amyotrophic lateral sclerosis, Huntington's, Alzheimer's, and Parkinson's (Skouta *et al*., 2018). Moreover, pathogenesis leading to carcinogenesis has been attributed to oxidative stress, as proliferating cancer cells have been found to produce high levels of ROS, due to oncogenic mutations that result in abnormal metabolism. It is suggesting that ROS behave as second messengers in intracellular signalling cascades, which prompt and maintain the oncogenic phenotype of cancer cells (Domenico *et al*., 2012) (Aslantürk and ÇelİK, 2013). Research reveals that oxidative stress chaperone different viral infections, which include HBV, HCV, hepatitis delta virus (HDV), herpes, and others. The infection with HIV-1 has been found to directly induce mitochondrial dysfunction and pronounced oxidative stress through deregulation of oxidative stress pathways, which further escalate the production of ROS. Moreover, HIV-1 infection does not only elevate oxidative stress but also reduces the total antioxidant capacity in people living with HIV (Ivanov *et al*., 2016) this promiscuous nature of HIV-1 infection has to be overcome so that people living with HIV are not further exposed to wide spectrum of infections, which ultimately leads to other chronic diseases.
Various studies have revealed that plants are rich in antioxidants (Boora *et al*., 2014) (Njoya *et al*., 2017), which have been found to be safe to use in preventing free radicals. The radical scavenging ability of medicinal plants may have significant applicability as therapeutic agents and prevention of diseases (Boora *et al*., 2014).

This study evaluated the scavenging ability of *F. multiflora* extract on nitric oxide (NO) production. The NO is a radical generated from L-arginine through the enzyme action of vascular endothelial cells, certain neuronal cells, and phagocytes. NO plays several roles as an effector molecule in various biological systems such as, neuronal messenger, antimicrobial, vasodilatation, and antitumor activities. Low concentrations of NO are necessary for physiological functions. However, high concentrations of nitric oxide are related to several

carcinogenesis and inflammatory conditions such as, juvenile diabetes, arthritis, multiple sclerosis, and ulcerative colitis. The toxicity of NO is further escalated by reacting with superoxide radical, which produces a highly reactive peroxynitrite anion ($ONOO^-$). It has been reported that NO is directly scavenged by flavonoids obtained from plants (Boora *et al*., 2014). Skouta *et al*. (2018) indicated that plant derived natural products may be utilised as alternative radical scavengers capable of transforming the course of oxidative stress and related diseases.

2.6 Natural products as potential drug candidates

The use of plants in traditional medicine has been practiced for centuries with knowledge being conveyed through all generations and empirically used to avert or cure different ailments as well as to promote well-being and general health (Yusmazura *et al*., 2017). The widespread use of plants has gained the attention of the body of science, through which useful bioactive constituents have been extracted from a variety of plant materials (Lichota and Gwozdzinski, 2018). The composition of diverse bioactive substances enables medicinal plants to possess curative abilities. Reports have shown that plant derived bioactive compounds exhibit inhibitory activities against numerous diseases such as: cancer, osteoporosis, malaria, Parkinson's disease, hyperglycaemia, leishmania, hypertension and diabetes (Njoya *et al*., 2017) (Alshehri, 2020). Some bioactive constituents derived from medicinal plants have shown strong inhibition against major human infections such as: Herpes viruses, Hepatitis B and C viruses, Dengue viruses, and HIV (Rebensburg *et al*., 2016). Due to the curative nature of medicinal plants they are considered a major source of traditional medicines, modern medicines, nutraceuticals, and pharmaceutical intermediates (Alshehri, 2020).

The curative nature of plant species is greatly attributed to phenolic compounds, which is a diverse form of secondary metabolites consisting of an aromatic ring with one or more hydroxyl groups. Phenolic compounds isolated from plants include simple phenols, lignins and lignans, flavonoids, xanthones, tannins, and coumarins (Anantharaju *et al*., 2016). Several studies have revealed the correlation between the increase in the activity of the compound with the increase in the number of hydroxyl group. This has been reported for anticancer activity (Anantharaju *et al*., 2016), anti-HIV activity (Denaro *et al*., 2020) as well as radical scavenging activity (Oksana *et al*., 2012). Phenolic compounds have the potential to combat various diseases that are associated with oxidative stress (Anantharaju *et al*., 2016). With *F. multiflora* belonging to a plant family that has been reported to be a rich source of phenolic compounds (Ekalu *et al*., 2019) (Fechine *et al*., 2020) it is hoped that *F. multiflora* will exhibit inhibitory activity towards HIV and/or cancer.

2.6.1 Natural products with anti-HIV-1 activity

The accessibility of antiretrovirals in developing regions like sub-Saharan Africa has been regulated by economic, political, and commercial factors. These barriers together with the limitations brought by HAART have heightened the incidences of HIV-1 resistance and the transmission of HIV-1 resistant strains among individuals (Kurapati *et al*., 2016). There is therefore an urgent need to search for alternative regiments for developing regions and plant-derived natural products have presented themselves as a promising effective and affordable therapeutic agent towards HIV-1 (Salehi *et al*., 2018). Plant-derived natural products have long-standing recognition as great sources of anti-HIV-1 therapies, as some have been found to exhibit good inhibitory activity against several HIV-1 processes that include viral entry, reverse transcription, replication, integration, virus maturation, and virion budding (Han *et al*., 2011). The standard screening procedure of plant materials commences with *in vitro* analysis of crude extracts and a good fraction of extracts have demonstrated inhibitory activity against HIV-1, proving to be potential agents for the development of antiretrovirals.

Plant extracts have demonstrated anti-HIV-1 activity targeting different stages of the replication cycle. Some of these examples include *Hemidesmus indicus* extracts and *Punica granatum* leaves, bark and peel extracts which exhibited inhibition against HIV-1 RT (Salehi *et al*., 2018) (Sanna *et al*., 2021). *Ocimum labiatum* and *Rhus paviflora* extracts showed inhibitory activity against HIV-1 PR (Kapewangolo *et al*., 2017) (Modi *et al*., 2013). Mode of action studies revealed that *Cistus incanus* (Ci) (Pink Rockrose) participate in viral entry inhibition by targeting the viral envelope proteins, thus preventing the primary attachment of the virus to host cells. Ci extracts also demonstrated broad inhibition against different isolates of HIV, which include a clinical virus isolate that has been found to exhibit multiple resistance against antiretrovirals (Rebensburg *et al*., 2016).

As plants are further being screened, there is a gradual increase of plant isolated compounds and their derivatives being discovered. These compounds have demonstrated inhibitory activity against HIV-1 and other neurocognitive disorders as a result of bioactive constituents contained in plants (Kurapati *et al*., 2016) (Subramaniam *et al.*, 2019). A variety of bioactive compounds including coumarins, terpenoids, xanthones, flavonoids, alkaloids, and polyphenols with anti-HIV-1 RT activity, achieve their ability by resembling nucleoside analogues either in structural shape or form. They seem to be close homologues sharing functional and structural similarities with FDA approved HIV-1 RT inhibitors, NNRTI. Some of these chemical compounds include, 1,5-dicaffeoylquinic acid, apigenin, agathisflavone, artemisinin, amentoflavone, gallic acid,

angoletin, baicalein, ellagic acid, betulinic acid, lupeol, mangiferin, luteolin, oleanolic acid, punicalin, p-cymene, rosmarinic acid, stigmasterol, shikimic acid, ursolic acid, α-bergamotene, xanthohumol and γ-terpinene (Chinsembu, 2019).

Nomilin and limonoid compounds isolated from the *Citrus bergamia* seeds were reported to inhibit the activity of HIV-1 RT. Derivatives of betulinic acid, a triterpenoid isolated from the methyl alcohol extracts of the leaves of *Syzigium claviflorum* together with novel betulinic acid derivatives have been employed as potent anti-HIV agents. These compounds have been in phase I and II studies of anti-HIV drug development strategies. A single oral dose of Bevirimat, a derivative of a betulinic acid-like compound was found to be well tolerated and showed a dose-dependent suppression in viral load (Han *et al*., 2011). Michellamines isolated from *A. korupensis* leaves are atropisomeric alkaloids with great inhibition activity against HIV-1 RT, demonstrated through blocking HIV-1 fusion phase and inhibiting HIV-1 RT mutants. Michellamines also showed inhibition activity against HIV-2 RT that is found particularly around Cameroon (Chinsembu, 2019). Discovering novel anti-HIV-1 targets from natural products derived from plants is an urgent necessity to treat HIV-infected people and to further attack HIV reservoirs to achieve complete viral suppression for the annihilation of HIV/AIDS (Kurapati *et al*., 2016).

2.6.2 Natural products with anticancer activity

The unbearable side effects of non-specific cytotoxicity of conventional cancer drugs and exorbitant costs spent towards treating cancer encourage the search for effective alternative anticancer regiments. Natural products contribute a large fraction of the current pharmacopoeia as they have provided many effective anticancer agents with over 50% of these agents having progressed to clinical trials (Yusmazura *et al*., 2017). If would often make one wonder as to why the search does not cease if a good fraction of natural products has yielded effective anticancer agents. One reason for the continued search is that some natural agents with good inhibition in *in vitro* studies, are found to be inactive in animal studies due to dosage issues (Wang *et al*., 2013), leading to agents not progressing to clinical studies. Therefore, it is imperative to analyse plants which have not yet been screened for anticancer properties, as there is a constant rise in the prevalence of different types of cancers (Lichota and Gwozdzinski, 2018).

Anticancer properties of plants are conferred by bioactive compounds, such as saponins, alkaloids, phenolics, flavonoids, tannins, and other phytoconstituents (Alshehri, 2020). There

has been a continuous rise in the search for natural compounds for anticancer drug development. About 50 000 of natural compounds were discovered in 2006, whereas, in 2014, approximately 326 000 natural compounds were discovered and among these, about 170 000 compounds were not only found to be toxic towards cancerous cells but also towards normal cells (Lichota and Gwozdzinski, 2018) and as a result, those compounds were ruled out. Diverse groups of compounds have shown cytotoxicity properties through various modes of action, such as, tumour cell growth inhibition, DNA damage, apoptosis induction and topoisomerases I and II inhibition (Lichota and Gwozdzinski, 2018).

Poor prognosis of most synthetic cancer drugs have largely initiated the hunt for natural drugs for cervical cancer treatment (Chakraborty, 2019). Many natural compounds have been recognized as rich sources of antioxidants, anticancer agents, or antitumor agents, like paclitaxel. Moreover, natural compounds are considered to be more suitable alternatives in treating cervical cancer as opposed to using platinum-based drugs, which have demonstrated severe side effects (Wang *et al*., 2013). Some novel natural compounds have been reported to exhibit potent anti-cervical cancer activity compared to known conventional agents. Such compounds include tanshinone IIA (Tan IIA), an isolate of *Salvia miltiorrhiza*, Bunge (Danshen) that caused the mitotic cells to move to apoptosis state quicker than taxol or vincristine (Wang *et al*., 2013). Other reports have demonstrated that crude extracts and isolated compounds can possess anti-cervical cancer properties in both *in vitro* and *in vivo* studies. One example is *Justicia spicigera* extracts, which is used for the empirical treatment of cervical cancer in Mexico. Kaempferitrin, a compound isolated from *J. spicigera* extracts, has demonstrated the ability to induce high cytotoxic effects against HeLa cells both *in vitro* and *in vivo*. This compound also demonstrated antitumor properties with preventive effects on the development of tumour (Wang *et al*., 2013).

Extracts found to possess anticancer activity towards multiple cell-lines have been reported, such example is the ethanolic extracts of fresh leaves of *Acacia tortilis* growing in Saudi Arabia, which showed significant cytotoxic activity against kidney carcinoma (HEK-293), human liver cancer (HepG2), and human breast cancer (MCF-7) cell lines. The cytotoxic activity of *A. tortilis* extracts is attributed to phenolic compounds which influence the specific mechanisms, including the cell cycle, cell death, and apoptosis (Alshehri, 2020). Extracts of *Hedyotis diffusa* have been found to possess antitumor properties against cancers of the colon, lung, liver, pancreas, and brain. Another example is with the methanolic extracts of *Morus alba*

leaves, which demonstrated the antiproliferative effect on colon carcinoma, pulmonary carcinoma, and breast adenocarcinoma cell lines (Khan *et al*., 2020).

Plants with the ability to treat several ailments have also been reported, examples include *Ardisia japonica*, which is one of the common traditional medicines used in China. The *A. japonica* has drawn global attention with a multitude of chemical compounds, pharmacological properties, and antibacterial activity. This plant exhibits anticancer properties, anti-HIV activity and is used in the treatment of trauma, pancreatic, bronchitis conjunctivitis, pneumonia and other types of cancer (Chen *et al*., 2018). Lupeol compound isolated from *Guiera senegalensis* induces growth inhibition and apoptosis in liver cancer-HepG2 cell lines, decreases levels of ROS, restores antioxidant enzyme activities in the liver and exerts anti-hepatitis B virus activity (Chinsembu, 2019). Xanthohumol compound isolated from *Humulus lupulus* possesses anticancer properties, drastically reduces oxidative stress, decreases inflammation and regulates some of pathways linked to apoptosis and proliferation (Fernández-García *et al.*, 2018).

Research studies involve harvesting different parts of the plant to explore the diversity of bioactive constituents and their properties. Therefore, the conservation of plants is crucial in ensuring sustainability and to avoid extinction. It is important for the body of research to not only be concerned about exploring plants for their useful properties but to also be environmentally conscious so as not to exploit plant materials. Lichota and Gwozdzinski (2018) elaborated on different methods which have been developed to produce paclitaxel without having to collect from natural products, one method involves plant cell fermentation. Another method involves the production of paclitaxel from Taxus, which unfortunately was not environmentally sustainable and not economically feasible. However, one method of synthesizing paclitaxel 10-deacetylbaccatin III precursors through the bioengineering sector proved to be more effective and feasible. Docetaxel and paclitaxel are widely used as monotherapies and in combination with other drugs that have the antiproliferative ability towards cancer cells, as well as drugs that participate in cell apoptosis. Therefore, it is necessity to advance techniques associated with the isolation of compounds from natural products to attain higher yields and still be conscious about the environment (Lichota and Gwozdzinski, 2018).

2.7 *Fockea multiflora*

This study investigated a plant from the *Fockea* genus, belonging to the family of Apocynaceae under the subfamily of Asclepiadoideae (Thiede *et al*., 2010). Apocynaceae represent a large family characterized by great morphological variability of flowering plants that includes vines, herbs, shrubs, stem and succulents (Alshehri, 2020). Due to the large number of secondary metabolites isolated from different parts of the plant of the family representatives, Apocynaceae presents itself as an important source of bioactive compounds. These compounds include: alkaloids, cardioactive and cyanogenic glycosides, saponins, tannins, coumarins, triterpenoids and phenolic acids (Fechine *et al*., 2020). *Calotropis procera*, one of the family representatives, has been reported to possess biological activities which include antibacterial, anti-inflammatory, analgesic, anticancer, antioxidant, purgative, anthelmintic, expectorant and sedative (Ekalu *et al*., 2019) (Fechine *et al*., 2020). A plant of the same genus *Fockea angustifolia* was reported to be rich in saponins, flavonoids, coumarins and was found to have anticancer properties (Stuurmann *et al*., 2016).

Fockea multiflora is commonly known as the python vine, elephant vine, python kambro, pythonrankle, pythonwein or the creeper (Thiede *et al*., 2010) (www.worldofsucculents.com). The existing information on *F. multiflora* has been retrieved from phylogenic studies. The functions of *F. multiflora* ranges from treating backaches, ornamental purposes, edible leaves and poisoning large predators (tropical.theferns.info/viewtropical.php?id=Fockea+multiflora). *F. multiflora* never appears to grow independently, as branches are found mostly twisting around other trees or rarely forming a self-supporting crown (Thiede *et al*., 2010). The vine can reach up to 10 - 15 meters and tubers are about half a meter in diameter as shown in **Figure 6** (www.worldofsucculents.com).

Figure 6| ***Fockea multiflora*** **plant** (Photo by Bart Wursten)
https://worldofsucculents.com/fockea-multiflora-python-vine/

F. multiflora grows in arid regions of southern Africa, Namibia, Angola, Botswana, Zambia, Zimbabwe, Mozambique, Malawi and Tanzania (Thiede *et al*., 2010), making it available to the large sub-Saharan population living with HIV prevalence. As of 9th of November 2022, there was no known scientific research conducted on *F. multiflora* as queried on Google Scholar, MDPI, Hindawi, Science Reports and PubMed. This study aimed at investigating the anti-HIV, anti-cancer and antioxidant activity of root and leaf extracts of *F. multiflora*.

2.8 Hypothesis

Based on the above, the hypothesis for the study was:

H_1: *Fockea multiflora* extracts should inhibit HIV-1 enzyme activity *in vitro* and exhibit additional anti-proliferative and antioxidant abilities.

H_0: *Fockea multiflora* will not inhibit HIV-1 enzyme activity in vitro nor exhibit additional anti-proliferative and antioxidant abilities.

2.8.1 Aims

The aim of the study was to investigate the bioactivity of roots and leaves of *Fockea multiflora* extracts against the activity of HIV enzymes as well as the proliferation of cancer cells and for antioxidant activity

Objectives

The objectives followed to achieve this aim were to:

- Evaluate the cell cytotoxicity 50% (CC_{50}) of *Fockea multiflora* extracts towards PBMCs and TZM-bl cell lines using MTT and Neutral Red Uptake.
- Investigate the inhibitory effect of *Fockea multiflora* extracts on HIV-1 Reverse Transcriptase and Protease.
- Determine the anti-proliferative effect of *Fockea multiflora* extracts in cancer cell lines using MTT and real-time cell analysis (RTCA).
- Evaluate the antioxidant ability of *Fockea multiflora* extracts through the nitric oxide scavenging assay.

3. MATERIALS AND METHODS

Extracts of *F. multiflora* were obtained from Dr Nanhapo from the University of Namibia and section **3.1** and **3.2** elaborates how the collection and extraction process was conducted.

3.1 Sample collection

Prior to collection, the plant was identified and authenticated as *F. multiflora*. Then the leaves and roots were collected from Ondeihaluka village in Ohangweni region. Collected samples were washed with distilled water, air dried and ground into fine powder using a blender.

3.2 Extracts Preparation

Leaves and roots of *F. multiflora* were extracted with chloroform, diethyl ether and distilled water. A hundred grams (100 g) of ground powder samples of *F. multiflora* were extracted with 400 mL of each solvent for 24 hours with no heat applied. The filtrates extracted with diethyl ether and chloroform were each concentrated using rotary evaporator at 30˚C to remove the solvent through a process known as lyophilization which gently freezes the product. The water filtrate was subjected to a freeze-dryer in a high-pressure vacuum to extract water in a form of vapour. The concentrated filtrates were dried at room temperature in the fume hood. These extracts were shipped to our lab in a form of sticky pastes in sealed tubes where they were stored at 4˚C (Ingle *et al*., 2017) (Tuama and Mohammed, 2019). *F. multiflora* extracts were weighed and dissolved in DMSO (see calculations in **Appendix II, Formula 1**) then extracts were spun in a vertex and kept in -20˚C. The amount of DMSO used to dissolve the extracts was too negligible for DMSO to be featured in experiments as a vehicle control. **Figure 7** shows the abbreviated version of *F. multiflora* extracts and the solvents used during extraction.

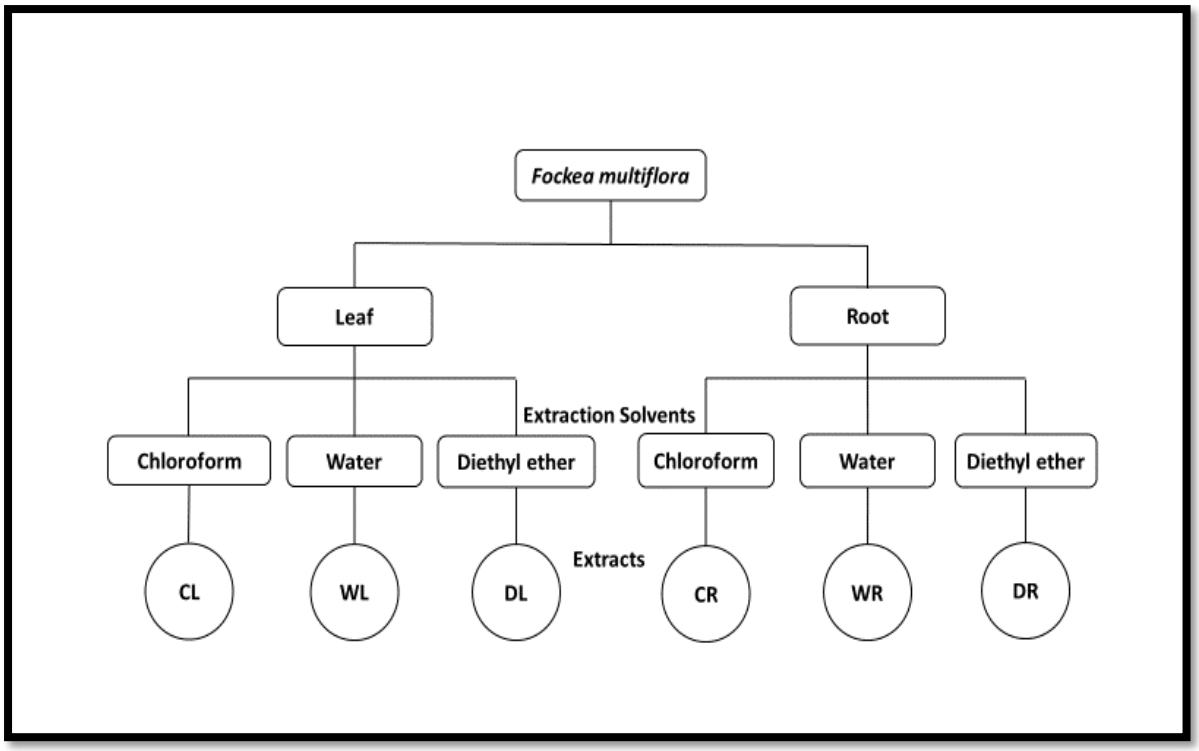


Figure 7| **Diagrammatic scheme showing the extraction of *F. multiflora***. The first letter of the solvent and part of the plant are being used as abbreviations representing each extract.

The choice of solvents used for this study was based on the selectivity triangle for solvents by Snyder and Kirkland (1974) published in the book titled "Introduction to Modern Liquid Chromatography". The selectivity triangle organizes various solvents into groups (I – VIII) of clusters of similar selectivity, this aids in selecting the appropriate solvent to use for the chromatography (**Figure 8**). The further apart the choice of solvent to be used for the sample extraction process, the better the comparable selectivity and the stronger the attraction strength of the solvent and sample molecules (Johnson and Vitha, 2011).

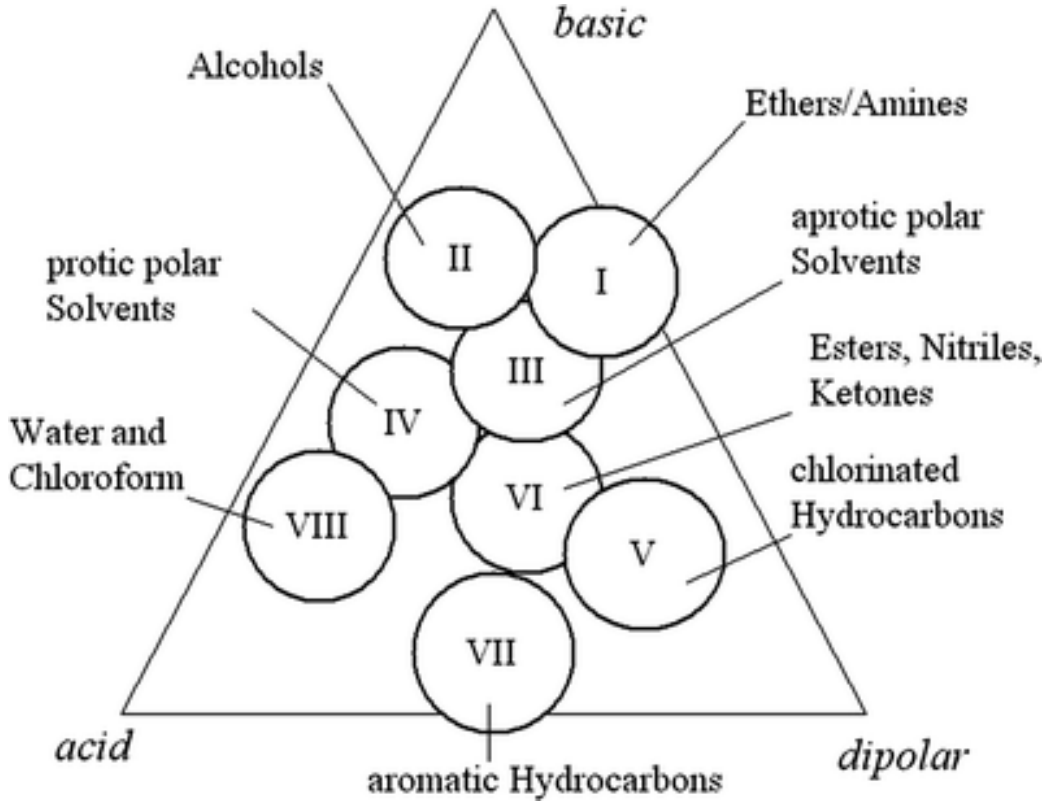


Figure 8| Selectivity solvent triangle by Snyder and Kirkland. The triangle organizes solvents into groups of similar selectivity. Source: "Quantitative Thin-Layer Chromatography" A Practical Survey. Authors: B. Spangenberg, C.F. Poole and C. Weins. 2011 (pp 81-103).

The chosen solvents for the extraction of *F. multiflora* being chloroform (is an acid donor solvent found in group VIII) and diethyl ether (is a basic acceptor solvent found in group I) are far apart from each other and this guarantees the highest difference in selectivity.

3.3 Cell Viability Assay

Cell viability refers to the number of healthy or live cells in a sample. The assays for cell viability or cytotoxicity are used to test the toxicity of a drug, compounds, extracts and/or chemical agents in different cell lines (Kamiloglu *et al*., 2020). When the tested substance is said to be cytotoxic, it means that the substance has interfered with either the attachment, growth, proliferation or caused death of the cells (Mensah *et al*., 2019). Several cell viability assays are based on various functions, which include enzyme activity, cell adherence, cell membrane permeability, adenosine triphosphate (ATP) production, co-enzyme production, and nucleotide uptake activity (Kamiloglu *et al*., 2020). The MTT and neutral red uptake (NRU) assay were the chosen assays for this study.

3.3.1 Media preparations and cell culture

Two types of media were used for this study, the first being Roswell Park Memorial Institute (RPMI) 1640 and Dulbecco's modified eagle's medium (DMEM). The RPMI media was used for the isolation and culturing of PBMCs and DMEM media was used for the culturing of TZM-bl, HeLa, HepG2 and Vero cells. RPMI media consisted of RPMI powder L-glutamine, sodium bicarbonate, gentamycin 50 µg/mL, antibiotic/antimycotic solution 100X [penicillin 10 000 U/mL; streptomycin 10 000 µg/mL; amphotericin B 25 µg/mL] in $dddH_2O$ at pH 7.2 filtered through a 0.2 µm stored at 4˚C. DMEM media consisted of DMEM powder with L-glutamine, sodium bicarbonate powder, sodium pyruvate 100 mM, HEPES buffer 1M and gentamycin 50 µg/mL in $dddH_2O$ at pH 7.2 filtered and store in the same manner as RPMI. Thawing media (20% media) was prepared from plain media by adding 20% foetal bovine serum (FBS) with 1% antibiotic/antimycotic solution. Culturing media (10% media) was prepared by adding 10% FBS with 1% antibiotic/antimycotic solution.

The TZM-bl was the first adherent cell line used in this study, it is derived from a HeLa cell clone engineered to express CD4, CCR5 and CXCR4. The cells are highly permissive to infection by most strains of HIV, SIV and SHIV, including primary or molecularly cloned viral isolates (Sarzotti-Kelsoe *et al*., 2014). The TZM-bl cells were thawed with 20% DMEM media, then centrifuged at 1200 rpm for 5 minutes. After centrifugation, the supernatant was discarded, and the cells were resuspended with thawing media then transferred into a 72 cm^2 tissue culture sterile flask with thawing media. The flask was incubated at a 90% humidified atmosphere of 5% CO_2 at a temperature of 37°C. The following day, media was changed to 10% culturing media. Cells were monitored each day and the cells were trypsinized upon reaching ~80% confluency. Media in the flask was completely removed, the cells were washed with 10mL of 1% phosphate buffer saline (PBS). Trypsin (Sigma (3 mL) was used to remove cells from the flask, this was achieved by allowing the cells to rest in trypsin for 30 seconds followed by discarding of trypsin and incubation of the flask for 3 minutes at 90% humidified atmosphere of 5% CO_2 at a temperature of 37°C. The DMEM media (10%) was added into the flask to halt the trypsinization process, the mixture was transferred to a falcon tube and centrifuged at 1200 rpm for 5 minutes. The supernatant was discarded, cells were resuspended with 10% DMEM to a concentration of 1 x 10^5 cells/mL. The cells were counted using a haemocytometer and a dilution of 10x cells and trypan blue. Then the cells were seeded at a concentration of 1 x 10^5 cells/mL in a 96-well plate overnight, this is done only on adherent cells to allow for the attachment of cells to the base of the wells. On the following day, the cells were treated with a

two-fold serial dilution of *F. multiflora* extracts of 250, 125, 63, 31, 16, 8 and 4 µg/mL. Treated cells were plated alongside media containing wells and wells containing untreated cells and plates were incubated for 72 hours at a 90% humidified atmosphere of 5% CO_2 at a temperature of 37°C.

This study also investigated Peripheral blood mononuclear cells (PBMC), suspension cells which are considered as the major cells in the immunity of the human body, as they contain various types of cells like lymphocytes, macrophages, or monocytes. Toxic reactions target PBMCs leading to severe side effects such as, toxicity and suppression of the immune system. The PBMCs are regarded as a significant tool to use for predicting the dosage for new drug compounds. For these reasons, PBMCs form an important tool for researching the toxic nature of chemical compounds and new drugs (Pourahmad and Salimi, 2015). The PBMCs were isolated from healthy donors and maintained in plain and 10% RPMI media.

Ethical approval NAS041/2019 was granted for extracting PBMCs from the blood of consenting healthy donors was obtained. The process was conducted by an occupational nurse at the University of Pretoria, South Africa. Blood diluted with plain RPMI media on a 1:1 ratio was layered on ficoll (Separation Scientific, SA), a hydrophilic polysaccharide that segregates blood layers. A ratio of 2:1 of diluted blood to ficoll was performed, followed by the gradient centrifugation at 3000 rpm for a duration of 30 minutes. This process separates blood into a layer of plasma at the top followed by a layer of PBMCs, a fraction of polymorphonuclear cells (neutrophils and eosinophils) and finally a layer of erythrocytes at the bottom (Pourahmad and Salimi, 2015).

The PBMCs were pipetted into a 50 mL falcon tube then plain media was added to approximately 30 mL and the mixture was centrifuged at 2200 rpm for 10 minutes. Supernatant was discarded, cells resuspended with 5 mL of Ammonium Chloride Potassium (ACK) (NH_4Cl 150 mM, $KHCO_3$ 10 mM and EDTA 0,1 mM at pH between 7.2 – 7.4). The mixture was allowed to rest, after 5 minutes approximately 15 mL of plain media was added to halt the lysing of cells and then centrifugation at 1200 rpm for 10 minutes. The next step was the was the discarding of supernatant and resuspension of cells with 5 mL of 10% RPMI media. The counting of PBMCs was conducted using a haemocytometer and the concentration was adjusted to $1x10^6$ cells/mL for seeding purposes. Phytohemagglutinin (PHA) (4 µg/mL), a lectin responsible for clumping blood cells together, was used to stimulate PBMCs for 2 hours at a 90% humidified atmosphere of 5% CO_2 at a temperature of 37°C. PBMCs were treated

with varying concentrations (250 - 4 µg/mL) of *F. multiflora* extracts. Unlike adherent cells, PBMCs were plated and treated on the same day then incubated for 72 hours. Treated PBMCs were plated alongside wells containing media only and wells containing untreated cells.

3.3.2 MTT Assay

The MTT assay is a colourimetric test for cell proliferation and survival, which was developed by Mosmann in 1983 and adapted by Cole in 1986 for measuring the chemosensitivity of human lung cancer cell lines. The assay is based on the conversion of MTT into formazan crystals (**Figure 10**) by the dehydrogenase system of metabolically active cells. The intracellular reducing power is mainly provided by NAD(P)H which is derived from dehydrogenase activity in mitochondria, endoplasmic reticulum, and plasma membrane (Chan *et al*., 2015) (Kamiloglu *et al*., 2020).

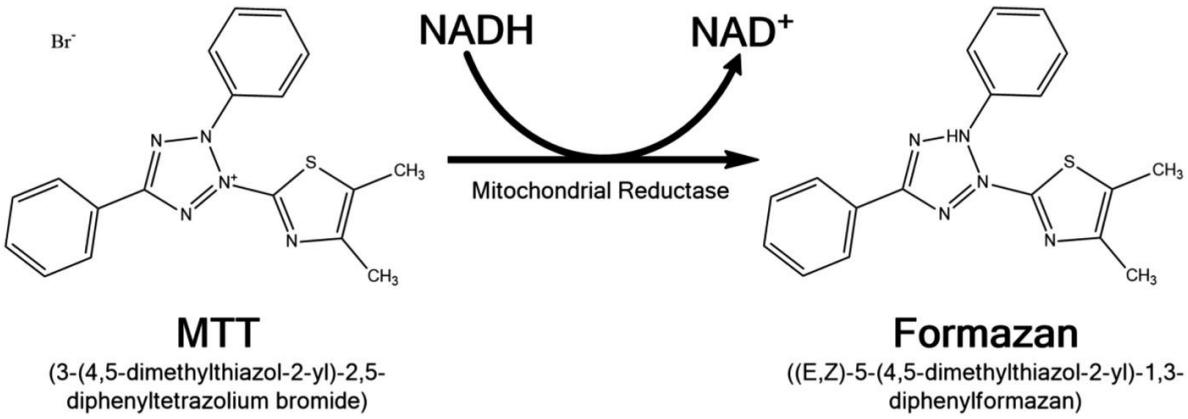


Figure 10| The conversion of MTT to formazan crystals. The yellow tetrazole water-soluble salt, MTT is reduced to a purple formazan salt by succinate dehydrogenase (Kamiloglu *et al*., 2020).

Method: Post treatment with the various extracts, the 96-well plates were centrifuged at 1200 rpm for 5 minutes after 72 hours of incubation and 160 µL was removed from each well to make room for the MTT solution that is to be added. MTT working solution was prepared by mixing 5 mg/mL of MTT and 10% media in a ratio of 1:5 [DMEM (for adherent cells) or RPMI (for suspension cells)] and 100 µL of the working solution was added to each well in the dark as MTT is light sensitive. The plates were incubated overnight covered with foil at a 90% humidified atmosphere of 5% CO_2 at a temperature of 37°C. The insoluble purple formazan crystals were solubilised by adding 100 µL of acidic isopropanol to each well. The solubilisation solution was prepared by adding 1mL of 1M HCl to 9mL of isopropanol. The plates were allowed to rest for 15 minutes at room temperature then absorbance was read at

540 nm, with a reference wavelength of 690 nm. Then the effects of *F. multiflora* extracts on the cell percentage viability was calculated (**Appendix II, Formula 4**).

Although MTT assay is a simple, rapid, economic, sensitive, and accurate test to determine *in vitro* deleterious effects of several substances (Figueiró *et al*., 2017) and the most used assay in our laboratory. However, due to the interferences between the reducing agents of the extracts and MTT indicators, the Neutral Red Uptake (Abcam, UK) assay had to be considered. The interference was possibly due to extracts reacting chemically with the MTT indicator and this is eliminated in NRU assay as the protocol involves the washing of cells before and after the dye is added (Chan *et al*., 2015).

3.3.3 Neutral Red Uptake Assay

The assay developed by Borenfreund and Puerner (1984) is also one of the most used colourimetric cytotoxicity assays. NRU is based on the ability of viable cells to take up the supravital dye, neutral red by non-ionic passive diffusion and retain it in the lysosomes. The incorporated dye is then extracted from the viable cells using an acidified solution (Aslantürk, 2018). The NRU assay is sensitive and readily quantifiable as the neutral red dye does not accumulate in the lysosomes of dead or damaged cells (Chan *et al*., 2015). NRU depends on the capacity of cells to maintain pH gradients through ATP production. The net charge of the dye is zero at physiological pH and this enables the dye to penetrate the cell membranes. Inside the lysosome there is a proton gradient to maintain a pH lower than that of the cytoplasm. The dye trapped by the lysosome becomes charged and is unable to leave the interior of the organelle. When the cell dies or pH gradient is reduced, the dye cannot be retained, therefore, damaged lysosomes decrease the amount of dye retained by the culture. The integrity of lysosomal is a highly sensitive indicator of cell viability. The assay can quantitate cell viability and measure cell replication, cytostatic effects or cytotoxic effects depending on the seeding density (Aslantürk, 2018) (Figueiró *et al*., 2017).

Method: The same culturing and plating method described in section (**3.3.1**) was performed in this assay. After 72 hours of incubation, the 96-well plates were centrifuged at 1200 rpm for 5 minutes and culture media was gently removed. Then the cells were washed with 200 µL of 1X washing solution (1-part 10X washing solution and 9-parts distilled H_2O). The washing process was carefully conducted to avoid disturbing the cell monolayer. The washing solution was gently removed and 150 µL of neutral red staining solution (1-part 100X neutral red staining solution to 99-parts cell culture medium) was added to each well in the dark and

incubated for 2 hours at 90% humidified atmosphere of 5% CO_2 at a temperature of 37°C. After incubation, the staining solution was completely removed and 250 µL of 1X washing solution was used to wash the staining solution, then the solution was gently removed. 150 µL of 1X solubilization solution (2X solubilization solution diluted 2-fold using 100% methanol) was added to each well. The plates were allowed to rest at room temperature and the absorbance was read as described for the MTT assay.

3.4 HIV-1 Enzyme Inhibition

3.4.1 HIV-1 Reverse Transcriptase Inhibition Assay

The assay is based on the *in vitro* ability of RT enzyme to synthesize DNA by utilizing the hybrid poly (A) x oligo $(dT)_{15}$ as a template and primer. The use of [^{3}H]- or [^{32}P]-labelled nucleotides is evaded, as the activity of RT incorporates digoxigenin- and biotin-labelled nucleotides in DNA molecule. **Figure 9** depicts a sandwich-like protocol that entails biotin-labelled DNA binding to the surface of streptavidin-coated microplate modules. In the next step, an antibody to digoxigenin which is conjugated to peroxidase (anti-DIG- POD) binds to the digoxigenin-labelled DNA. This is then followed by the addition of the peroxidase substrate [2,2′-azino-bis-(3-ethylbenzthiazoline-6-sulphonic acid)] ABTS leading to the substrate being cleaved by the peroxidase enzyme, thereby producing a coloured reaction product. The detection and quantification of the synthesized DNA as a parameter for RT activity can be determined using a microplate reader, where the absorbance of the samples directly correlate to the level of RT activity (www.sigmaaldrich.com).

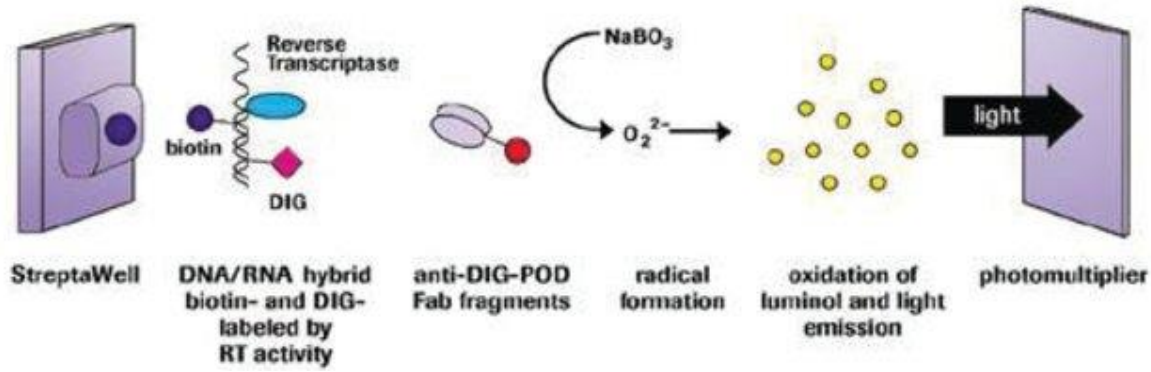


Figure 9| HIV-1 Reverse Transcriptase inhibition assay. Elaborating a sandwich-like protocol for the detection and quantification of the synthesized DNA (www.sigmaaldrich.com).

Method: The anti-HIV-1 RT activity of *F. multiflora* extracts was assessed using a colourimetric RT assay kit (Roche Diagnostics, Germany). The assay was performed according to the manufacturer's instructions and as previously described by (Kapewangolo *et al*., 2013).

F. multiflora extracts initially dissolved in DMSO were tested at three concentrations (10, 25 and 100 µg/mL). Each concentration of crude extracts diluted with lysis buffer (20 µL) was added in microtubes, 20 µL of reaction mixture [comprising of template/primer hybrid poly (A) x oligo $(dT)_{15}$ reconstituted with redistilled water and nucleotide diluted with incubation buffer (Tris-HCl 46 mM, with DDT, biotin-dUTP and dTTP)] and lastly, 20 µL of recombinant HIV-1 RT enzyme (2 ng/µL). Lysis buffer with reaction mixture without HIV-1 RT enzyme was used as the negative control. A microtube for the positive control was prepared, which consisted of the reaction mixture, lysis buffer and the enzyme but lacked any inhibitor. A preparation of the known inhibitor control was conducted and it consisted of reaction mixture, enzyme and doxorubicin (50 µg/mL) a known HIV-1 RT inhibitor (Kapewangolo *et al*., 2013). Properly labelled microtubes were incubated at 37˚C for one hour. Microplate modules precoated with streptavidin were properly inserted into the strip frame and after incubation, the samples were transferred into microplate modules and covered with a foil then incubated for another hour at 37˚C.

The solution was discarded completely, microplate modules were rinsed five times with 250 µL of washing buffer and modules were dried properly. Then 200 µL of anti-DIG-POD working dilution was added (200 mU/mL) and modules were covered with foil and incubated for an hour at 37˚C. The solution was discarded, and the rinsing process was done as previously described. The ABTS substrate solution was prepared immediately before use, where one tablet of ABTS was dissolved in 5 mL of substrate buffer. Then 200 µL of ABTS substrate solution was added and incubated at room temperature away from light until the green colour necessary for the photometric detection had developed (10 - 30 min). The absorbance was read at 405 nm (reference wavelength of 490) using SpectraMax® Paradigm® Multi-Mode Detection Platform plate reader (Molecular Devices, USA). The percentage inhibition of *F. multiflora* extracts was then calculated (**Appendix II, Formula 2**).

3.4.2 HIV-1 Protease Assay

The assay is based on the *in vitro* ability of HIV-1 PR enzyme to cleave HIV protease substrate-1 (Sigma Aldrich, USA), a synthetic peptide sequence: Arg-Glu(EDANS)-Ser-Gln-Asn-Tyr-Pro-Ile-Val-Gln-Lys(DABCYL)-Arg. The substrate contains the cleavage site (Tyr-Pro) with two covalently modified amino acids for the detection of the cleavage. The first modification that was performed on glutamic acid residue involves addition of a fluorescent chemical compound EDANS, 5-(2-aminoethylamino)-1-naphthalene sulfonate. The second modification performed on lysine residue involves an acceptor chromophore DABCYL, 4'-

dimethylaminoazobenzene-4-carboxylate. The modified amino acids are positioned on opposite sides of the cleavage site. During spatial orientation, the energy emitted from EDANS is quenched by DABCYL. However, when the peptide is cleaved by the HIV protease, the DABCYL is no longer proximal to EDANS and emission at 490 nm can is then detected (www.sigmaaldrich.com).

Method: The prepared protease assay buffer comprised of 1 M sodium chloride, 0,1 M sodium acetate, 1 mg/mL BSA, and 1 mM EDTA dissolved in redistilled water at pH 4.7. *F. multiflora* extracts having been previously dissolved in DMSO were diluted with protease assay buffer and tested at three concentrations (10, 25 and 100µg/mL). The protease substrate reconstituted in DMSO to a concentration of 1000 µg/mL was diluted with protease assay buffer to 10 µg/mL and shielded from light. The protease enzyme (Bachem, Switzerland) was diluted in assay buffer to a final concentration of 1 µg/mL. was thawed in ice and diluted with protease assay buffer to a concentration of 1 µg/mL. The known HIV-1 protease inhibitor, acetyl pepstatin (AP) (Sigma, Germany) at 10 µg/mL was used as a control. **Table 1** depicts the plate layout of the reaction performed in a black costar 96-well plate, which included the background wells with extracts and protease assay buffer.

Table 1| HIV-1 PR inhibition assay plate layout

	Negative control/Blank (µL)	**Untreated positive control (µL)**	**Positive control (known inhibitor) (AP-10 µg/mL)**	**Samples conc. (µg/mL)**		
				100 µg/mL	**25 µg/mL**	**10 µg/mL**
Protease assay buffer	51	49	39	48.5	36.8	44
Enzyme		2	2	2	2	2
Substrate	49	49	49	49	49	49
Inhibitor			10	0.5	12.3	5
Final volume	100	100	100	100	100	100

The addition of HIV protease substrates was performed in the dark and once all reagents were added the plate was covered with foil and incubated at 37˚C for one hour. The fluorescence was measured at wavelength settings of the spectrofluorometer detection of 355 nm excitation and 460 nm emission using SpectraMax® Paradigm® Multi-Mode Detection Platform plate

reader (Molecular Devices, USA). The percentage inhibition of *F. multiflora* extracts was then calculated (**Appendix II, Formula 2**).

3.5 Anticancer Assay

3.5.1 Through Cell Viability Assay

The anticancer profile of *F. multiflora* was first evaluated in HeLa and HepG2 cells using cell viability assays. HeLa is a cervical cancer cell line derived from a biopsy of a cervical tumour taken from Henrietta Lacks, an African American woman. Henrietta's cells were taken in 1951 became the first human cells to grow well in a lab. HeLa cell line contributed to the development of a polio vaccine, the discovery of human telomerase and other advances (Callaway, 2013). HepG2 is a human liver cancer cell line derived from the liver tissue of a 15-year-old Caucasian male with hepatocellular carcinoma. HepG2 cells display morphological and functional differentiation *in vitro* (www.phe-culturcollections.org.uk).

The NRU assay described in section **3.3.3** was first conducted in HeLa and HepG2 cells, the assay proved not to work with these cancer cell lines as ambiguous data was obtained. Numerous failed attempts (**Appendix IV**, **troubleshooting 1**) with the anticancer evaluation of *F. multiflora* then led to MTT assay (described in section **3.3.2**) with little modification. This involved the washing of cells with 1X PBS before and after adding the MTT dye, adopted from section **3.3.3**. The modified MTT assay worked well with these cancer cell lines. With all the attempts made with this evaluation using cell viability assay another assay had to be selected to confirm the data obtained. Therefore, real-time cell analysis (RTCA) was chosen as a confirmatory assay.

3.5.2 Through Real-Time Cell Analysis

The RTCA is based on monitoring the behaviour of cells when an electric current is applied through the microelectrodes embedded at the base of the E-plates. This technique was identified by Giaever and Keese in 1984. The impedance of electrons in RTCA offers quantitative information about the viability, number, migration, and morphology of the cells (Şener *et al*., 2017). This special feature of RTCA overcomes the limitation posed by endpoint assays of not revealing cellular changes between time points throughout the experiment (Stefanowicz-Hajduk and Ochocka, 2020). The RTCA technique is advantageous in the sense that it is a dynamic, label-free, real-time, and cell-based microelectronic biosensor system with numerous applications in drug development strategies, cancer research, toxicology, virology, and microbiology (Şener *et al*., 2017).

Before cells are added, electric current flows freely through the culture medium in an RTCA system. Current flow is impeded once cells begin to attach and proliferate and this action permits the system to provide a sensitive readout of cells. Adherent cells insulate the surface of the electrodes, alter the ionic medium of the electrode solution, thereby increasing the impedance. The rate of cell proliferation and adhesion is directly proportional to the rate of impedance (Şener *et al*., 2017). The values of impedance are expressed as the Cell Index (CI), which is regarded as a measure of adhesion. In the absence of cells, the CI is zero and as cells start to adhere, the CI increases the greater the Cell Index values, the greater the level of adhesion (**Figure 11**). Conversely, when the CI decreases, the net adhesion also decreases (Kho *et al*., 2015).

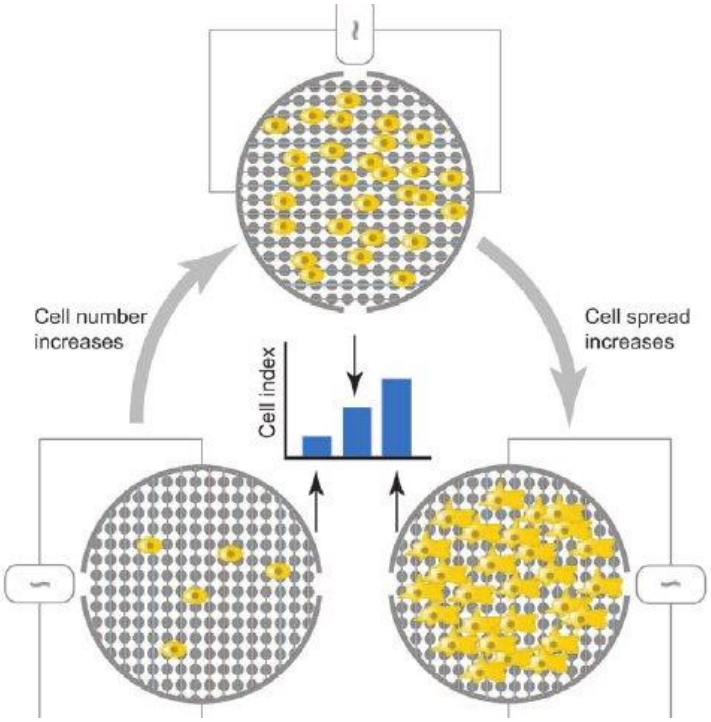


Figure 11| RTCA impedance-based detection of cell viability. The well from the left depicts cells starting to adhere which increases the cell number thereby increasing the CI value (Limame *et al*., 2015).

The software data is generated and collected as the experiment is running, this ensures that the effect of the tested sample in cells is always monitored throughout the experiment. The advantage of the RTCA system over MTT is that the system generates a full proliferation profile in real time in a single experiment and allows for the determination of IC_{50}/EC_{50} values. These advantages of RTCA assist in better understanding the toxicity profiles which helps in selecting the best compound candidates in early drug development before *in vivo* studies

(Stefanowicz-Hajduk and Ochocka, 2020). Therefore, any response that induces a change in cell morphology can be investigated using xCELLigence technology (Kho *et al*., 2015).

In this study, the RTCA was performed in HeLa and HepG2 for further assessment of the potential antiproliferative activity of *F. multiflora* extracts. As some anticancer drugs have originated from plants, this has led to an increase in anticancer research being directed towards plant-derived natural products. Therefore, investigating the anticancer effect of *F. multiflora* extracts may be useful in contributing to the treatment of cervical and liver cancer.

Method: The assay was conducted according to the manufacturer's instructions (Roche Diagnostics, Germany). Briefly, culture medium (100 µL) was added onto the 16-well E-plates and the plates were inserted in the xCELLigence instrument for the background reading to be recorded. Titration was conducted on both cell-lines, where varying concentrations of cells (50 000, 40 000, 30 000, 20 000, 10 000, 5 000, 2 500 cells/mL) were seeded and 30 000 cells/mL was selected as an ideal seeding concentration for HeLa cells and 20 000 cells/mL was selected for HepG2 cells. Cell count was then conducted as previously described in the MTT assay and the cells seeded overnight. On the following day, the cells were treated with 100, 50 and 25 µg/mL of *F. multiflora* extracts and 10 µg/mL of cisplatin was used as a positive control for antiproliferation. Media containing wells were included as negative controls. The effect of *F. multiflora* extracts on the proliferation of HeLa and HepG2 cells was monitored in real-time for 3 days.

3.6 Nitric Oxide Assay

Free radicals are highly reactive species, essential for the maintenance of normal physiological function. They partake in reactions of radical addition, hydrogen abstraction, bond scission as well as annihilation reactions targeting the macromolecules like proteins, lipids, and DNA. Antioxidants are necessary for the prevention of the oxidative stress that occurs naturally in tissues by chelating metal ions, quenching free radicals and acting as electron donors. Therefore, it is crucial to investigate naturally occurring antioxidants of plant origin, which could contribute in the development of novel drugs and reduce the susceptibility of chronic diseases (AsokKumar *et al*., 2009). Because nitric oxide (NO) has a short half-life of a few seconds or less *in vivo*, the Griess Reaction measures the accumulating nitrite, which is an indicator of NO in the supernatant. The Griess reaction (**Figure 12**) involves the two-step diazotization reaction in which acidified NO_2^- produces a nitrosating agent, which reacts with an aromatic amine, sulfanilic acid to produce the diazonium ion. This ion is then coupled to N-

(1-naphthyl) ethylenediamine to form a highly coloured diazo dye, the chromophoric azo-derivative. Levels of NO are indirectly measured by converting nitrate (NO_3^-) to nitrite (NO_2^-) through the action of nitrate reductase. Nitrite is then detected colourimetrically as an azo dye product of the Griess Reaction (Njoya *et al*., 2017) (Skouta and Mor, 2018) (Váradi *et al*., 2019).

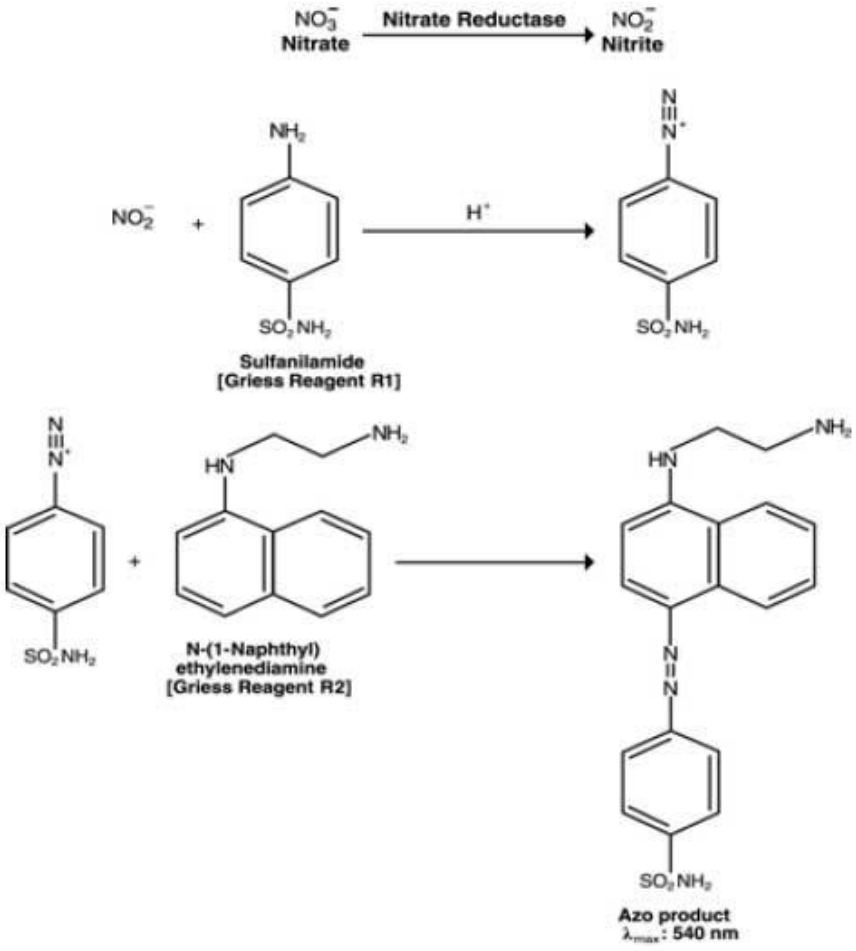


Figure 12| The Griess Reaction depicting nitrate being converted to nitrite by nitrate reductase. This reaction involves the diazotization reaction where a nitrosating agent produced by the acidified NO_2^-, reacts with sulfanilamide which is then combined with N-(1-naphthyl) ethylenediamine to produce the chromophoric azo-derivative (Titheradge, 1998).

Method: A protocol described by (Wu and Yotnda, 2011) was modified for this assay. A protocol obtained from (www.promega.com) for the determination of the Nitrite Standard Reference curve was also adopted for this assay. Nitric oxide production by HepG2 and HeLa cells were detected by measuring the nitrite accumulated in the supernatant that was treated with and without *F. multiflora* extracts and ascorbic acid which was used as a positive control. HepG2 and HeLa cells were seeded at a density of 1×10^5 cells per well in 96 well plates as

described in section (**3.3.1**). The supernatant (50 µL) from each well was transferred into new 96-well plates. Griess reagents were prepared from scratch for each conducted experiment as reagents are to be used within 8 hours to avoid oxidation.

The standard stock of sodium nitrite 0.1M was diluted to 100µM in DMEM media in a ratio of 1:1000, then a serial dilution in the same medium was prepared. A nitrite standard reference curve was prepared for each experiment to ensure accurate NO_2^- quantitation. Three columns for the Nitrite Standard reference curve were designated in a new 96-well plate (with 50 µL of supernatant), where 50 µL of DMEM media was dispensed into the wells in rows B–H. A 100µl of nitrite solution (100µM) was added in row A, then two-fold serial dilutions were performed (50µL/well) in triplicate to generate the Nitrite Standard reference curve (100, 50, 25, 12.5, 6.25, 3.13 and 1.56µM). The last row contained media only. The final volume in nitrite standard reference curve wells was 50µL. Another set of 3 columns was designated for ascorbic acid, a positive control for antioxidant chosen for this assay. Ascorbic acid dissolved in DMSO (20 000 µg/mL) was diluted with DMEM media to make two-fold serial dilutions in triplicate of concentrations 250, 125, 63, 31, 16, 8 and 4 µg/mL.

Griess reaction allows for the sulfanilamide and N-1-naphthylethylenediamine dihydrochloride (NED) to compete for nitrate as described above, and when the two components are added sequentially greater sensitivity is achieved. Therefore, sulfanilamide solution (50µL) was dispensed to all wells including those containing the dilution series for the Nitrite Standard reference curve and ascorbic acid. Then NED Solution (50µL) was immediately dispensed to all wells. The plates were incubated at room temperature for 10 minutes, shielded from light. A magenta colour was immediately observed in the highest concentrations of nitrite standard reference curves. The absorbance was read within 30 minutes at 550 nm on a microplate reader. The nitrite quantity was determined from a sodium nitrite standard curve. The nitrite standard reference curve was developed by plotting the average absorbance values of the Nitrite Standard concentrations as a function of "Y" with nitrate concentrations as a function of "X". Then the average absorbance value for extracts as well as their concentrations were determined by comparing with the nitrite standard reference curve.

3.7 Data Analysis

The absorbance values obtained from the spectroscopic evaluation of *F. multiflora* extracts were analysed in Microsoft Excel and formulas are shown in (**Appendix II**). The GraphPad Prism 7,0 software (GraphPad, USA) was used to determine the CC_{50} and IC_{50} values and these

were presented as mean ± standard error. *F. multiflora* extracts were tested in triplicate. The level of significance was estimated using analysis of variance (ANOVA), Dunette's post hoc test in GraphPad Prism 7 to determine *p*-values for the differences observed between *F. multiflora* extracts and positive controls or untreated cells. Results were considered significantly different when $p < 0,05$.

4. Results

For plant crude extracts and/or isolated compounds to be considered as anti-HIV or anti-cancer therapeutic agents, they must undergo screening to elucidate the efficacy and the effective concentrations. This study elucidated the inhibition activity of *F. multiflora* extracts on two of the crucial enzymes which are involved in the HIV-1 replication life cycle, namely, HIV-1 reverse transcriptase and protease. The study also investigated the ability of *F. multiflora* extracts to confer inhibition on the proliferation of the cervical and liver cancer cells with the intent of identifying the anticancer ability of these extracts. Since crude extracts contain various constituents that may interfere with some of the viability assays, therefore, an assay considered to be more ideal for crude extracts had to be employed in this study to draw a better line of conclusion on the results obtained.

4.1 Effects of *F. multiflora* on cell viability and proliferation

Levels of toxicity described by (Mensah *et al*., 2019) were adopted in this study to analyse the cytotoxicity data. Levels describe anything above 50 µg/mL as low cytotoxicity, (30 – 50 µg/mL) as moderate cytotoxicity, (10 – 30 µg/mL) as high cytotoxicity and below 5 µg/mL as very high cytotoxicity. The concentration initially used to test *F. multiflora* extracts in PBMCs obtained from healthy donors was 4000 µg/mL and the two-fold serial dilution of 1000 – 16 µg/mL was used for treatment. However, this concentration did not work as cells appeared to be highly viable in all tested concentrations, then adjustments were made leading to 1000 µg/mL and the serial dilution of 250 - 4 µg/mL being used for treatment. A dose-dependent curve was obtained with the newly adjusted concentration, which was employed for all cytotoxicity analysis in this study.

4.1.1 CC_{50} Values for Extracts

The cytotoxicity concentration of *F. multiflora* extracts in PBMCs and TZM-bl cells were determined using MTT and NRU assay respectively. The obtained CC_{50} values are shown in **Table 3** below. The percentage cell viability graphs for both cell lines are shown in **Appendix I** (**Figure A1** and **A2**).

Table 2| CC_{50} for *F. multiflora* extracts in PBMCs and TZM-bl cells using MTT and NRU respectively. Values are expressed as (µg/ml ± SEM).

***F. multiflora* extracts**	**PBMCs CC_{50} using MTT**	**TZM-bl CC_{50} using NRU**
CR	33.08 ± 0.10	193.1 ± 0.04
DR	47.95 ± 0.26	117 ± 0.03
WR	100.30 ± 0.10	>250
WL	44.34 ± 0.09	>250
CL	12.14 ± 0.12	80.4 ± 0.06
DL	10.39 ± 0.26	68.9 ± 0.07

High cytotoxicity of *F. multiflora* in PBMCs (highlighted in rusty shade) was observed with the leaf extracts DL and CL with CC_{50} values of 10.39 ± 0.26 and 12.14 ± 0.12 µg/mL respectively. Moderate cytotoxicity of *F. multiflora* also in PBMCs (highlighted in peach shade) was demonstrated by extract CR, WL, and DR with CC_{50} of 33.08 ± 0.10, 44.34 ± 0.09 and 47.95 ± 0.26 µg/mL respectively. Low cytotoxicity was demonstrated by extract WR with a CC_{50} of 100.30 ± 0.10 µg/mL. The interaction between MTT and the extracts of *F. multiflora* gave false results with TZM-bl cells in such a way that the reliability of MTT assay was questionable (see **Appendix 1**, **Figure A2**). However, this was not observed with PBMCs as dose-dependent curve was obtained. Therefore, the NRU assay was considered for TZM-bl cells and a dose-dependent curve was obtained, which is shown in **Appendix 1** (**Figure A3**). The obtained results from NRU revealed a low cytotoxic profile of *F. multiflora* in TZM-bl cells. The CC_{50} values obtained for extracts WR and WL in NRU assay were way above the highest tested concentration and are represented as (>250) in the table.

4.2 HIV-1 Enzyme Inhibition

HIV-1 enzyme inhibition assays are crucial in discovering anti-HIV-1 agents that can render the HIV-1 replication phase inactive. The predicted concentration of an inhibitor that can produce half (50%) maximal activity was denoted as IC_{50}. Extracts can be considered for pharmaceutical studies if they exhibit minimum or no toxicities with a low IC_{50}. Levels of activity adopted from (Ntlhamu *et al.*, 2021) were considered for HIV-1 enzyme inhibition data analysis. The levels are defined as follows: below 25 µg/mL was considered as high activity, 25 - 50 µg/mL good activity, 50 - 100 µg/mL moderate activity and above 100 µg/mL low activity.

4.2.1 Inhibition of HIV-1 Reverse Transcriptase activity

The HIV-1 reverse transcriptase enzyme is responsible for commencing the viral replication process of the HIV-1 life cycle, this compels anti-HIV studies to place HIV-1 RT at the forefront of the investigation. It is, therefore, crucial to evaluate *F. multiflora* extracts for their ability to inhibit HIV-1 RT activity. The investigation was carried out by testing six extracts of *F. multiflora* for their inhibitory effect on RT activity at three selected concentrations (10, 25 and 100 µg/mL). **Figure 13** represents the percentage inhibition values for each extract.

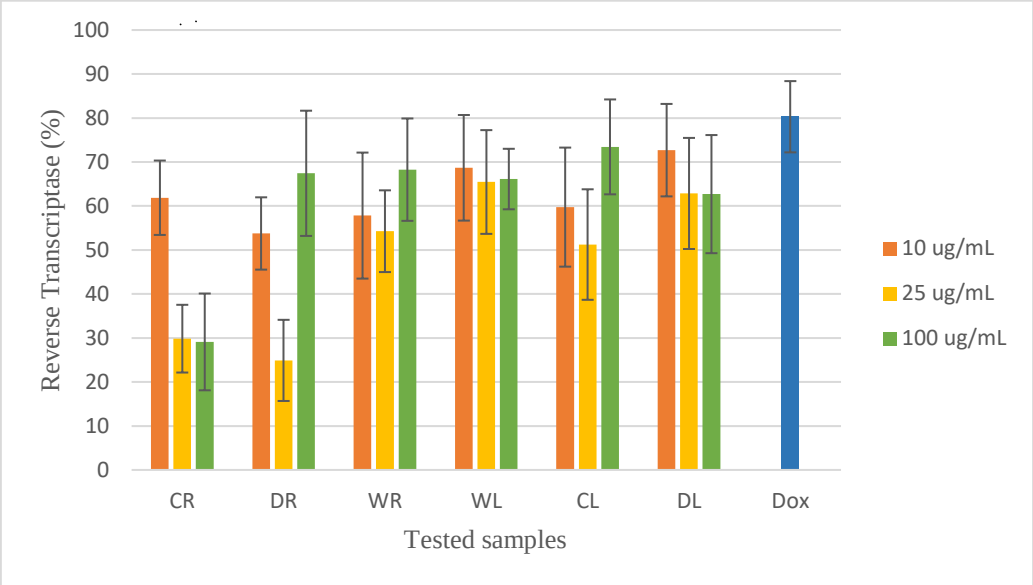


Figure 13| Inhibitory effect of *F. multiflora* extracts on HIV-1 Reverse Transcriptase. Data represent averages of triplicates with $n = 4$. Error bars represent SEM. Doxorubicin was used as a positive control.

Extracts WR, WL, CL and DL showed percentage inhibition values above 50% at all tested concentrations, while the CR surprisingly showed inhibition only at the lowest tested concentration (10 µg/mL). At 10 and 100 µg/mL, extract DR showed high percentage inhibition values of 54% and 67% respectively. HIV studies usually consider enzyme inhibition of ≥50% as significant activity (Ellithey *et al.*, 2014). An inverse response was observed with extracts CR and DL, with highest tested concentrations exhibiting less inhibition compared to the lowest tested concentration. The intermediate tested concentration (25 µg/mL) for all extracts exhibited lower HIV-1 RT inhibition activity compared to the lowest tested concentration (10 µg/mL). Some of the root extracts were found to be statistically significant (CR: $p = 0.0085$ and DR: $p = 0.0406$).

4.2.2 Inhibition of HIV-1 Protease activity

The final phase of HIV-1 replication depends on the HIV-1 protease enzyme to produce mature infectious virus particles, which makes this enzyme a crucial target for HIV inhibition studies. Extracts were tested at the same concentration as described in section **4.2.1** and **Figure 14** represent the percentage inhibition values for the various extracts.

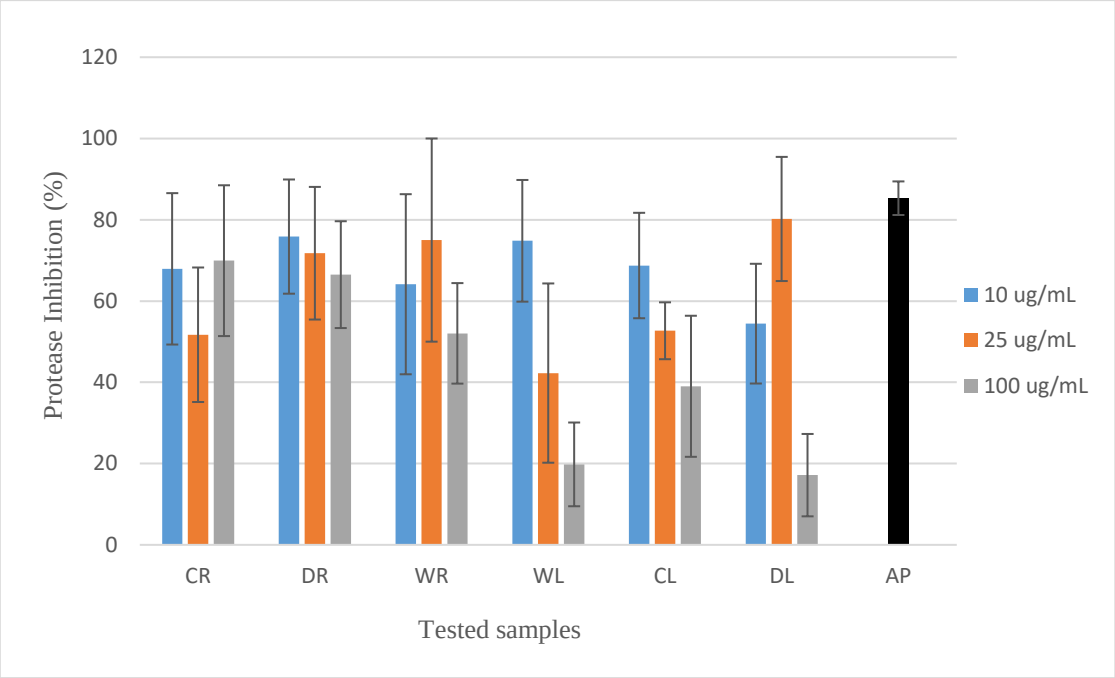


Figure 14| Inhibitory effect of *F. multiflora* extracts on HIV-1 Protease. Data represent averages of triplicates (n = 5). Error bars represent SEM. Acetyl pepstatin (AP) was used as a positive control.

All root extracts showed percentage inhibition values above 50% in all tested concentrations and CR and WR were found to be statistically significant (p = 0.0250 and 0.0283, respectively). The leaf extracts exhibited relatively low inhibition. An inverse dose response was observed with extracts DR, WL, and CL. The intermediate tested concentration (25 µg/mL) of extract WR and DL exhibited high percentage inhibition of 75% and 80% respectively, while 10 and 100 µg/mL concentrations of the same extracts exhibited lower inhibition compared to the intermediate tested concentration. Notably, the intermediate tested concentration (25 µg/mL) of extract CR, DR, WL, and CL demonstrated lower percentage inhibition compared to the lowest tested concentration (10 µg/mL).

The obtained data from HIV-1 enzyme inhibition assays was expressed as IC_{50} ± standard error of the mean (SEM), shown in **Table 4** with high IC_{50} values for both assays (highlighted in peach shade).

Table 3| The predicted inhibitory concentrations of *F. multiflora* for HIV-1 RT and PR Assays. Extract CR gave the highest inhibition for both assays. IC_{50} for DR in PR assay and WR in RT assay was undetectable as all the tested concentrations resulted in percentage inhibition values way above 50%.

***Fockea mulfiflora* extracts**	**HIV-1 RT IC_{50}**	**HIV-1 PR IC_{50}**
CR	14.02 ± 0.30	10
DR	35.50 ± 0.89	<10
WR	<10	<10
WL	<10	22.63 ± 0.08
CL	<10	38.42 ± 0.08
DL	<10	33.17 ± 0.46

The highest predicted IC_{50} of *F. multiflora* for HIV-1 PR activity was observed with extract CR (0.02 ± 14.98 µg/mL), followed by WL extract with estimated IC_{50} of 22.63 ± 0.08 µg/mL. Extract CL and DL showed good inhibition of HIV-1 PR activity. The anti-HIV-1 RT assay revealed that predicted IC_{50} obtained from CL extract exhibited the highest inhibition activity with IC_{50} of 5.34 ± 0.89 µg/mL, followed by extracts CR with estimated IC_{50} of 14.02 ± 0.30 µg/mL (p = 0.0085). Extract DR demonstrated good inhibition of HIV-1 RT activity and was statistically significant (p = 0.0406). The HIV-1 PR assay revealed that extracts CR and WR were statistically significant (p = 0.0250 and 0.0283, respectively).

4.3 Anticancer Assay

The immunosuppression caused by HIV-1 infection leads to the development and progression of opportunistic infections and diseases such as cancer. HIV-1 has been classified as an indirect carcinogen (Lekoane *et al.*, 2020), therefore, the discovery of effective therapeutics with both anti-HIV-1 and anticancer activity could alleviate the burden caused by HIV. The anticancer inhibition activity of *F. multiflora* extracts was investigated on cervical (HeLa) and liver (HepG2) cancer cell lines. Initially, NRU assay was used for anticancer investigation, surprisingly, there was no dose-dependent curve obtained from any of the tested concentrations. As much as NRU assay is recommended as an ideal assay for crude extracts, however, our findings implied that this is not the case with the type of cancer cell lines investigated in this study. The protocol that finally worked for anticancer evaluation was that of MTT with washing steps employed from NRU assay.

4.3.1 Effects of *F. multiflora* extracts on the proliferation of HeLa using MTT assay.

Cervical cancer is regarded as one of the AIDS defining cancers, however, HAART plays no part in mitigating the development and/or progression of this type of cancer among HIV-infected individuals. Some plant-derived natural products have shown a dual curative ability for antiviral as well as anticancer, therefore, it is important to not only investigate *F. multiflora* extracts for anti-HIV-1 activity but for anticancer abilities as well. *F. multiflora* extracts of concentrations ranging from (4 – 250 µg/mL) were evaluated for their inhibition activity on the proliferation of the HeLa cervical cancer cell lines, and the percentage cell viability are depicted in **Figure 15** below.

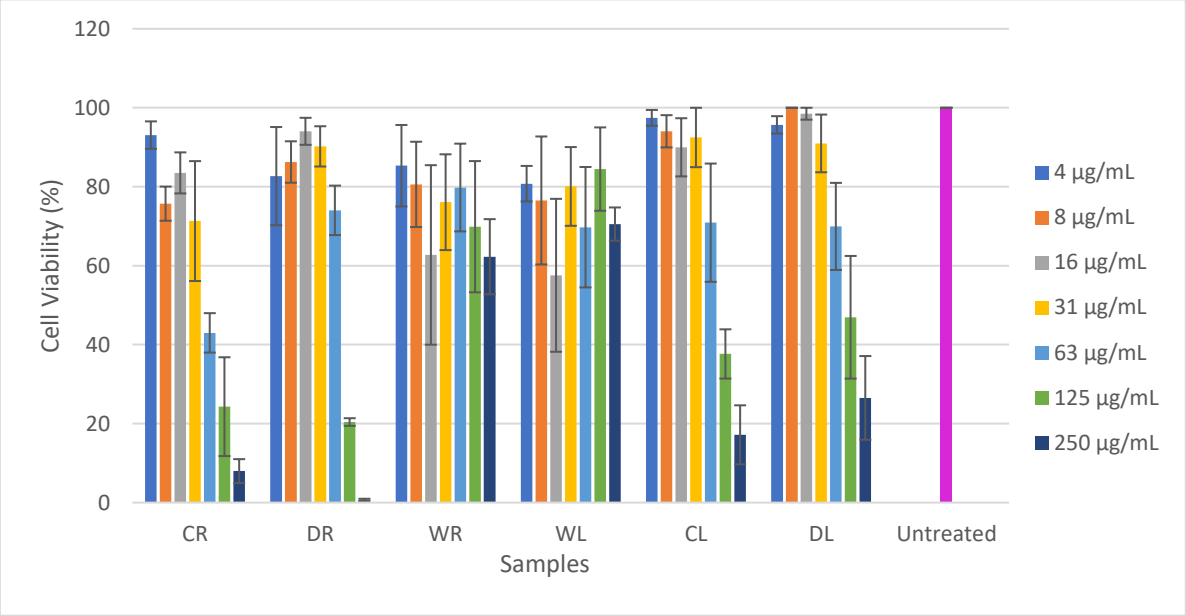


Figure 15| The inhibitory effects of *F. multiflora* extracts on the proliferation of HeLa cells. Extract CR was able to inhibit the proliferation of cervical cancer cell lines with an IC_{50} of 49.1 ± 0.07 µg/mL. Data represent averages of triplicates (n = 3) and error bars represent SEM.

The evaluation of the antiproliferative effect of *F. multiflora* through modified MTT assay gave dose-dependent curves for extracts CR, DR, CL, and DL. The highest tested concentrations (250 µg/mL and 125 µg/mL) for extract DR showed greatest antiproliferation, followed by extract CR, CL, and DL as they were able to lower the cell viability below 50%. As the trend with aqueous extracts to have no effect in cell viability was also noted in the HeLa cells, as none of the tested concentrations were able to inhibit cells below 50%. Moderate anticancer activity was observed with CR and DR extract giving IC_{50} of 50.49 ± 0.06 and 83.79 ± 0.05 µg/mL respectively. Low anticancer activity was observed with CL and DL extracts (IC_{50} >100

µg/mL), low tested concentrations (4 – 31 µg/mL) had little to no effect on proliferating cervical cancer cells as the viability of cells resembled that of untreated cells. Moreover, the antiproliferative capacity of water extracts (WR and WL) of *F. multiflora* exhibited better inhibition compared to low tested concentrations (4 – 31 µg/mL) of CL and DL extracts. All tested extracts of *F. multiflora* were found to be statistically significant with *p*-values ranging from 0.0012 to 0.0187).

4.3.2 Effects of *F. multiflora* extracts on the proliferation of HepG2 using MTT assay.

Although liver cancer is regarded as non-AIDS-defining cancer, it is often diagnosed at an advanced stage in HIV-positive people presumably due to the prolonged duration of ARV intake. As the people living with HIV population is aging, there has been an increase in liver cancers cases and over the years this type of cancer has become a growing cause of mortality among people who are infected with HIV (Dhokotera *et al*., 2019) (Sun *et al*., 2021). As a result of this information, the liver cancer cell line had to be considered in the anticancer assay. Therefore, the antiproliferative activity of *F. multiflora* extract was evaluated on liver cancer cell line, HepG2 and the obtained percentage cell viabilities are shown in **Figure 16** below.

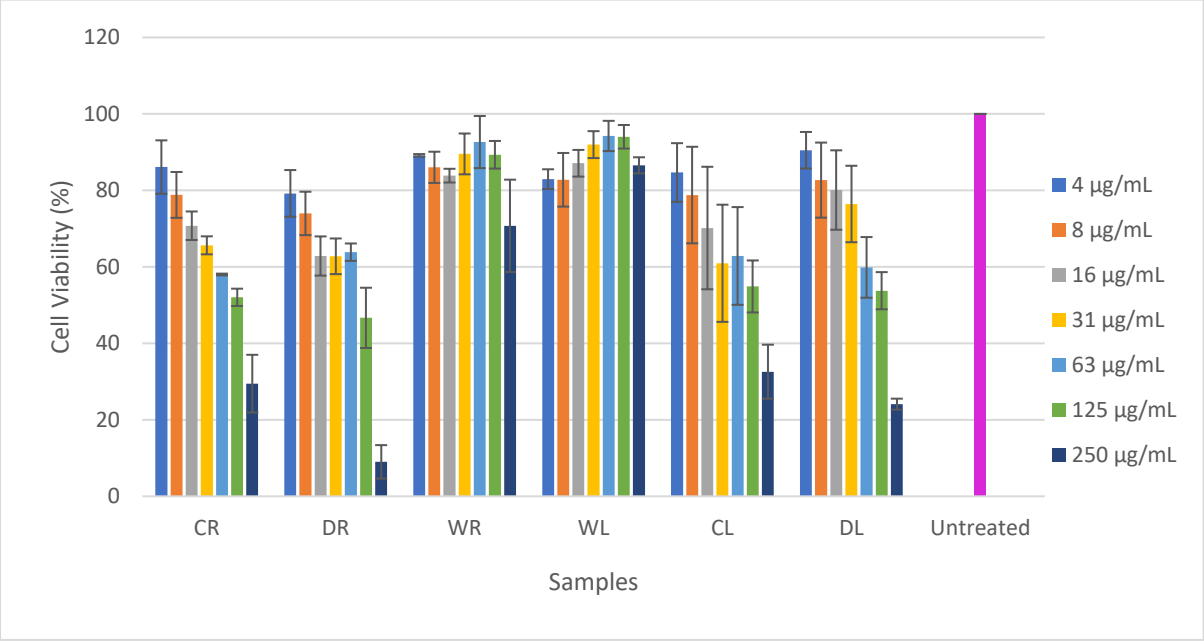


Figure 16| The inhibitory effects of *F. multiflora* extracts on the proliferation of HepG2 cells. None of the extract was able to inhibit the proliferation of cervical cancer cell-lines. Data represent averages of triplicates (*n* = 3) and error bars represent SEM.

The evaluation of the antiproliferative effect of *F. multiflora* through modified MTT assay when compared to untreated cells depicted that most extracts were able to inhibit HepG2 cells. Extracts CR, DR, CL, and DL gave dose-dependent curves and the highest tested concentrations (250 µg/mL) of these extracts inhibited HepG2 cells below 50%. Moderate anticancer activity was observed with extract DR with an IC_{50} of 57.77 ± 0.16 µg/mL, followed by CR with IC_{50} of 92.88 ± 0.07 µg/mL. Extract CL and DL exhibited low activity with IC_{50} values above 100 µg/mL. All extracts were found to be statistically significant (p = 0.0001). Aqueous extracts of *F. multiflora* (WR and WL) could not inhibit the proliferation of HepG2 cells below 70%.

4.3.3 Real-Time cell analysis

A quantitative method of monitoring changes in cell adhesion was used as a confirmatory assay to further investigate the inhibitory capacity of *F. multiflora* extract towards HeLa and HepG2 cells using the RTCA device. What sets RTCA apart from other cell viability assays used in this study is that a continuous readout of cell adhesion can be monitored from the moment the cells begin to attach and that the CI determination is based on the entire population of cells. Therefore, the results obtained from RTCA are less likely to be subject to bias (Hamidi *et al*., 2018). Cell seeding density optimisation shown in **Figure 17** was conducted and 30 000 cells/mL was selected as the concentration to be use for the treatment as it gave CI values between 1 and 1.5. Untreated cells, media, and cisplatin (10µg/mL) were tested alongside *F. multiflora* extracts.

4.3.3.1 Impedance-Based Growth Curves of HeLa Cells

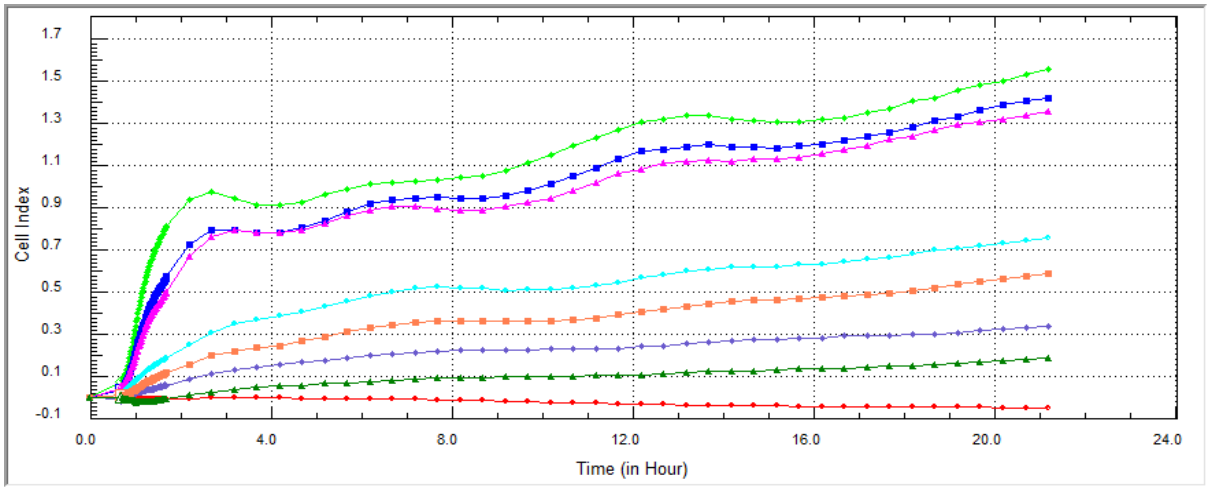


Figure 17| RTCA cell titration growth curves of HeLa cells ----- 50 000 ----- 40 000 ----- 30 000 ----- 20 000 ----- 10 000 ----- 5 000 ----- 2 500 ----- media were established according to the number of cells seeded in E-plates to obtain the ideal cell number to use for the treatment of HeLa cells. As CI values were recorded it was noted

that cell densities above 20 000 cells/mL proliferated higher than other seeding concentrations. Therefore 30 000 cells/mL was chosen as the optimal cellular density to work with as it gave CI value between 1 and 1,5 after 20 hours.

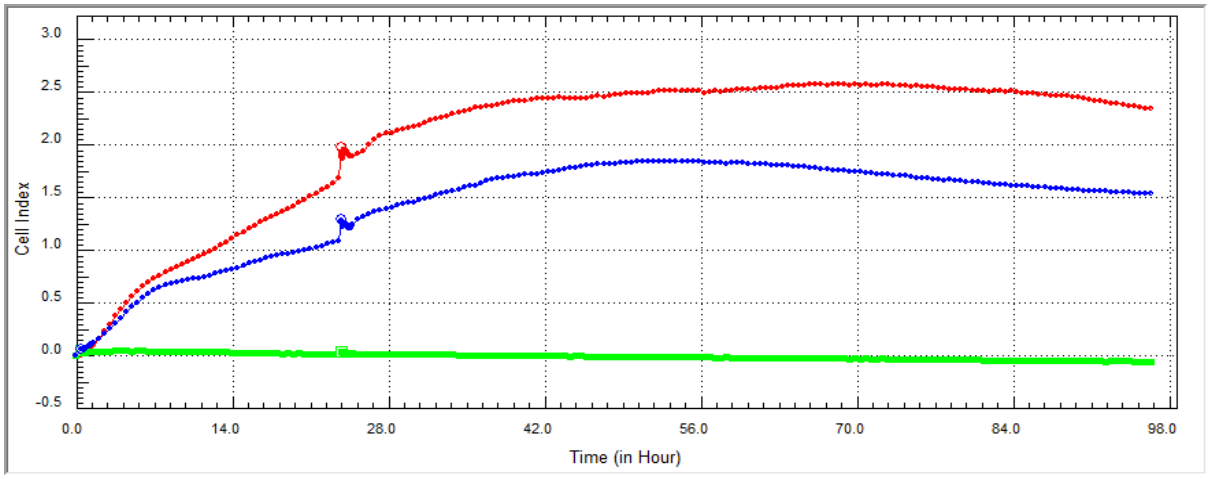
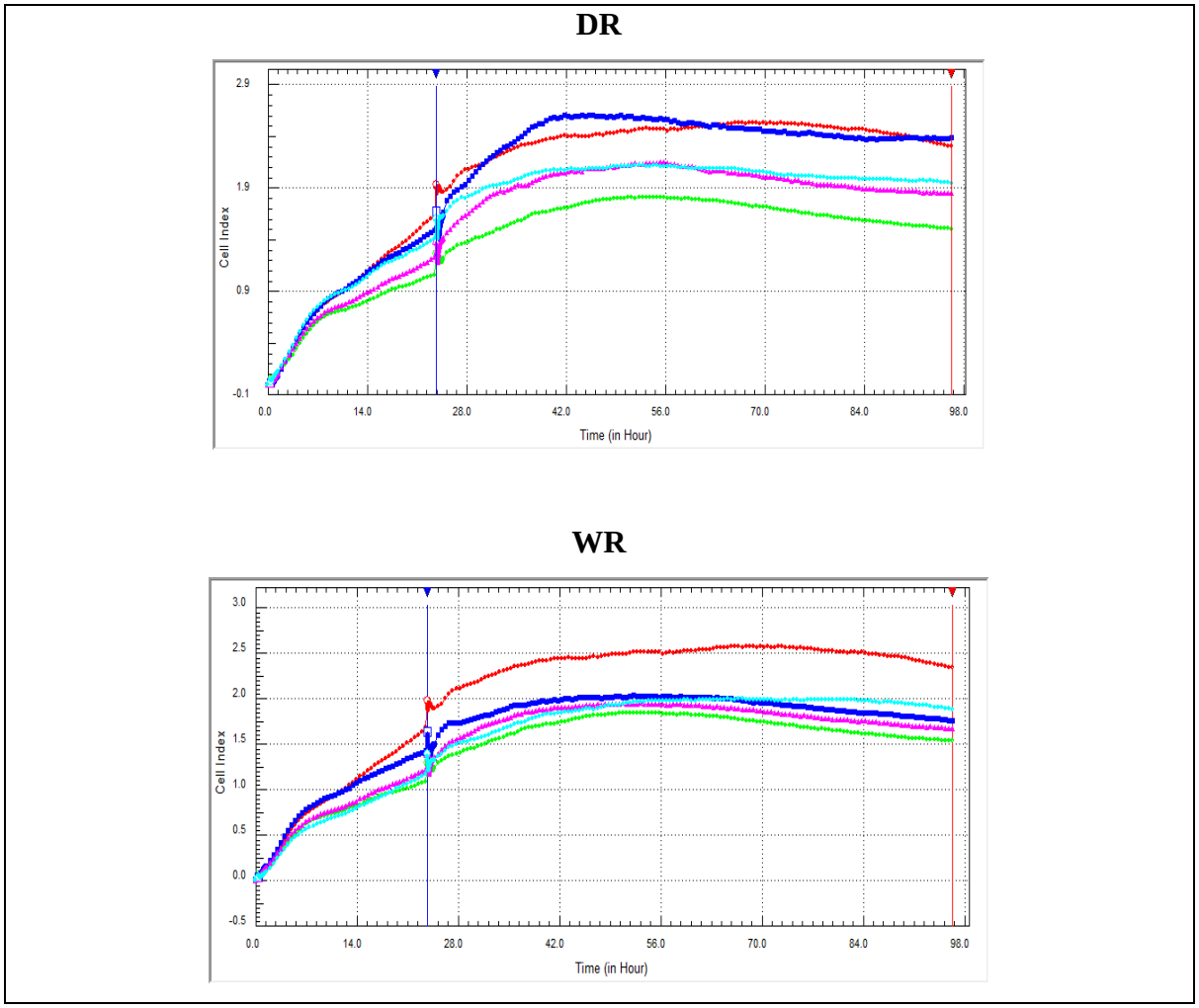
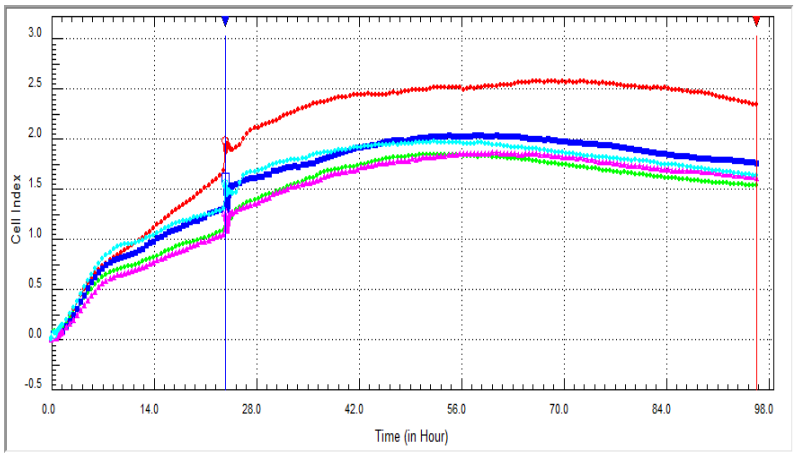


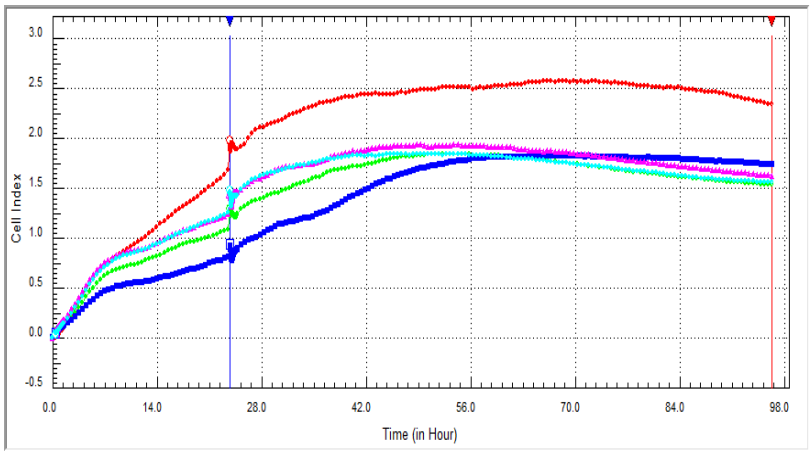
Figure 18| RTCA growth curves of HeLa cells, cisplatin, and media. ----- Cells only ------ Cisplatin ------ Media only *F. multiflora* extracts with (100; 50 and 25 µg/mL) concentrations were used to treat HeLa cells. Untreated cells and cisplatin were used as controls. The observed peaks between 14 and 28 hours are due to the experiment being put on a halt to add varying concentrations of *F. multiflora* extracts.



WL



CL



DL

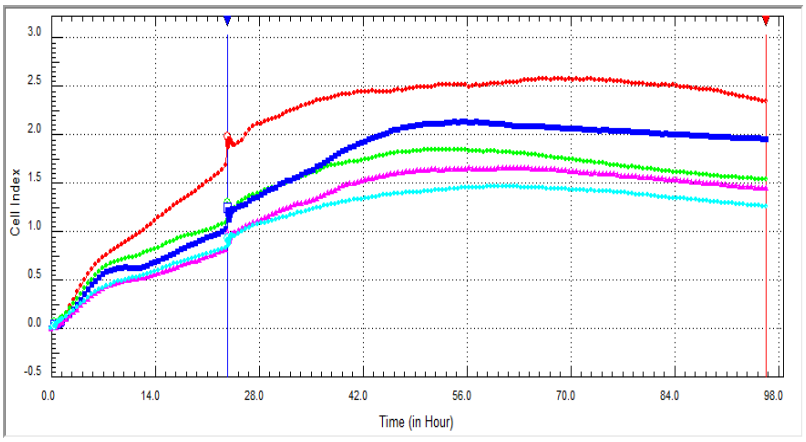


Figure 19| Impedance-based growth curves of HeLa cells treated with *F. multiflora* extracts using RTCA. **---- Cells only ------ 100 µg/mL ----- 50 µg/mL ----- 25 µg/mL ----- Cisplatin**. Most extracts (even low concentrations) showed impedance curves like that of cisplatin, except for DR at 100 µg/mL which demonstrated

a curve like untreated cells. The IC_{50} derived from these treatments are representative of three independent experiments, each performed in triplicates.

The effect of the antiproliferation of *F. multiflora* extracts in HeLa cells was observed between 56 and 96 hours of treatment. The highest tested concentration (100 µg/mL) for all extracts exhibited less antiproliferative ability compared to 25 and 50 µg/mL, except for extract DR where the growth curve of 100 µg/mL resembled that of untreated HeLa cells indicating that this concentration could not inhibit the proliferation of HeLa cells. Extract CL exhibited an inverse dose-dependent curve, with the lowest tested concentration conferring greater inhibition to the level of cisplatin. The greatest antiproliferative effect was demonstrated by 25 µg/mL followed by 50 µg/mL of extract DL, as both tested concentrations inhibited HeLa cells far greater than cisplatin.

4.3.3.2 Impedance-Based Growth Curves of HepG2 Cells

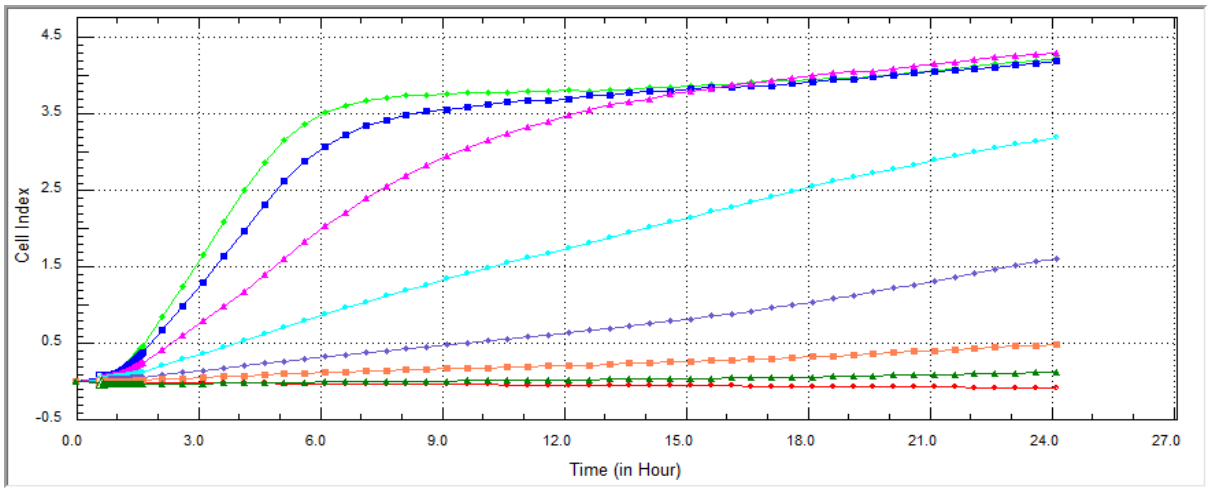


Figure 20| Cell titration growth curves of HepG2 cells ----- **50 000** ----- **40 000** ----- **30 000** ----- **20 000** ----- **10 000** ----- **5 000** ----- **2 500** ----- **media** were established according to the number of cells seeded in E-plates (2500 – 50 000 cells/mL) to obtain the ideal cell number to use for the treatment of HepG2 cells. Seeding concentration of 20 000 cells/mL was chosen as the optimal cellular density.

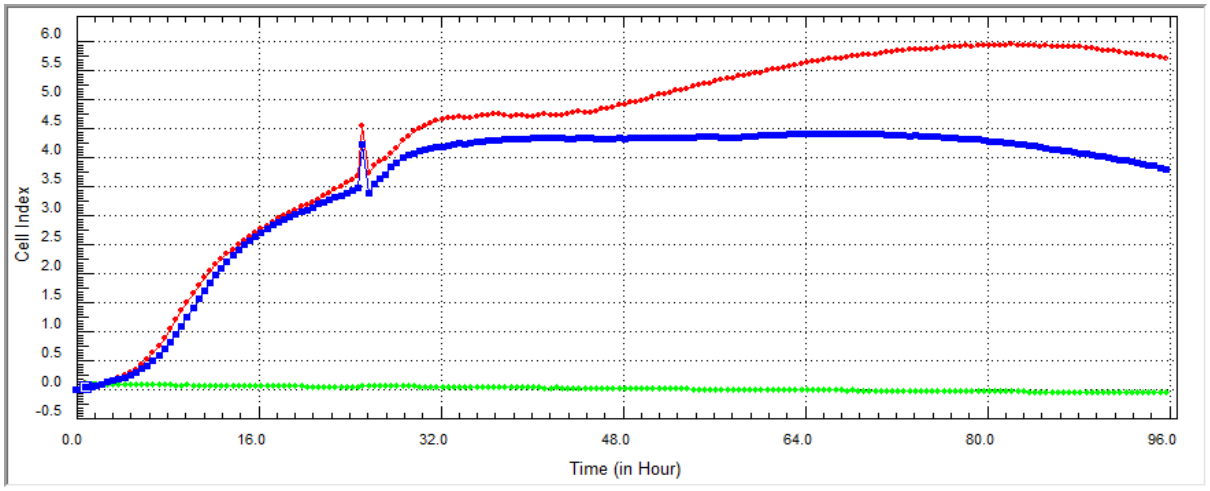
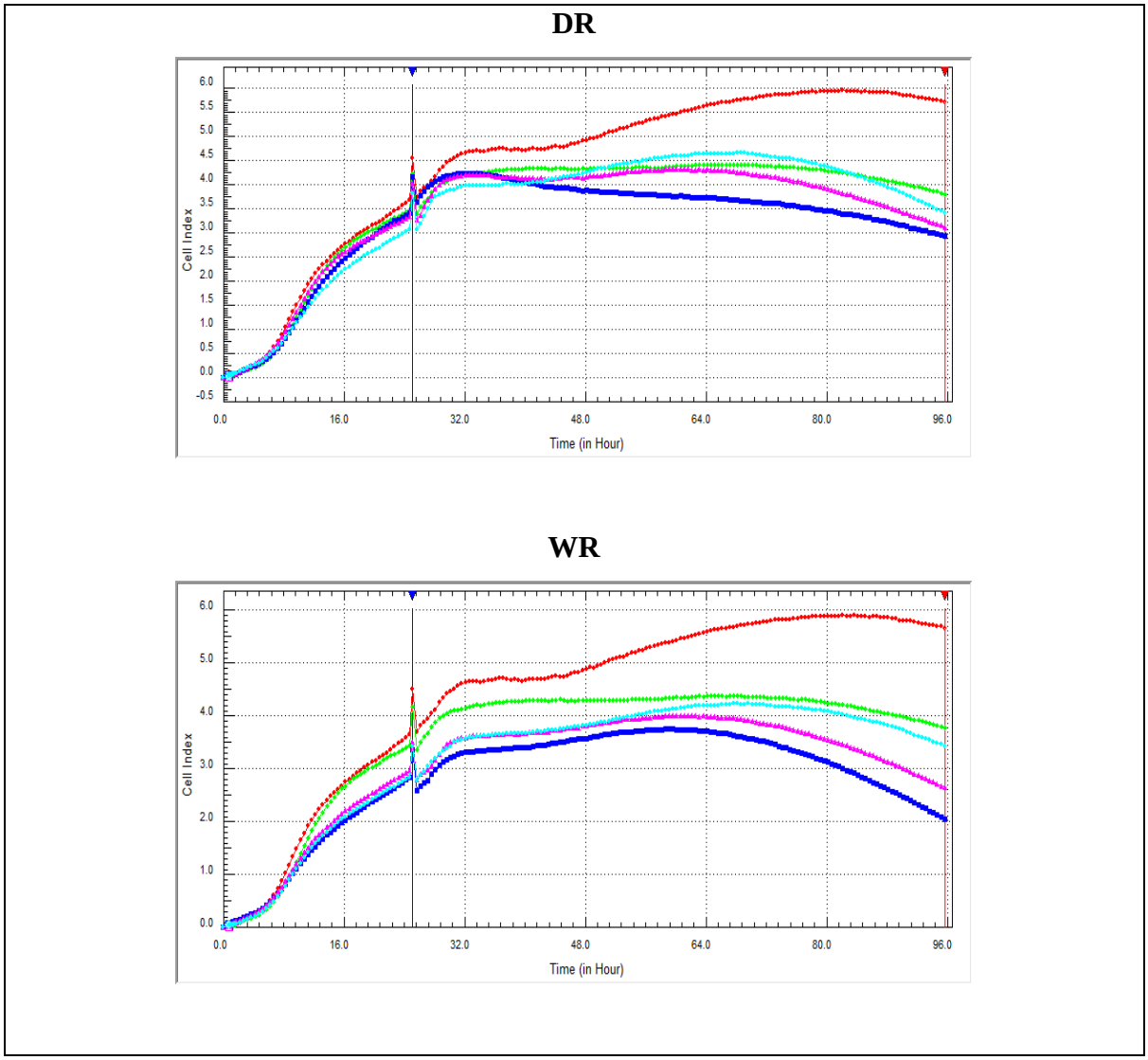
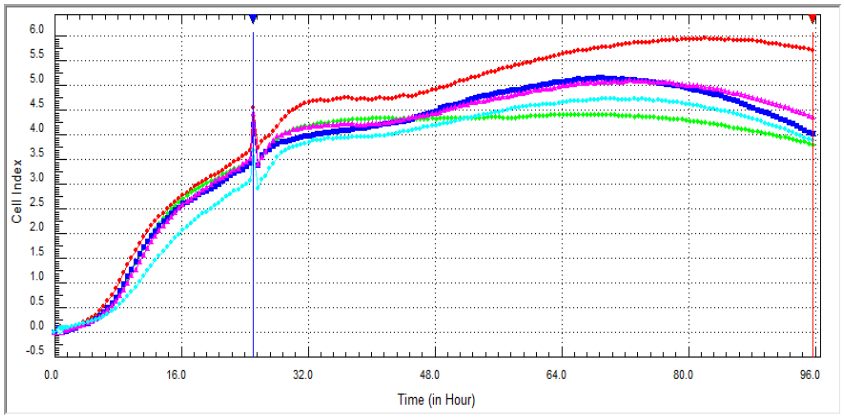


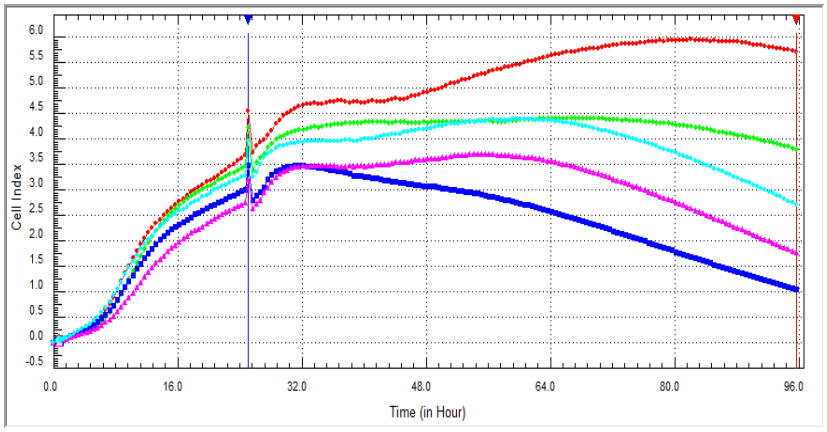
Figure 21| RTCA growth curves of HepG2 cells, cisplatin and media ----- Cells only ------ Cisplatin ------ Media only *F. multiflora* extracts with (100; 50 and 25 µg/mL) concentrations were used to treat HepG2 cells. Untreated cells and cisplatin were used as controls. The observed peaks between 16 and 32 hours are due to the experiment being put on halt to add varying concentrations of *F. multiflora* extracts.



WL



CL



DL

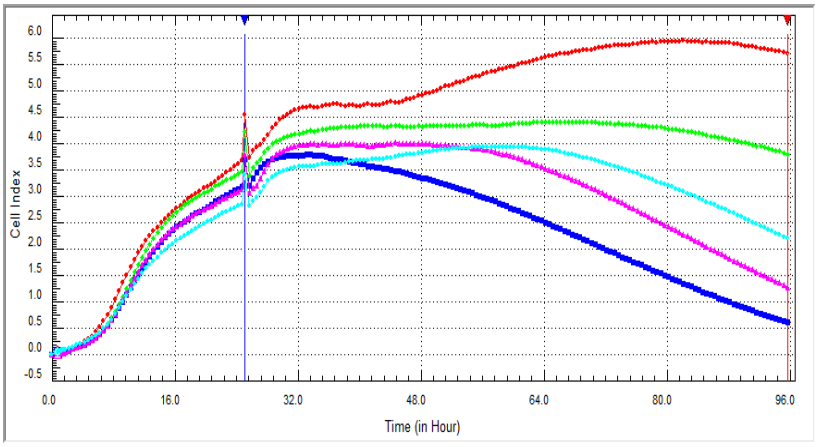


Figure 22| Impedance-based growth curves of HepG2 cells treated with *F. multiflora* extracts using RTCA. ----- **Cells only** ------ **100 µg/mL** ----- **50 µg/mL** ----- **25 µg/mL** ----- **Cisplatin**. Extracts DR, WR, CL and DL showed a dose-dependent curve with greater antiproliferative activity compared to cisplatin. Extract WL treatment showed a proliferative profile like that of cisplatin. The IC_{50} derived from these treatments are representative of three independent experiments, each performed in triplicates.

A dose-dependent curves of the antiproliferative capacity of extracts DR, WR, CL and DL were observed to be more than that of cisplatin, shown in **Figure 22**. The lowest tested concentration (25 µg/mL) of extract WL showed greater antiproliferative ability than 50 and 100 µg/mL. *F. multiflora* extracts WR, CL and DL for all tested concentrations demonstrated greater inhibition than cisplatin. The antiproliferative effect of the highest tested concentration (100 µg/mL) of extract CL and DL was observed in the early stages of treatment (32 hours). The antiproliferative effects of *F. multiflora* aqueous extracts were demonstrated at the later stage of treatment (from 64 hours for WR and from 80 hours for WL). The special feature of sensitive gold microelectrodes embedded within each well in RTCA E-plates allows the real-time recording of the strength of cell attachment of the entire population of cells (Lebourgeois et al., 2018) as a result, IC_{50} values that were not detected in MTT assay were predicted by the real-time assay as shown in **Table 5** below.

Table 4| The predicted IC_{50} of HeLa and HepG2 cells from MTT and RTCA. IC_{50} for WL was undetectable as all the tested concentrations resulted in percentage inhibition values way above 50%. Extract CR ran out when RTCA was conducted and the IC_{50} could not be detected.

***F. multiflora* extracts**	**HeLa IC_{50} MTT**	**HeLa IC_{50} RTCA**	**HepG2 IC_{50} MTT**	**HepG2 IC_{50} RTCA**
CR	50.49	×	92.88	×
DR	83.79	81.87	57.77	49.75
WR	>250	35.53	>250	41.28
WL	-	74.68	>250	67.36
CL	99.4	63.32	109.1	78.50
DL	118.8	72.02	102.1	66.17

The highlighted (peach shade) IC_{50} from RTCA shown in **Table 5** reveal lower values compared to those obtained from MTT. RTCA was able to predict IC_{50} for WL and WR extract in both cell lines, these could not be detected by the end-point assay, MTT. Extract WL (highlighted in blue) was observed to be toxic while none of the concentrations of *F. multiflora* was able to kill liver cancer cells below 50% and this was ruled as false-positive. The toxic profile of extract WR in HeLa cells (with an IC_{50} of 35.53 µg/mL) was revealed by RTCA, which gave an IC_{50} that was recorded as above the final tested concentration in the MTT assay.

4.3.4 Selectivity Index

The safety of *F. multiflora* extracts toward Vero cells, a monkey kidney cell line was also evaluated to determine the selectivity index (SI) of the extracts. The selectivity index was calculated from the ratio of the extract IC_{50} in Vero cells over the IC_{50} values obtained in the cancer cells, that is, HeLa and HepG2 cells (**Appendix II, Formula 5**). Selective toxicity is an important parameter to detect anticancer potential *in vitro*, as cancer therapy should only target cancerous cells and not affect non-cancerous cells (Njoya *et al*., 2017). An extract is considered to have high selectivity towards cancer cells if the SI value is greater than 3 (Chothiphirat *et al*., 2019) (López-lázaro, 2015).

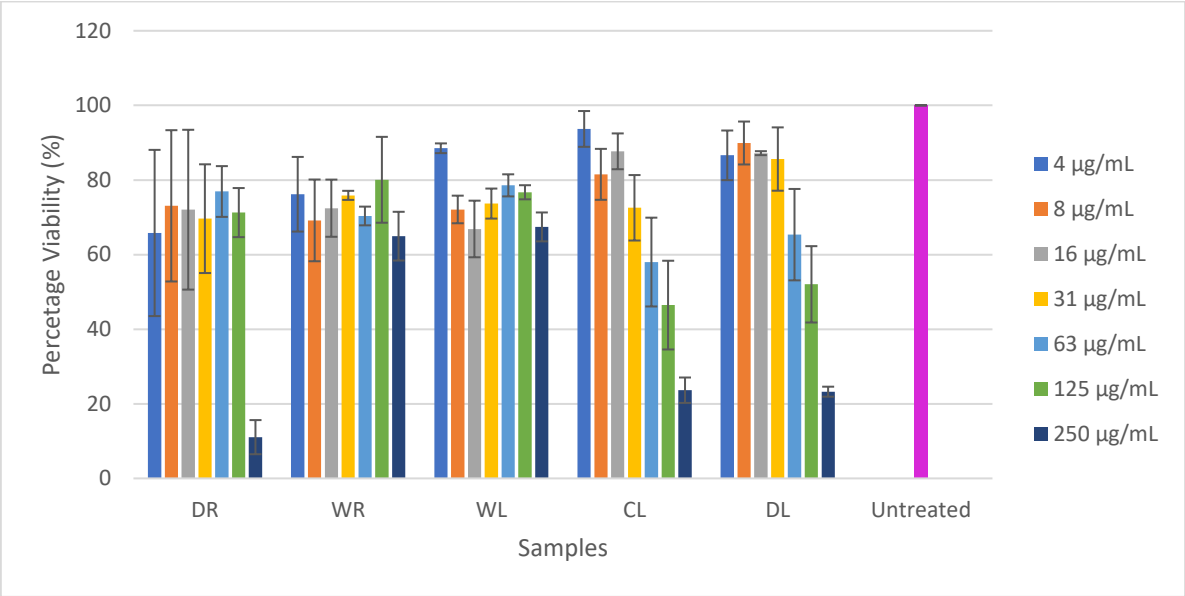


Figure 23| The effect of *F. multiflora* extracts on the proliferation of Vero cells. None of the extracts were able to inhibit the proliferation of Vero cells. Data represent averages of triplicates (n = 3) and error bars represent SEM.

The proliferative ability of *F. multiflora* extracts on Vero cells shown in **Figure 23** above was analysed using the modified MTT assay. Dose-dependent curves were observed with two of the leaf extracts, namely, CL and DL. Again, extract WR and WL were observed to be non-toxic at all tested concentrations. Extract DR followed the trend observed with aqueous extracts, as most tested concentrations were found to be non-toxic towards Vero cells except for 250 µg/mL which inhibited Vero cells way below 50%.

Table 5| The predicted IC_{50} values of the extracts in Vero cells and Selectivity Index towards HeLa and HepG2 cells. (-) not detected.

F. multiflora extracts	**Vero IC_{50}**	**Selectivity Index (SI)**	
		HeLa	**HepG2**
DR	130.8 ± 0.46	1.6	2.6
WR	>250	>7	>6
WL	>250	>3.3	>3.7
CL	89.65 ± 0.05	1.4	1.1
DL	112.7 ± 0.06	1.6	1.7

The levels of cytotoxicity described in section **4.1** were adopted to analyse the cytotoxic effect of *F. multiflora* in Vero cells. Low cytotoxicity was demonstrated by extract CL with IC_{50} of 89.65 ± 0.05 µg/mL. The lowest cytotoxicity was exhibited by extracts WR and WL with IC_{50} way above the final tested concentration (250 µg/mL) and both extracts were selective towards both HeLa and HepG2 as the obtained SI values were above 3 (**Table 6**). Extract WR showed greatest selective cytotoxicity against HeLa and HepG2 cells with SI values of >7 and >6 respectively. Extract WR and WL show promise of being further developed for anticancer activity through fractionation.

4.4 Antioxidant Assay

Nitric oxide is a pro-inflammatory mediator and in the absence of antioxidants, the overproduction of NO leads to inflammation diseases, tissue damage and cancer. Natural products have been reported to be great sources of antioxidants and excellent anti-inflammatory agents during oxidative stress (Boora *et al*., 2014) (Njoya *et al*., 2017). Therefore, the radical scavenging ability of *F. multiflora* extracts was evaluated on the NO production in cancer cell lines. Vitamin C, a natural antioxidant found in many fruits and vegetables was used as a positive control for this assay.

4.4.1 Inhibitory effect of *F. multiflora* extracts on the NO production in HeLa cells

The increasing number of incidences of cervical cancer among HIV-positive women compels research to be directed towards the factors contributing to the development and/or progression of cervical cancer. This assay monitored the inhibitory effect of *F. multiflora* on the production of NO as one of the key players in the development of cancer. HeLa cells were treated with

varying concentrations (4 – 250 µg/mL) of *F. multiflora* extracts and vitamin C and untreated cells were used as positive and negative control respectively.

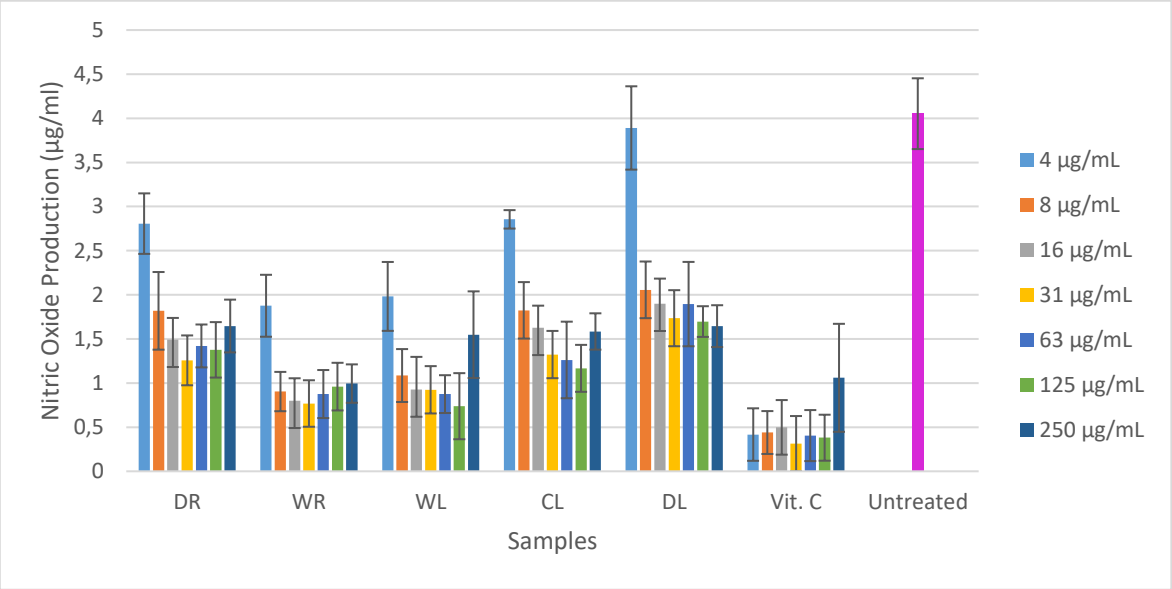


Figure 24| Inhibitory effect of *F. multiflora* extracts on the production of nitric oxide in HeLa cells. *F. multiflora* extracts were tested against untreated cells and Vitamin C. Data represent averages of triplicates (*n* = 4) and error bars represent SEM.

Aqueous extracts of *F. multiflora*, WR and WL showed greater inhibition compared to other extracts (**Figure 24)**. The lowest tested concentration (4 µg/mL) notably did not have that much effect on the inhibition of the production of NO, especially DL extract which was observed to be almost as high as untreated cells. Surprisingly, the highest tested concentration (250 µg/mL) for most extracts appeared to have less inhibition effect compared to intermediate tested concentrations. Vitamin C drastically reduced the production of NO in HeLa cells and the similar trend observed with the 250 µg/mL concentration in other extracts was also noted with vitamin C. The comparison between untreated cells and extracts of *F. multiflora* revealed the inhibitory capacity of extracts against the production of NO in HeLa cell lines.

4.4.2 Inhibitory effect of *F. multiflora* extracts on the production of nitric oxide in HepG2 cells

There has been a growing number of liver cancer cases among the population of people living with HIV due to the prolonged use of antiretrovirals. The search for novel anticancer agents with many curative abilities is the need of the hour and will be of great benefit to be used in conjunction or be incorporated with currently existing antiretrovirals. With that said, this assay had to be featured in this study, to evaluate whether *F. multiflora* extracts possess the ability to

inhibit the production of NO in liver cancer cells, HepG2. The assay was conducted alongside untreated HepG2 cells and vitamin C as negative and positive control respectively.

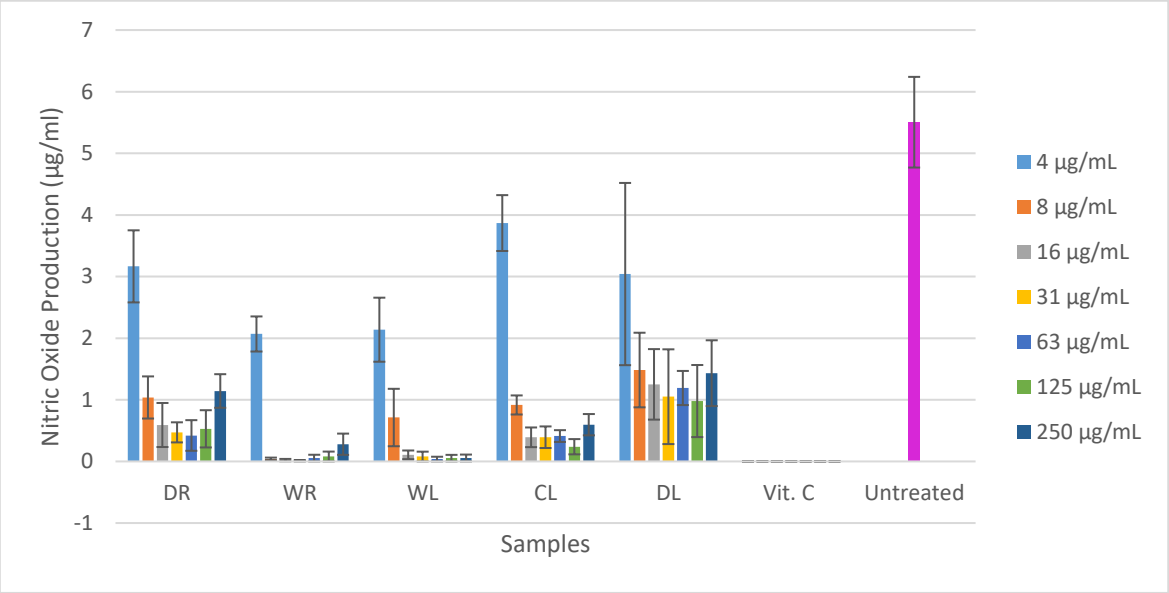


Figure 25| Inhibitory effect of F. multiflora extracts on the production of nitric oxide in HepG2 cells. *F. multiflora* extracts were tested against untreated cells and Vitamin C. Data represent averages of triplicates (n = 3) and error bars represent SEM.

Aqueous extracts, WR and WL reduced NO production to almost undetectable levels (**Figure 25**). Even the lowest concentration (4 µg/mL) exhibited inhibition greater than those of other extracts at the same tested concentration. Least inhibition was observed with DL as none of the extracts were able to inhibit nitric oxide below 1 µg/mL. Vitamin C inhibited nitric oxide production to undetectable levels. It was also noted that *F. multiflora* extracts drastically reduced the production of NO in HepG2 cells to almost undetectable levels with extract WR and WL. All *F. multiflora* extracts exhibited the highest degree of inhibition activity in HepG2 proliferation. The highest IC_{50} was observed with DL (IC_{50} of 0.001 ± 1.21 µg/mL) and DR (IC_{50} of 0.23 ± 0.30 µg/mL). All extract were found to be statistically significant (p = 0.001).

5. Discussion

5.1 Cell viability and proliferation

One of the main functions of bioactive constituents contained in plants is to provide defence against invading pathogens and herbivores, through a certain level of toxicity. As a result, cytotoxicity studies form the basis of natural product research, by determining the toxicity profiles of extracts so that they do not pose health risks to humans. The use of different cell lines to evaluate the cytotoxicity profile of plant extracts allows for promising extracts with good inhibitory activity to progress beyond *in vitro* studies and this is useful in the discovery of good leads with low toxic profiles to be considered for drug development strategies (Mensah *et al*., 2019). Therefore, investigating the cytotoxicity profile of *Fockea multiflora* was of paramount importance as the basis of this study.

The extracts of *F. multiflora* were investigated for toxicity in two types of cell lines, TZM-bl cells and PBMCs. The MTT assay was performed in PBMCs, this assay is characterized by the reduction of tetrazolium salt into purple formazan crystals. The NRU assay that is characterized by viable cells incorporating the neutral red dye in their lysosomes was performed in TZM-bl cells. Both assays provide a quantitative measurement of viable cells after the treatment (Chan *et al*., 2015). The lowest cytotoxicity obtained in PBMCs was exhibited by extract WR with a CC_{50} of 100.30 ± 0.10 µg/mL, as most of the tested concentrations for this extract did not inhibit PBMCs below 50% (**Appendix II, Figure A1**), this indicates the safety profile of WR extract. The leaf extracts of *F. multiflora* gave the highest cytotoxicity profile with a CC_{50} of 10.39 ± 0.26 µg/mL for DL and 12.14 ± 0.12 µg/mL for CL extract.

Testing samples (plant extracts, compounds) in PBMCs indicate how the toxicity profile of samples will impact the human immune system, as high levels of toxicity can lead to several serious adverse events which can suppress the immune system. What makes PBMCs an ideal tool for assessing the toxicity profile is that these cells form a major component of the immune system that become exposed to invading pathogens and infections (Pourahmad and Salimi, 2015). With DL and CL extract of *F. multiflora* exhibiting the highest cytotoxicity towards PBMCs suggests that these extracts may possess the additional benefits to confer inhibition in anticancer studies as tested samples are expected to be toxic towards cancer cells.

The use of two different cytotoxicity assays in this study was due to the false-positive results obtained in the evaluation of the cytotoxicity profile of *F. multiflora* extracts in TZM-bl cells

using MTT assay. It was observed under the microscope that the cells were dead in some wells with the highest tested concentrations, yet there were purple formazan crystals in those wells indicating the presence of viable cells. Moreover, the absorbance values obtained from those wells also confirmed a significant portion of viable cells. This led to questioning whether MTT assay was an ideal protocol to use for *F. multiflora* extracts or not.

Based on the literature, several factors have been reported to interfere with the MTT assay; natural products are counted among these factors. Studies reveal that isolated compounds of flavonoids (kaempferol and resveratrol), antioxidants (ascorbic acid, N-acetylcysteine and vitamin E) and plant extracts (*Hypericum perforatum* and *Cimicifuga racemose*) have been found to directly reduce the MTT to formazan (Stefanowicz-Hajduk and Ochocka, 2020). The interaction between plant extracts and the MTT indicator is due to the presence of reducing agents contained in plant extracts (Chan *et al*., 2015). Another reason for the interference has been attributed to inactive or injured mitochondria being able to produce the same amount of formazan as the fully operative mitochondria, this is often due to some bioactive constituents conferring a reparative ability on injured mitochondria. One study reported on the (-)-epigallocatechin-3-gallate (EGCG), a polyphenol compound found in green tea, which demonstrated the ability to repair injured mitochondria and enhance the activity of succinate dehydrogenase (Wang *et al*., 2010). Reports have also been made of non-mitochondrial dehydrogenases and flavin oxidases conferring the same reducing ability towards tetrazolium salts as the mitochondrial succinate dehydrogenases, which is responsible for the conversion of a yellow water-soluble tetrazolium dye into purple formazan crystals in MTT assays (Bruggisser *et al*., 2002) (Stefanowicz-Hajduk and Ochocka, 2020).

The interesting observation noted in this study was that either the mitochondrial reparative ability conferred by antioxidants or the reducing agents in *F. multiflora* extracts seem to be cell specific as the MTT assay worked as expected with the PBMCs. Aslanturk (2018) pointed out that the type of cell lines used tend to affect the performance of the cytotoxicity assays, as was the case with the MTT indicators and *F. multiflora* extracts in TMZ-bl cells. These findings amplify the importance of carefully analysing the MTT results so as not to overlook the interactions between reducing agents and the tetrazolium salts. To circumvent this interference, NRU assay was employed to evaluate the cytotoxic effect of *F. multiflora* extracts.

The NRU assay was suggested as the ideal assay to use in determining the cytotoxicity of crude plant extracts, as the possibility of extracts reacting with NRU indicator is excluded. The protocol involves the washing of cells with PBS before and after adding the neutral red dye.

This ensures the elimination of reducing agents that could have caused interference in the reaction, making the assay more reliable (Chan *et al*., 2015). Upon conducting NRU assay for the determination of cytotoxic profile of *F. multiflora* extracts in TZM-bl cells, dose-dependent curves were obtained. The CC_{50} values revealed a low cytotoxicity profile of all *F. multiflora* extracts towards TZM-bl cells. The water extracts for both roots and leaves appeared to remain viable through all the tested concentrations, indicating a non-toxic profile of these extracts. It was interesting to note this observation as some societies in African regions already have *F. multiflora* readily available and included in their diets. The processing method often used by these societies is that of boiling the plant and with the non-toxic profile obtained with water extracts of *F. multiflora* means that people should not be subjected to undesirable side effects and/or toxicity. This information provides the safe benefits of including *F. multiflora* in the diet and it also expands the herbal medicine value for sub-Saharan African regions with limited access to essential medical resources. The non-toxic prolife of water extracts of *F. multiflora* suggests the potential of these extracts possibly conferring inhibitory activity in HIV-1 enzyme inhibition studies.

5.2 HIV-1 Enzyme Inhibition

With the advancement in pharmacognosy and technology along with the trends of a more health-conscious public, natural products have become a wealth of resources for researchers to discover novel and effective antiviral drugs. This is mainly due to the reduced adverse effects and cost-effectiveness of natural products on a commercial scale (Salehi *et al*., 2018). Evidence of plant extracts exhibiting anti-HIV-1 activity has been demonstrated throughout literature (Chinsembu, 2019) (Kurapati *et al*., 2016) (Subramaniam et al., 2020). The vast potential of bioactive constituents in plants has proven to be a useful benefit in anti-HIV-1 studies and this was the first report of *F. multiflora* root and leaf extracts being investigated for anti-HIV-1 activity. The extracts were investigated against HIV reverse transcriptase and protease.

5.2.1 HIV-1 Reverse Transcriptase inhibition

Reverse transcriptase is one of the most crucial enzymes in the HIV lifecycle as it transcribes its RNA code to synthesise viral DNA that becomes integrated into the host DNA to produce new virus progeny. For this reason, the enzyme has become a target for the screening and development of antiretroviral drugs against HIV (Ntlhamu *et al*., 2021). A diverse array of phytochemicals including proteins, terpenoids, coumarins, xanthones, alkaloids, flavonoids, polyphenols, and polysaccharides have been reported to be responsible for making HIV- 1 RT

less active. With plants possessing these phytochemical constituents enables them to be regarded as valuable sources of new active leads that may be developed further into anti-HIV drug candidates (Chinsembu, 2019). The ability of *F. multiflora* extracts to act as HIV-1 RT inhibitors was evaluated at three concentrations: 10, 25 and 100 µg/mL. Following the steps of other researchers who have also worked with three testing concentrations like Kapewangolo *et al*., (2017), proved to be beneficial in providing a room for comparability of the obtained data.

F. multiflora extracts displayed anti-HIV-1 RT activity, with extracts WR, WL, CL and DL exhibiting percentage inhibitions above 50% at all tested concentrations. This confirms the prediction previously made (**5.1**) with water extracts of *F. multiflora* exhibiting anti-HIV-1 activity due to their non-toxic profile. The greatest inhibition capacity was observed with CL extract exhibiting a percentage inhibition of 73% at 100 µg/mL (estimated IC_{50} of 5.34 ± 0.89 µg/mL). Leaf extracts exhibited relatively high HIV-1 RT inhibition activity compared to root extracts. Interestingly, *F. multiflora* exhibited a pronounced HIV-1 RT inhibition activity at low tested concentration (10 µg/mL) compared to intermediate concentration (25 µg/mL) for all extracts. Extracts CR, WL and DL demonstrated higher HIV-1 RT inhibition activity at 10 µg/mL than at 100 µg/mL.

The variation in anti-HIV-1 RT activity observed with *F. multiflora* could be attributed to the presence of metal ions that are distributed in varying quantities in different parts of the plants. Metal ions found in plants form an essential part of medicinal bioactive constituents. Reverse transcription being a key aspect of the retroviral life cycle, the enzyme facilitating the reaction, reverse transcriptase requires divalent cations like magnesium or manganese to function (Filler and Lever, 1997). Although HIV-1 RT uses Mg^{2+} as a co-factor, however alternative divalent cations such as Mn^{2+}, Cu^{2+}, Co^{2+} and Zn^{2+} can also be used for polymerase activity. It has been established that Zn^{2+} is a potent inhibitor for several viral polymerases and studies have shown the inhibitory effect of Zn^{2+} on HIV-1 RT activity *in vitro*. Reports of manganese inhibiting HIV-1 RT activity has also been shown (Bessong and Obi, 2006) (Achuthan and Destefano, 2015). Animal model studies have reported the inhibitory effect of Zn^{2+}-based drugs on the HIV replication. Bessong and Obi (2006) reported that even a slight increase in the concentration of manganese ions is capable of inhibiting HIV-1 RT activity *in vitro* (Bessong and Obi, 2006) (Bolton *et al*., 2002).

This information implies that any part of the plant and any tested concentration can confer high anti-HIV-1 RT activity in correlation with high levels of metal ions contained. As observed

with all extracts tested at 10 µg/mL demonstrating higher inhibition than the intermediate tested concentration, this could be due to metal ions being highly concentrated and active at a low tested concentration, or the bioactive compounds that have been proven to confer inhibitory properties on HIV-1 RT activity (e.g., flavonoids) were highly active at 10 µg/mL. In the case of *F. multiflora* leaf extracts exhibiting high inhibition, could point to the leaves of *F. multiflora* containing high quantities of metal ions and compounds known to make HIV-1 RT inactive.

The plant family of Apocynaceae accounts for less than 2% of 52.6% of the plant species with anti-HIV-1 RT activity. Other plant families like Lamiaceae, have gained more interest among researchers and were found to account for 13.7% of 52.6% plant species with anti-HIV-1 RT activity (Chinsembu, 2019). It is, therefore, crucial to expand the search for anti-HIV-1 RT agents among Apocynaceae plants. The results obtained with HIV-1 RT assay places *F. multiflora* (CL extract: IC_{50} of 5.34 ± 0.89 µg/mL) at the same level as plants that have high activity against HIV-1 RT, such plants include *Caesalpinia coriaria* and *Terminalia sericea* with IC_{50} of 6.24 µg/ml and 7.2 µg/ml, respectively (Chinsembu, 2019). The inhibitory activity demonstrated by extract CL of *F. multiflora* reveals the potential of this extract to be considered for further studies towards the development of anti-HIV-1 RT drug candidates derived from plants.

Water extracts for both roots and leaves of *F. multiflora* demonstrated percentage inhibition between 54 and 68%. The anti-HIV-1 RT activity coupled with non-toxic profile of these extracts will greatly benefit people living with HIV in the sub-Saharan African regions who already have *F. multiflora* growing in their own backyard. The ability of *F. multiflora* water extracts to curb the activity of the first enzyme that initiates the HIV-1 replication cycle suggests that people living with HIV will achieve a certain level of viral suppression from the consumption of *F. multiflora*. As literature revealed how HIV-1 reverse transcriptase diverges to get way from the action of inhibitors that are being administered. It is, therefore, imperative to investigate the inhibitory effect of another crucial enzyme in HIV-1 replication, HIV-1 protease, to decipher whether *F. multiflora* has the capacity to inhibit the proviral DNA that evaded the action of HIV-1 RT inhibitory effect through RT divergence.

5.2.2 HIV-1 Protease

HIV-1 protease is essential for virus maturation that promotes the infection of other CD4+ cells, making HIV-1 PR a major therapeutic target for HIV/AIDS treatment. Extracts of *F. multiflora* were investigated for anti-HIV-1 PR activity and all root extracts exhibited higher inhibition (>50%) compared to leaf extracts at all tested concentrations. Extract CR demonstrated the highest anti-HIV-1 PR activity with an estimated IC_{50} of 0.02 ± 14.98 µg/mL. Water extracts of *F. multiflora* were again given a closer look as they tie to the essence of this study and they were observed to exhibit high inhibition of HIV-1 PR activity with percentage inhibition of 75% for extract WR and WL tested at 25 µg/mL and 10 µg/mL, respectively. Reports of water extracts of other plants exhibiting anti-HIV-1 PR activity have been made, examples of such plants include *Cassia garrettiana* with 94.6% inhibition, *Pseudotsuga menziesii*, *Hypoxis sobolifera* and *Bulbine alooides* with >50% inhibition (Omoruyi *et al.*, 2020). The ability of water extracts to exhibit anti-HIV-1 PR activity is suggested to be due to high polar solvent capacity of water, which allows for the extraction of a wide range of polar compounds (Omoruyi *et al.*, 2020), thereby promoting the availability of active compounds necessary for the inhibitory effect of HIV-1 PR. These findings suggest that *F. multiflora* will provide maximum inhibition of HIV-1 PR activity for people living with HIV in sub-Saharan African regions.

The inhibition of HIV-1 PR activity among plant species is greatly attributed to the presence of phenolic compounds, which include flavonoids and polyphenolic compounds (Anantharaju *et al*., 2016). Flavonoid compounds like C_{60} fullerene have been reported to have high affinity for the active site of HIV-1 PR, as the radius of the compound is almost the same as that of the cylinder of the enzyme. Additionally, the hydrophobic amino acids contained in the catalytic site are compatible with the C_{60} fullerene that is also hydrophobic. These properties of flavonoid compounds increase the chances of interaction with the active site of HIV-1 PR (Akhtar *et al*., 2019), thereby making these compounds suitable inhibitors to the enzymatic action of HIV-1 PR activity. With water extracts of *F. multiflora* demonstrating high percentage inhibition against HIV-1 PR means that a good portion of hydrophobic compounds could not be extracted by water, as a result, the compounds with high affinity for the active site of HIV-1 PR were retained in water extracts of *F. multiflora*.

Another attributing factor for plant species to possess inhibition activity lies on the structures of phenolic compounds, as explained in the literature that the increase in the inhibition activity corresponds to the increase in the quantity of hydroxyl group. The more hydroxyl groups a

compound contains the greater the inhibition activity (Anantharaju *et al*., 2016). This was evident with taxifolin, a flavanone compound that exhibited inhibition activity against HIV-PR owing to the hydroxyl group in C-3' position, while another flavonone compound, aromadendrin could not inhibit HIV-PR as it lacked the hydroxyl group (Denaro *et al*., 2020).

The cytotoxicity profile of extracts has been established as another contributing factor to the inhibition to HIV-1 PR activity (Palshetkar *et al*., 2020). This indication prompted us to trace back as far as PBMCs to decipher the correlation between the cytotoxic profile and inhibitory effect of *F. multiflora* extracts. A certain level of anti-HIV-1 PR activity was observed with highly cytotoxic extracts (CL and DL), as well as moderate cytotoxic extracts CR, DR, and WL towards PBMCs. Indeed, extracts CR and WL exhibited high anti-HIV-1 PR activity with IC_{50} of 0.02 ± 14.98 and 22.63 ± 0.08 µg/mL, respectively. While this correlation could be verified by most extracts of *F. multiflora,* however, WR exhibited the least cytotoxicity towards PBMCs (CC_{50} = 100.3 ± 0.10 µg/mL) yet was found to confer greater anti-HIV-1 PR activity (between 52 and 75%) far better than the highly cytotoxic extract of *F. multiflora* (CL: 52% at 25 µg/mL and 69% at 10 µg/mL). Additionally, the cytotoxicity effect of *F. multiflora* in TZM-bl cells through MTT revealed that none of the extracts were cytotoxic. Therefore, the findings obtained with *F. multiflora* do not fully support the correlation suggested by Palshetkar *et al*., (2020).

The interesting observation noted with the anti-HIV-1 PR assay is that the inhibitory effect of *F. multiflora* extracts was highly active at low tested concentrations (10 and 25 µg/mL) with the highest exhibited by DL extract (80% at 25 µg/mL) followed by DR (76% at 10 µg/mL). *F. multiflora* at the highest tested concentration (100 µg/mL) revealed that the inhibition activity was lowered for root extracts and completely inactive for leaf extracts as the percentage inhibition was below 39%. The data obtained with *F. multiflora* reveals the unique property of these extracts to express the ability to confer inhibition against the activity of HIV-1 PR at a concentration as little as 10 µg/mL. Data also points to the varying quantities of the active constituents being synthesized in different parts of the plant. As observed with leaf extracts of *F. multiflora* exhibiting less inhibition indicate low synthesis and availability of active constituents in the leaves of *F. multiflora*.

Other studies have reported on extracts that were tested at low concentrations to exhibit high inhibition, one example was with a study conducted on 18 medicinal plants in China, Japan, and Indonesia, where the authors reported HIV-1 PR inhibition of greater than 70% at

concentrations between 25 and 250 µg/mL. Among the examined extracts, *Belamcanda chinensis*, *Magnolia fargesii*, *Paeonia suffruticosa*, *Phellodendron amurense*, and *Terminalia chebula* were the most active at 25 µg/mL (Omoruyi *et al*., 2020).

The Apocynaceae family has been reported to possess both anti-HIV-1 RT and PR activity. For instance, *Hoodia gordonii* exhibited protease inhibition activity with an IC_{50} of 97.29 ± 0.01 and 63.76 ± 9.01 µg/mL for the ethanol and ethyl acetate extracts, respectively (Kapewangolo *et al*., 2016). The dual inhibition property was also observed with *F. multiflora* as leaf extracts exhibited high anti-HIV-1 RT activity and root extracts demonstrated high inhibition against the activity of HIV-1 PR, with estimated IC_{50} values of 5.34 ± 0.89 µg/mL for extract CL and 0.02 ± 14.98 µg/mL for CR extract. What these findings mean for the societies in sub-Saharan Africa who are greatly impacted by HIV-1 infection is that *F. multiflora* should impede the virus at different stages of the replication cycle. This will ensure inhibition in the case where some virions escape the action of *F. multiflora* anti-HIV-1 RT activity through divergence. The anti-HIV-1 PR activity of *F. multiflora* will then thwart the conversion of immature virions to infectious and mature virions, thereby offering maximum inhibition to curb the replication of HIV-1. The ability of *F. multiflora* to act as dual inhibitors for HIV-1 reveals the wide spectrum of bioactive compounds that will significantly benefit people living with HIV in sub-Saharan Africa and contribute to HIV-1 drug development strategies. With the rise of people living with HIV presenting with cancer, it would be more beneficial for their wholistic treatment if *F. multiflora* can also exhibit anticancer properties.

5.3 Anticancer assay

Despite significant efforts made through anticancer therapies, cancer remains an aggressive killer worldwide. Moreover, synthetic conventional chemotherapeutic agents that are currently being used clinically have not succeeded in fulfilling expectations despite the considerable cost of their development. Targeted cancer therapies have significantly prolonged the lives of cancer suffers; however, they are not devoid of unbearable side effect (Yan *et al*., 2011). Plants are endowed with tremendous bioactive compounds capable of treating cancer of which some have been approved by FDA. As the focal point of anticancer studies is aimed at discovering new, effective, and affordable anticancer drugs, this study therefore deepened the search into elucidating the anticancer profile of *F. multiflora* extracts.

The inhibitory effect of *F. multiflora* extracts on the proliferation of HeLa cells revealed that CR was the only extract that exhibited high inhibition (IC_{50} of 50.49 ± 0.06 µg/mL). However,

this extract did not meet the guideline set limit for crude extracts allotted by the National Cancer Institute (NCI, USA), which acknowledges an IC_{50} < 20 µg/mL as potential anticancer agents (Vijayarathna and Sasidharan, 2012) (Srisawat *et al*., 2013). Water extracts of *F. multiflora* were observed to be ineffective in inhibiting the proliferation of HeLa cells, as cells maintained high viability at all tested concentrations with IC_{50} for WR extracts observed to be above the highest tested concentration (250 µg/mL).

Although the family of Apocynaceae has had major contribution in anticancer studies, with plants such as *Carissa carandas*, *Carissa spinarum*, and *Calotropis procera* having been recognised as potential anticancer agents (Alshehri, 2020). However, the guideline set limit rules out *F. multiflora* to be counted among these plants as none of the extracts prepared in this study were able to exhibit an IC_{50} of 20 µg/mL and below on the proliferation of HeLa cells through MTT. This lack of inhibition is a setback for HIV positive and negative women who are subjected to cervical cancer in the sub-Saharan African countries. These regions are populated with a high burden of cervical cancer which is often diagnosed at a later stage. Moreover, sub-Saharan African regions are stricken by lack of access to necessary health care coupled with lengthy referral channels for treatment (Ba *et al*., 2021).

Plant extracts have demonstrated a broad spectrum of inhibition against multiple cancer cell lines (Alshehri, 2020) (Khan *et al*., 2020), it will therefore be restrictive to rule out *F. multiflora* as an anticancer potential candidate when it has only been tested in one cell line, cervical cancer. With anticancer studies having yielded about 400 compounds that have been isolated from medicinal plants found in African regions (Bayala *et al*., 2020), demonstrates a broad curative potential that the African grown plants possess towards different cancers. Therefore, it is crucial to expand the cell line panel in search of anticancer potential in plant materials. With that said, *F. multiflora* extracts were further investigated for inhibitory effect on the proliferation of a liver cancer cell line, HepG2 cells.

The results obtained from the treatment of HepG2 cells with *F. multiflora* extracts through MTT revealed that DR extract (estimated IC_{50} of 57.77 ± 0.16 µg/mL) exhibited higher inhibition of cancer cell line proliferation compared to other extracts. The least cytotoxic profile was demonstrated by water extracts of *F. multiflora*, which exhibited an IC_{50} above the highest tested concentration. These findings reveal that none of the extracts tested against HepG2 cells can be considered for further anticancer studies.

Apocynaceae family is known for being a great source of alkaloid compounds, that are responsible for conferring the antiproliferative properties in plant materials. One example of

these plants is *Catharanthus roseus*, whose isolated compound, vincristine compound was granted approval by FDA for anticancer treatment (Njoya *et al*., 2017) (Seca and Pinto, 2018). Reports have also been made of Apocynaceae plants that lack anticancer activity even though they tested positive for alkaloids, one example is *Fockea angustifolia*, which was tested against renal (TK10), breast (MCF-7) and human melanoma (UACC-62) cancer cell lines (Stuurmann, 2016). As much as the alkaloids contained in *F. angustifolia* could not inhibit any of the chosen cell lines, does not mean that inhibition cannot be achieved with other types of cell lines. Therefore, ruling out plant extracts as inactive when tested in a limited panel of cell lines would deny plant materials a platform to fully express their potential.

Anticancer studies also require extracts to be tested against normal cells to evaluate the safety of extracts, where a selectivity index (SI) greater than 3 is considered as highly selective (Chothiphirat *et al*., 2019). Therefore, *F. multiflora* extracts were tested against Vero cells and it was revealed that none of the extracts were found toxic to non-cancer cells. Water extracts of *F. multiflora* were highly selective against both HeLa and HepG2 cells. However, due to the NCI guidelines not being met by any of *F. multiflora* extracts, even the SI value as high as that demonstrated by WR against HeLa cells (>7) carries no weight in granting this extract the potential of being considered for further anticancer studies.

An interesting point was made by López-lázaro (2015), stating that bioactive constituents may trigger selective cytotoxic effects at short exposure times and non-selective cytotoxic effects at long exposure times. The selectivity of tested constituents towards cancer cells could be missed if cell viability is assessed after a long incubation period (e.g., 72 hours), but could be detected after a short exposure time (e.g., 4 hours) (López-lázaro, 2015). As much as our standard lab incubation period is 72 hours, which could have resulted into the selectivity being missed, however, water extracts of *F. multiflora* showed high selectivity against both cancer cell lines that were chosen for this study.

Although conventional endpoint assays (such as MTT) are rapid, simple, and inexpensive screening methods, however, there are a lot of limitations experienced with such assays. The first one being the optimization of the entire experiment to select the best time for plate reading, which could prove to be an issue when the selected endpoint time is too short as other cell lines tend to proliferate later. Another limitation with endpoint assay is that of the presence of toxic substances in low concentrations being able to trigger cell activity (Stefanowicz-Hajduk and Ochocka, 2020). To circumvent these limitations, the inhibitory effect of *F. multiflora* extracts

was evaluated in HeLa and HepG2 cells using real-time cell analysis (RTCA) as a confirmatory assay.

5.3.1 Real-Time cell analysis

The RTCA technique measures the effect of tested samples in cells, reflecting the cellular proliferation in real time, growth and morphological changes before and after adding the samples (Stefanowicz-Hajduk and Ochocka, 2020).

The data obtained with *F. multiflora* reveal that the water extracts (WR and WL) exhibited inhibition towards HeLa cells from 56 to 96 hours and the growth curves were observed to resembles that of cisplatin (**Figure 19**). This antiproliferative property of aqueous extracts was not observed in MTT results, as none of the tested concentrations inhibited HeLa cell proliferation below 50%. The RTCA revealed that extract DR tested at 100 µg/mL could not confer inhibition on the proliferation of HeLa cells and the growth curve was observed to mimic that of untreated cells. However, low tested concentrations (25 and 50 µg/mL) of DR were able to inhibit HeLa cells. The notable observation made with data from RTCA is that low tested concentrations (25 and 50 µg/mL) exhibited more antiproliferative capacity compared to 100 µg/mL and extract DL at low tested concentrations conferred even greater antiproliferative ability than cisplatin. This capacity of extracts DR and DL to confer antiproliferative property even though they are from different parts of the plant could be attributed to the solvent used in the extraction of these extracts, diethyl ether. The stable nature of diethyl ether solvent and its ability to extract numerous compounds such as alkaloids, terpenoids, coumarins, which have been established as compounds that contain the antiproliferative property. Additionally, the low boiling point nature of diethyl ether also plays a major role in preventing the degradation of extracted compounds by heat (Abubakar and Haque, 2020). These properties of diethyl ether serve to preserve the extracted compounds from being denatured or inactive.

Monitoring the treatment of HeLa cells in real time proved to reveal the properties of *F. multiflora* extracts which were undermined by the MTT assay. As IC_{50} obtained from RTCA for extract CL was 63.32 µg/mL while the one obtained from MTT was 100.4 µg/mL and the difference between these is substantial. Not only did RTCA reveal IC_{50} values of extracts that were not detected with MTT but it also revealed the toxicity profile of WR extract with IC_{50} of 35.53 µg/mL. Moreover, RTCA gave lower IC_{50} values than those obtained with MTT assay.

The treatment of HepG2 cells with *F. multiflora* showed a concentration-dependent antiproliferative activity with extracts DR, WR, CL and DL exhibiting inhibition greater than that of cisplatin (**Figure 22**). After 64 hours of treatment with *F. multiflora*, extract DR, WR and WL depicted a gradual decline in the Cell Index, this indicated that cells were being compromised by the extracts. On the other hand, extract DL and CL depicted a progressive decline in the proliferation of HepG2 cells after 32 hours of treatment, while other extracts started compromising cells after 62 hours. These observations provide a firmer ground to conclude that a point made by López-lázaro, (2015), which stated that compounds may trigger selective toxicity effects at short exposure times and non-selective toxicity effects at long exposure times, is only true for endpoint assays and not cells monitored in real time as the entire duration of treatment with *F. multiflora* extracts was 96 hours. The data obtained from RTCA depict that the selected time frame for the treatment does not determine the inhibitory effects of *F. multiflora* extracts, however, it is the ability of active compounds contained in extracts conferring inhibition faster than other extracts.

The data obtained from RTCA excessively demonstrated the benefits of this assay, which strengthens the importance of considering a cytotoxicity confirmatory assay. As it was noted that RTCA revealed lower IC_{50} values lower than those obtained from the MTT assay. The same observation was observed in a study conducted on the bioactivity of metallodrugs, where RTCA revealed different values than those obtained from MTT (Gama, 2013). End-point assays may be faster and cheaper, yet they bear a limited view on the studied samples. As research work is put out there for further studies, therefore, presenting only endpoint cytotoxicity data is like telling an incomplete story. This could prove to be an issue when *in vivo* studies are considered and could be the reason why most research studies fail to even make it to clinical studies. The difference between IC_{50} values obtained from MTT and RTCA in this study revealed how extracts may be ruled out as inactive when in fact they do confer inhibition, which is why it is crucial to factor-in a confirmatory assay.

This study revealed that none of *F. multiflora* extracts meet the NCI criteria for consideration as anticancer agents. However, RTCA has demonstrated the inhibitory effect of some of *F. multiflora* extracts, which may still reduce the progression of cervical and liver cancer in sub-Saharan African societies who are suffering from these cancers. To further expand the curative profile of *F. multiflora* the antioxidant property of extracts was evaluated.

5.4 Antioxidant assay

The extracts of *F. multiflora* were finally evaluated for their effects on the production of nitric oxide radical, a proinflammatory mediator (Njoya *et al*., 2017). Literature has revealed the promiscuous behaviour of HIV-1 infection to interfere with the antioxidant defence systems (Ivanov *et al*., 2016) and if the system is compromised this results in the overproduction of free radicals which include nitric oxide. The chronic exposure to nitric oxide is associated with inflammatory conditions that lead to the development of various cancers (Boora *et al*., 2014). The interplay between HIV-1 infection, the development of cancers and the interference with the antioxidant defence system highlight the importance of evaluating the scavenging capacity for the wholistic treatment. The extracts of *F. multiflora* were investigated for the potential to scavenge the nitric oxide produced by HeLa and HepG2 cells.

5.4.1 Antioxidant assay of NO production in HeLa cells

Water extracts of *F. multiflora* (WR and WL) inhibited the production of nitric oxide in HeLa cells far greater than other extracts (**Figure 24**). It was noted that the lowest tested concentration was not that much effective in inhibiting the nitric oxide production, especially extract DL tested at 4 µg/mL was observed to almost be at the same level as untreated cells. Another observation was made with the highest tested concentration (250 µg/mL) of DR, WR, WL and CL, which showed a reduction in the strength of the inhibitory effect of this concentration. Additionally, Vitamin C drastically reduced the production of NO, however, vitamin C demonstrated the same trend that was observed with *F. multiflora* extracts tested at highest concentration. This trend could be an indication of the inhibition effect being due to the antioxidative profile and/or nature of the tested sample rather than the increase in the concentration. This was confirmed in the study conducted by (Kapewangolo *et al*., 2015) on the *Ocimum labiatum*, where an intermediate concentration of 25 µg/mL inhibited NO to undetectable levels.

The intermediate tested concentrations of *F. multiflora* for all extracts were the ones that exhibited greater inhibitory effect towards the production of nitric oxide in HeLa cells. The estimated IC_{50} were too low to be detected and all tested extracts were found to be statistically significant ($p = 0.0001$).

5.4.2 Antioxidant assay of NO production in HepG2 cells

The treatment of HepG2 cells with *F. multiflora* revealed that extracts exhibit high inhibition with estimated IC_{50} ranging from 0.001 ± 1.21 to 2.02 ± 0.09 µg/mL and all extracts were found

to be statistically significant ($p = 0.0001$). The water extracts of *F. multiflora* demonstrated the greatest inhibitory effect inhibiting the production of NO to almost undetectable levels (estimated IC_{50} of 0.43 ± 0.07 µg/mL for WL and 2.02 ± 0.09 µg/mL for WR). This unique capacity of water extracts to confer greater inhibitions have been reported, one report is the study conducted on *Padina tetrastromatica* where water extract inhibited the NO production at 16.6 ± 1.8 µM while methanolic extracts inhibited at 24.7 ± 2 µM both at the tested concentration of 100 µg/mL (Subramaniam *et al.*, 2019).

The link between the production of nitric oxide and inflammation has been reported in the study of *O. labiatum*, where the ability of diterpenoids was confirmed to be responsible for the anti-inflammatory abilities through nitric oxide inhibition (Kapewangolo *et al*., 2015). This could suggest the possibility of *F. multiflora* to be a potential source of diterpenoids, which may have greatly contributed to the inhibition effect of the extracts.

The data obtained with antioxidant evaluation of *F. multiflora* indicate that extracts stand a better chance of providing holistic curative properties which will enhance the antioxidant defence system and contribute to reducing inflammatory conditions thereby alleviating susceptibility to cancer development. The treatment of HepG2 cells with *F. multiflora* was observed to be highly inhibited compared to HeLa cells, these findings will be beneficial to women in sub-Saharan Africa who are suffering from cervical cancer and even greater news for people living with HIV who suffer from liver cancer that has been a cause of deaths among people living with HIV due to a prolonged intake of HAART.

6. Conclusion

This study revealed for the first time that the extracts of *F. multiflora* possess anti-HIV-1 reverse transcriptase and protease activity which can inhibit different stages of the HIV-1 life cycle thereby increasing chances of curbing the viral infection. *Fockea multiflora* demonstrated moderate anticancer activity towards cervical and liver cancer cells. Extracts of *F. multiflora* also exhibited high scavenging ability against the production of nitric oxide radical species. Water extracts of *F. multiflora* proved to be prominent as they demonstrated inhibitory properties in all evaluation points of this study, making water to be the best solvent in preparing *F. multiflora* extracts.

This study has presented promising evidence of the curative capacity of *F. multiflora* and the findings will be of great benefit in the drug discovery field. Moreover, the presented data will strengthen the value of *F. multiflora* in the sub-Saharan African regions, where there is less access to health care facilities. The escalating numbers of people living with HIV/AIDS who are at high risk of developing cancers could greatly benefit from further research into *F. multiflora*, a plant that is readily available in their own backyard and which is already part of their diet.

ANSWERS TO RESEARCH QUESTIONS

The hypothesis for this case study was that "*Fockea multiflora* extracts should inhibit HIV-1 enzyme activity *in vitro* and exhibit additional anti-proliferative and antioxidant abilities". To confirm this possibility made at the beginning of this investigation, certain questions had to be considered and they are as follows:

1. **Do *F. multiflora* extracts possess the ability to inhibit HIV-1 RT and PR?**

   Extract WR, WL, CL and DL of *F. multiflora* were able to inhibit HIV-1 RT with over 50% inhibition at all tested concentrations while CR managed to inhibit HIV-1 RT above 50% at 10 µg/mL and extract DR exhibited above 50% at 10 and 100 µg/mL. Extracts CR and DR were found to be statistically significant ($p = 0.0085$ and 0.0406, respectively).

   All root extracts (CR, DR and WR) were able to inhibit HIV-1 PR above 50% at all tested concentrations, while leaf extracts showed inhibition above 50% only at lowest and intermediate tested concentrations and extracts CR, DR, WR and CL. Extracts CR and WR were found to be statistically significant ($p = 0.0085$ and 0.0283, respectively).

2. **<u>What is the nature of the cytotoxicity of *F. multiflora* extracts in PBMCs and TZM-bl cells?</u>**

   F. multiflora extracts were found to exhibit low cytotoxicity towards TZM-bl cells as all CC_{50} were above 50 µg/mL. Extracts CL and DL of *F. multiflora* exhibited high cytotoxicity (10 – 30 µg/mL) towards PBMCs, while extract CR and DR exhibited moderate cytotoxicity (30 – 50 µg/mL) and WR gave low cytotoxicity with CC_{50} >50 µg/mL.

3. **<u>Can *F. multiflora* confer antiproliferative ability in cancer cell lines; HeLa and HepG2 and based on their SI values what is the safety profiles of the extracts?</u>**

   Data obtained from the end-point microscopy-based analysis revealed that extract WR and WL were not able to inhibit the proliferation of HeLa nor HepG2 cells. All the other extracts showed a dose-dependent inhibition towards both cell lines. None of the extracts of *F. multiflora* were able to inhibit the proliferation of cancer cells below 50%. Even though the selectivity index proved the safety profiles of extract WR and WL (SI above 3), none of the extracts met the NCI criteria for consideration as anticancer agents.

4. **<u>What is the effect of *F. multiflora* extract on cell proliferation in HeLa and HepG2 cells when comparing the end-point microscopy-based analysis and real-time cell analysis?</u>**

   The continuous readouts of the effect of *F. multiflora* extracts in HeLa and HepG2 cells in real time revealed an IC_{50} lower than those obtained from the end-point analysis. The proliferation of cancer cells revealed that some extracts DR (IC_{50} of 49.75 µg/mL) and WR (IC_{50} of 41.28 µg/mL)) exhibited moderate inhibition towards HepG2 cells and extract WR (IC_{50} of 35.53 µg/mL) also exhibited antiproliferation towards HeLa cells. Growth curves obtained from RTCA revealed that some extracts of *F. multiflora* exhibited antiproliferative capacity greater than cisplatin.

5. **Is *F. multiflora* able to scavenge the production of nitric oxide in HeLa and HepG2 cells?**

   Extracts WR and WL were able to inhibit NO production in HepG2 cells to almost undetectable levels. *F. multiflora* extract exhibited the highest inhibition with HepG2 compared to HeLa cells. The highest tested concentration (250 µg/mL) in most extracts appeared not to confer much inhibition, indicating that the scavenging ability of extracts was not due to toxicity.

Table 5| Summary of positive response from analysed *F. multiflora* extracts.

ACTIVITY	EXTRACTS
HIV-1 RT inhibition	WR, WL, CL, DL
HIV-1 PR inhibition	CR, DR, WR
Non-toxic to PBMCs	WR
Non-toxic to TZM-bl cells	CR, DR, WR, WL, CL, DL
Antiproliferative activity through RTCA in HeLa cells	WR
Antiproliferative activity through RTCA in HepG2 cells	DR, WR
Antioxidant activity	DR, WR, WL, CL, DL

Future studies could begin with a determination of metal ions in *F. multiflora* extract especially those that exhibited anti-HIV-1 RT activity, as metal ions have been reported to contribute to plant materials conferring anti-HIV-1 RT activity.

Fractionation of *F. multiflora* extracts must be done to eliminate constituent interference, as plant extracts consist of a mixture of phytoconstituents which have been proven to interfere with some of the assays. This will enhance the chances of identifying specific anti-HIV-1 and antioxidant compounds with a low cytotoxic profile. Moreover, fractionation will determine whether isolated active compounds are more effective than a pool of compounds synergistically or antagonistically working together in *F. multiflora* extracts.

The mechanism of action of the active compounds can then be considered for *in vivo* and possibly clinical studies.

Viral entry studies could also be performed expand the search for anti-HIV-1 properties of *F. multiflora* as this will increase the chances of the virus to be curbed in different stages of the HIV-1 replication cycle. With *F. multiflora* having proven to exhibit inhibitory activity against two of the crucial steps of HIV-1, adding viral entry inhibition activity will make *F. multiflora* to contribute greatly to drug discovery strategies in overcoming the issue of resistance with current conventional entry inhibitors.

Additionally, the anti-inflammatory potential of *F. multiflora* extracts could further be investigated as inflammation is responsible for inducing numerous pathological conditions including cancer.

This study could revisit the anticancer studies using fractions, and expand the types of cancer cell lines, as one of the crude extracts had an IC_{50} not too far from that set by NCI for promising drug discovery studies. Then the correlation between the scavenging and antiproliferative capacity of *F. multiflora* can be analysed, to evaluate the strength or intensity that the antioxidant profile contributes towards extracts conferring antiproliferative activity.

As *F. multiflora* has not been extensively investigated, that means a lot of areas of enquiry remain. Therefore, this study could bear more weight if collaboration could be done with:
(1) microbiology field to investigate the antimicrobial activity of *F. multiflora*.
(2) Diabetes field to investigate the wound healing property of *F. multiflora* as people in Africa have been reported to use this plant to heal backaches. This can be monitored either *in vitro* in tissue cultures or *in vivo* on mice.

Results obtained throughout this study have demonstrated how the roots of the plant can exhibit different activity from the leaves. It can formerly be concluded that every part of the plant has its special role, therefore all sections of the plant ought to be investigated. The latex part of *F. multiflora*, the bark and/or flowers, all should be equally investigated.

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Appendix I: Reagents, equipment, and software information

Reagents	Catalogue number	Company	Country
Acetyl Pepstatin (AP)	A4815	Sigma-Aldrich	Germany
Antibiotic/Antimytotic	A5955-100ML	Sigma-Aldrich	Germany
Cisplatin	P4394-100MG	Sigma-Aldrich	Germany
DMEM media	D5648-10X1L	Sigma-Aldrich	Germany
DMSO	D2650-5X5ML	Sigma-Aldrich	Germany
EDTA-anti-coagulant vacuette tubes	VGRV454021	Greiner Bio-one, Lasec	SA
Ficoll - Histopaque®-10771	10771-100ML	Sigma-Aldrich	Germany
Foetal-Bovine Serum	S181G-500	Celtic Molecular Diagnostics	SA
Gentamicin	G1272-10ML	Sigma-Aldrich	Germany
HEPES buffer	SH30237.01	Separations Scientific	SA
HIV-1 protease substrate	H6660-1MG	Sigma-Aldrich	USA
HIV-1 Reverse transcriptase colorimetric kit	11468120910	Roche Applied Science	Germany
NED solution	33461	Sigma-Aldrich	Germany
Neutral red assay kit – cell viability/cytotoxicity	Ab234039	Abcam	UK
Nitrite solution		Sigma-Aldrich	Germany
Phytohemagglutinin – protein (PHA)	L9017-5MG	Sigma-Aldrich	Germany
rec HIV-1 Protease enzyme (expressed in E. coli)	H-9040	Bachem	Switzerland
rec HIV-1 Reverse transcriptase enzyme (expressed in E. coli)	382129-500U	Merck	Germany
RPMI-1640 media	R6504-10X1L	Sigma-Aldrich	Germany
Sodium nitrite	67398	Sigma-Aldrich	Germany
Sodium pyruvate	SH30239.01	Thermo Fisher Scientific	USA
Sulfanilamide solution	00187	Sigma-Aldrich	Germany
Thiazolyl Blue Tetrazolium Bromide (MTT)	M2128-1G	Sigma-Aldrich	Germany
Trypan blue	C0775-25G	Sigma-Aldrich	Germany
Trypsin 0.25% (1X) Solution	SV30031.01	Sigma-Aldrich	UK

Appendix II: Data Analysis Formulae

Formula 1: Calculation of required volume of DMSO to dissolve extracts

$$\text{DMSO } (\mu\text{L}) = \text{Extracts(g) x } 50\ 000$$

Formula 2: Calculations of percentage enzyme inhibition

$$\%\text{Activity} = \frac{(\text{Ave absorbance extract}) - (\text{Ave absorbance blank})}{(\text{Ave absorbance positive}) - (\text{Ave absorbance blank})} \text{ x}100$$

$$\%\text{ Inhibition} = 100 - \%\text{ Activity}$$

Formula 3: Cell concentration calculation using haemocytometer

$$\text{Concentration} = \frac{\text{Live cells}}{\text{Number of quatrants counted}} \text{ x dilution factor x } 10^4$$

Formula 4: Cell percentage viability

$$\%\text{ Viability} = \frac{(\text{Ave OD extract}) - (\text{Ave OD blank})}{(\text{Ave OD untreated cells}) - (\text{Ave OD blank})} \text{ x } 100$$

$$\%\text{ Cytotoxicity} = 100 - \%\text{ Viability}$$

Formula 5: Selectivity index calculation

$$\text{SI} = \frac{\text{Vero cells IC}_{50}}{\text{HeLa/HepG2 cells IC}_{50}}$$

Appendix III: Cell Viability

Figure A1 and A2 represent the data obtained using MTT colorimetric dye, which revealed the interference of reducing agents with MTT indicators, while figure A3 represent the data obtained using NRU assay.

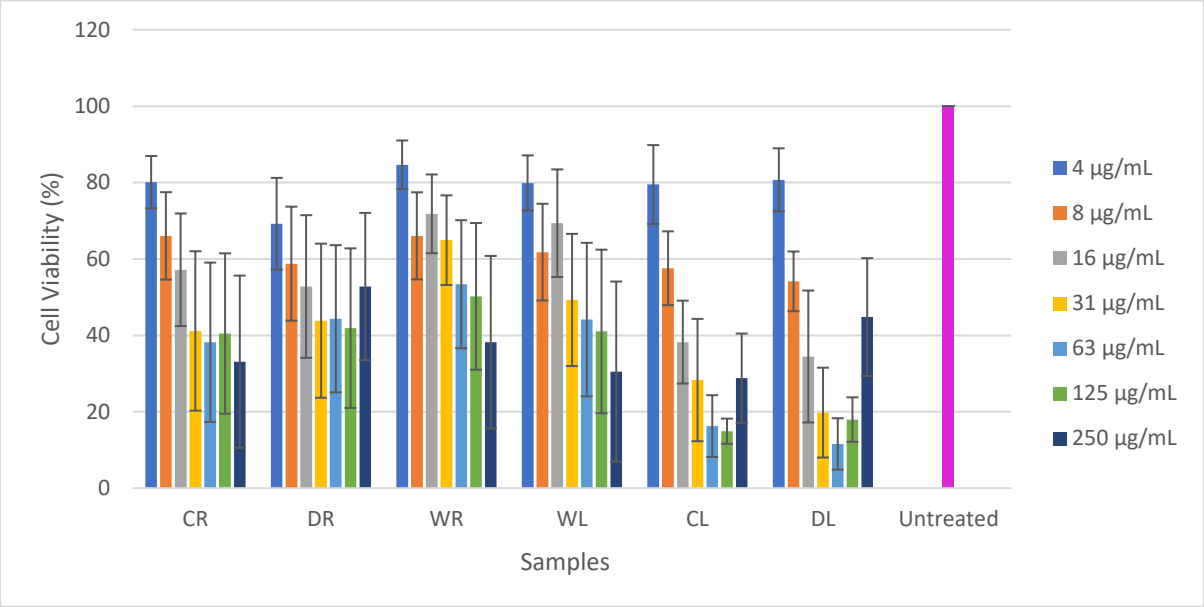

Figure A1| The effect of *F. multiflora* extracts in PBMCs using MTT assay.

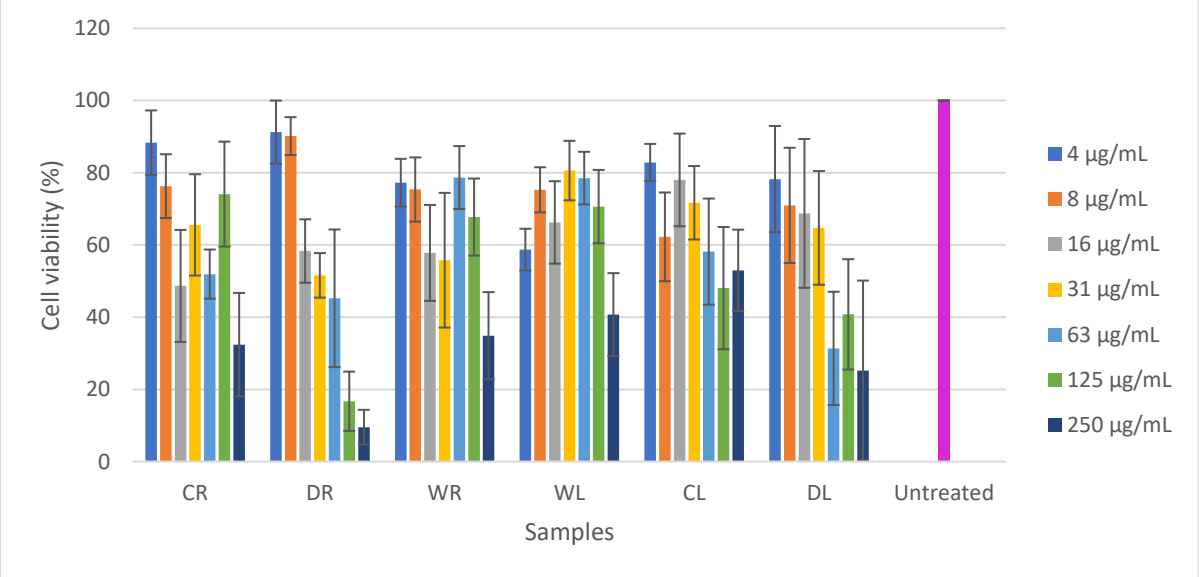

Figure A2| The effect of *F. multiflora* extract in TZM-bl cells using MTT assay.

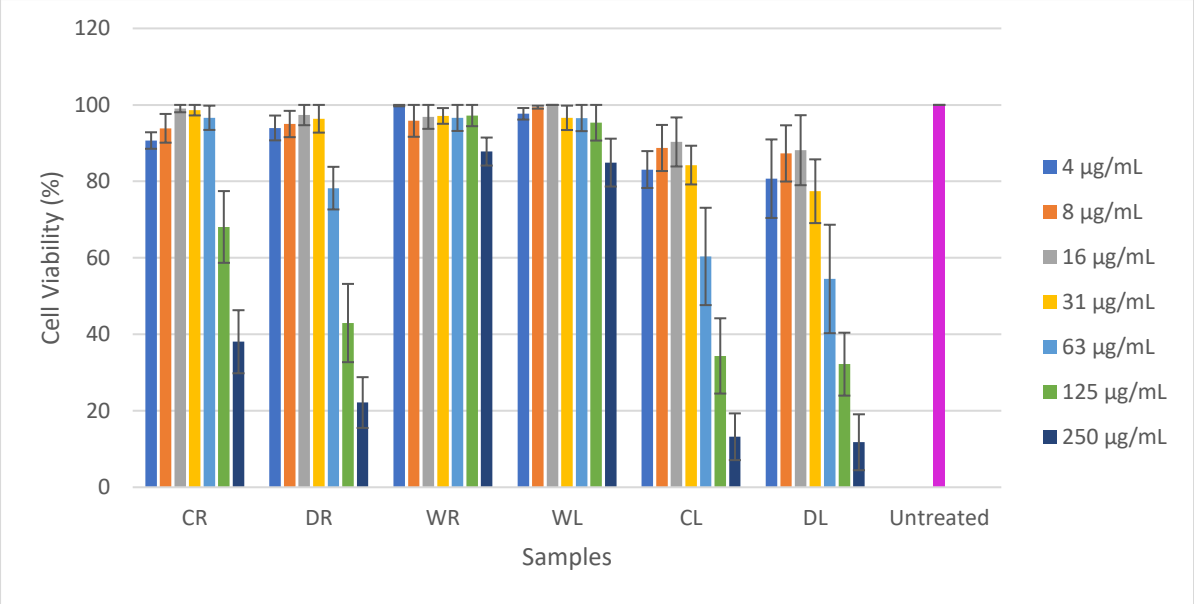

Figure A3| The effect of *F. multiflora* extracts in TZM-bl cells using NRU assay, depicting dose-dependent curves for the extract which was not obtained with the MTT assay.

APPENDIX IV: Troubleshoots

Troubleshoot 1: Cell Viability Assay

NRU assay for Hela and HepG2 cell lines revealed ambiguous results like that obtained from the initial MTT assay for TZM-bl cells. This interference may be attributed to the type of cell lines as the NRU assay conducted in TZM-bl cells gave dose-dependent curves.

Then MTT assay was conducted in HeLa and HepG2 cells and again ambiguous results were obtained. The MTT assay was conducted with modifications of double washing step with 1X PBS before and after addition of MTT dye. The modified steps were adopted from the NRU assay, and this proved to be effective as dose-dependent curves were obtained from analysed data.

Troubleshoot 2: Nitric Oxide Assay

The followed protocol prescribed by (Wu and Yotnda, 2011) and www.promegra.com was set to be read at 520 and 550 nm, however, the nitrite standard reference curve assumed an inverse trendline to that which was expected (**Figure A1**).

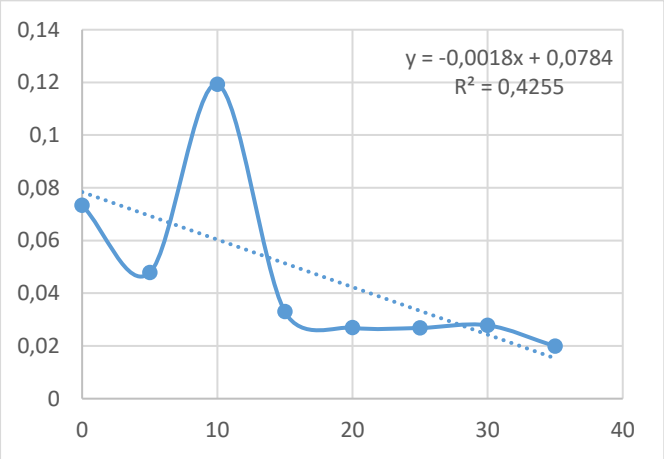

Figure A1| Depicting the ambiguous nitrite standard reference curve initially obtained.

- A study conducted by (AsokKumar *et al*., 2009) (Boora *et al*., 2013) (Skouta *et al*., 2018) revealed that the wavelength used for nitric oxide assay was 564nm. The effect of *F. multiflora* extract on the production of NO in HeLa cells was then evaluated using 564 nm, but this did not work.
- Parameter™ protocol guide stated that proteins in the media tend to interfere with the Griess Reaction, then the media was filtered. Again, this did not work.

- Some researchers have conducted Griess Reaction by mixing Griess Reagents instead of adding one after the other with incubation period of 5 to 10 minutes in between. So, the point taken from this was to add reagents subsequently (that is NED solution being added immediately after sulfanilamide solution). The absorbance was read at 540 and 690 nm. This method proved to be effective and was adopted as the protocol to be used for crude extracts in our lab.