CYTOTOXIC, ANTI-INFLAMMATORY, ANTIBACTERIAL AND ANTI-HIV EFFECTS OF *TABERNAEMONTANA ELEGANS* LEAVES AND FRACTIONS

by

MASHILO MAPHEPELE SARA

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PRIVATE BAG X5050

THOHOYANDOU

Supervisor : Prof AN Traore

Co-Supervisor: Prof N Potgieter

Co-Supervisor: Dr CP Anokwuru

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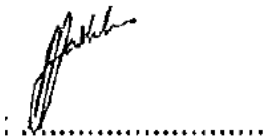
DECLARATION

I, MS Mashilo (Student # 11640422), hereby declare that this mini-dissertation submitted to the University of Venda for the degree Master of Science in Microbiology is my own work in design and execution and it has not been previously submitted by me or anyone else at this or any other Universities. The materials contained therein have been duly acknowledged.

Signature: 

Date: 11 June 2020

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DEDICATION

This is dedicated to:

To the late Lethuba Maphepele a' ntshi e tala and Mashilo Belly Pheladi a' Kanyane le Mologadi …..women of great wisdom…..thank you very much for the wisdom you both had instilled in me. To date, I am still guided by it. Dear father (Mashilo T. Doktor) your trust in me and my intelligence had immensely built up my self-efficacy and to that I will always be grateful. To my family of four siblings I would like to extend my sincere gratitude to you all for your Love and Support in everything I needed to accomplish this work.

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ABSTRACT

Cytotoxic, Anti-inflammatory, Antibacterial and Anti-HIV effects of *Tabernaemontana elegans* leaves and fractions

Mashilo MS, Traoré AN, Anokwuru CP and Potgieter N

Background: Plant-derived natural products continue to serve as a reservoir for the discovery of new medicines. In South Africa, the use of plants to treat many diseases is widely practiced. This is due to high cost of conventional drugs, cultural beliefs and assumed safety. *Tabernaemontana elegans* is a medicinal plant which is traditionally used for the treatment of a wide variety of ailments such as pulmonary diseases, chest pains, heart diseases, cancer, sexually transmitted infections and applied as a wash to wounds. However, there is not enough literature concerning the chemical constituents responsible for its ethnomedicinal properties. There have been no reports about the antimicrobial activity of the plant on selected opportunistic bacteria nor any reported anti-HIV and anti-inflammatory effects of *T. elegans* leaf fractions as far as literature is concerned.

Objective: The aim of the study was to determine the antimicrobial activity against opportunistic bacteria, the anti-HIV-1 reverse transcriptase effect, anti-inflammatory activity and cytotoxicity of *T. elegans.*

Methods: *T. elegans* leaves were collected, dried and ground into powder using a miller. Three solvents (hexane, dichloromethane and methanol) were used to successively extract compounds of different polarities. Column chromatography was used to isolated compounds from the dichloromethane and methanol extracts. Qualitative phytochemical and antioxidant (DPPH) screening was carried out on the extracts and fractions obtained. Antibacterial activity of the crude extracts and fractions was evaluated against *Escherichia coli*, *Pseudomonas aeruginosa, Staphylococcus aureus* and *Streptococcus pyogenes* using the Microdilution assay. The anti-HIV-1 RT activity was measured using a commercial ELISA kit. The crude extracts and fractions were assessed for anti-inflammatory potential by detection of nitric oxide (NO) production on stimulated macrophage cells and cytotoxicity of the

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plant was assessed on Vero cells as well as on HeLa cancer cells using the dual staining microscopy assay.

Results: Seven fractions were obtained from dichloromethane (DCM), C2 and methanol (MeOH), C3 crude extracts. Four fractions (FD1, FD2, FD3 & FD4) and 3 fractions (FM1, FM2 & FM3) resulted from DCM and MeOH crude extracts respectively. Phytochemical analysis revealed the presence of saponins, flavonoids, terpenoids and steroids. The crude extracts and all fractions displayed a free radical scavenging activity, wherein FM3 showed a profound free radical activity. For antibacterial activity, the lowest MIC noted was 0. 625 mg/mL from the crude extracts and fractions against *S. aureus*, *S. pyogenes, E. coli* and *P. aeruginosa.* Among all the strains, *S. pyogenes* was the most susceptible. Moreover, FD4 was the most active for antibacterial activity. FD3 showed strong anti-inflammatory potential, since it had the lowest NO reduction with the cell viability > 66%. FD4 showed strong anti-inflammatory activity with severe cytotoxicity at 50, 100 and 200 µg/mL. All extracts and fractions displayed anti-HIV-1 RT activity. FD1 exhibited a potent anticancer activity with less or no harm to Vero cells, whereas FD3, FD4, FM2, C2 and C3 were found to be toxic to both non-cancerous Vero cells and cancerous HeLa cells.

Conclusion: Our findings provide evidence that extracts and fractions from *T. elegans* leaves displayed antioxidant, antibacterial anti-inflammatory and anti-HIV-1 activities. Findings from this study further validates the traditional use of *T. elegans* leaf for the treatment of a wide range of diseases.

Keywords: Anti-inflammation, Cytotoxicity, Fractions, Reverse transcriptase, *Tebernaemontana elegans.*

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LIST OF ABBREVIATIONS

%	Percentage
°C	Degree celcius
µg/mL	Microgram per mililiter
µL	Microliter
ATCC	American Type Culture Collection
BEA	Benzene/ethanol/ammonium hydroxide
C2	Dichloromethane extract
C3	Methanol extract
CEF	Chloroform/ethyl acetate/formic acid
cm	Centimiter
CO_2	Carbon dioxide
DCM	Dichloromethane
DMEM	Dulbecco's Modified Eagle Medium
DMSO	Dimethyl sulfoxide
E. coli	*Escherichia coli*
EA	Ethyl acetate
ELISA	Enzyme-Linked Immunosorbent Assay
EMW	Ethyl acetate/methanol/water
Ex	Extract
FD1	First Dichloromethane Fraction
FD2	Second Dichloromethane Fraction
FD3	Third Dichloromethane Fraction
FD4	Fourth Dichloromethane Fraction
$FeCl_3$	Ferric chloride
FM1	First Methanol Fraction
FM2	Second Methanol Fraction
FM3	Third Methanol Fraction
g	Gram
hex	Hexane
HCl	Hydrochloric acid

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hr(s)	Hour(s)
INT	p-iodonitrotetrazolium violet
LPS	Lipopolysaccharide
MeOH	Methanol
mg	Miligram
MBC	Minimum bactericidal concentration
MIC	Minimum inhibitory concentration
mL	Mililiter
NaOH	Sodium hydroxide
nm	Nanometer
NO	Nitric oxide
Pen-strep	Penicillin-Streptomycin
PI	Propidium iodine
P. aeruginosa	*Pseudomonas aeruginosa*
®	Registered
RPMI	Roswell Park Memorial Institude
S. aureus	*Staphylococcus aureus*
S. pyogenes	*Streptococcus pyogenes*
T. elegans	*Tabernaemontana elegans*
TLC	Thin-Layer Chromatography
™	Trade Mark
WHO	World Health Organisation

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Chapter 1

GENERAL INTRODUCTION

1.1 BACKGROUND

Indigenous plants are widely used for alternative treatment globally for various types of ailments. To date, the usage of medicinal plants has been escalating exponentially and of note, billions of people in developing regions of the globe are utilizing medicinal plants as alternative medicine (Van Vuuren and Holl, 2017). It was estimated that 80% of the globe's population is depending on medicinal plants to alleviate various ailments (Savithramma et al., 2017). Notably, due to the emergence of widespread antibiotic resistance microorganisms, there is a great interest in identifying and characterizing bioactive compounds from natural products (Adebayo et al., 2015). Most importantly, in South Africa, traditional healers have made claims about the efficacy of some medicinal plants against HIV/AIDS treatment (Sigidi et al., 2017; Tshikalange et al., 2008) and these traditional healers use medicinal plants-based remedies for their trades (Netshivulana et al., 2018).

Tabernaemontana elegans Stapf a deciduous upright small tree belonging to the Apocynaceae family is used traditionally in Mozambique to treat cancer. In South Africa, the root decoction is used to treat pulmonary disease, chest pains, malaria, heart disease and as solution to wash wounds (Lim, 2014; Pallant et al., 2012). Moreover, this plant has been extensively studied on various cancerous cell lines (Koul, 2019; Paterna et al., 2017; Paterna et al., 2016; Mansoor et al., 2013). The root decoction is also known to have aphrodisiac properties (Pallant et al., 2012). *T. elegans* is rich in monoterpenoid indole alkaloids (Mansoor et al., 2009a) which are considered chemotaxonomically important (Bapela et al., 2014; Pallant et al., 2012). Additionally, some monoterpene and bisindole alkaloids (Mansoor et al., 2009a,b) were found to induce apoptosis in certain colon and liver cancer cells (Paterna et al., 2016).

Microbial infections remain a major menace to human health globally (Pallant et al., 2012). The selected opportunistic bacteria (*Escherichia coli, Pseudomonas*

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aeruginosa, Staphylococcus aureus, and Streptococcus pyogenes) in the present study are able to cause serious infections in immunocompromised patients. Moreover, there is an increase in drug-resistant *Staphylococcus* infections which poses a threat to the efficacy of antimicrobial therapy (Lee et al., 2007). *Pseudomonas aeruginosa* is a Gram-negative opportunistic human pathogen that preferentially infects patients with cancer or AIDS, patients immunocompromised by surgery, cytotoxic drug users or burn wound victims (Bosgelmez-Tinaz et al., 2005), people with cystic fibrosis, skin, eye, and genitourinary tract infections (Ugurlu et al., 2016).

Additionally, *P. aeruginosa* is inherently susceptible to a limited number of antibiotics (Merchant et al., 2018). Human Immunodeficiency Virus (HIV) is known for its ability to attack the immune system by damaging specific type of white blood cells, resulting in the weakening of the immune system (Boukandou et al., 2018; Akase et al., 2017; Deeks et al., 2013). Hence, opportunistic infections are known to be the cause of morbidity in patients suffering from HIV infection (Boukandou et al., 2018; Akase et al., 2017). More importantly, chronic inflammatory processes that promote the non-acquired immunodeficiency syndrome-related complications are observed in patients infected with HIV (Delgado-Velez and Lasalde-Dominicci., 2018).

1.2 PROBLEM STATEMENT

Vhavenda people use *T. elegans* roots mostly to treat chest pains, pulmonary diseases and as a wash for wounds. The roots of *T. elegans* have been studied extensively, however little is known about the chemical composition and pharmacological properties of *T. elegans* leaves as well as their safety. This plant's genera has been reported to be highly active against various *in vitro* assays (Silveira et al., 2017). Mostly in plant research different plant parts such as leaf, bark and or roots have different secondary metabolites which confer their biological activities and literature has shown that distribution of secondary metabolites such as phenolics differs from organ to organ (Anokwuru et al., 2015). To the best of our knowledge only a couple of studies based on the leaves of *T. elegans* were reported (De Wet et al., 2012; Mansoor et al., 2009a,b).

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Therefore, the study was undertaken in order to determine the biological propertities (antibacterial, anti-HIV-1 reverse transcriptase, anti-inflammatory activities and cytotoxicity) of *T. elegans* leaves. The increased number of mortality and morbidity is not only due to HIV infection, but also to opportunistic pathogens associated with the disease (Netshivulana et al., 2018; Weldegebreal et al., 2018). Annually, millions of deaths result from infections and pain-related diseases in developing countries (Netshivulana et al., 2018). The plant's roots as well as fractions have been tested against both laboratory bacterial strains and clinical isolates where the crude extracts and alkaloidal subfraction showed significant antibacterial activity (MIC≤256 µg/mL) against mycobacteria and Gram-positive bacteria (Pallant et al., 2012). Importantly, the *T. elegans* root fractions were found to be active against *Mycobacterium tuberculosis* (Luo et al., 2011).

Medicinal plants used by indigenous people around the globe are assumed to be safe and non-toxic. This safety is based on the evidence of non-clinical trials and their efficacy over time. However, scientific studies have shown that some substances contained in some medicinal plants have potentially toxic phytochemicals with toxic, carcinogenic and or genotoxic potential (Demma et al., 2009).

Therefore, the aim of the current study was to determine the antimicrobial activity against selected opportunistic bacteria, anti-HIV-1 reverse transcriptase activity, anti-inflammatory activity and cytotoxicity of *T. elegans* leaves.

1.3 OBJECTIVES

1.3.1 PRIMARY OBJECTIVE

- To evaluate the cytotoxic, anti-inflammatory, antibacterial and anti-HIV RT effects of *T. elegans* leave fractions.

1.3.2 SECONDARY OBJECTIVES

- To obtain crude extracts from *T. elegans* leaves using sequential extraction method with increasing polarity solvents;

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- To obtain fractions from the extracts of *T. elegans* leaves using column chromatography;
- To determine the antibacterial activity of the fractions using Minimum Inhibitory Concentration assay (MIC) and Minimum Bactericidal Concentration (MBC);
- To evaluate the HIV reverse transcriptase effect of the fractions using a RT ELISA kit;
- To determine the anti-inflammatory activity of the fractions by measuring the nitric oxide (NO) inhibition in tissue culture; and
- To determine the *in-vitro* cytotoxicity effects of the plant fractions against Vero and HeLa cancer cell lines using dual staining and fluorescence microscopy.

1.4 HYPOTHESIS

The null hypothesis (Ho): The *T. elegans* leaves and fractions are not effective against the selected opportunistic microorganisms, have no anti-inflammatory and anti-HIV properties as well as having no selective toxicity to cells, otherwise, there is no need for further studies on extracts as it is already known to have the activities.

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Chapter 2

LITERATURE REVIEW

2.1 MEDICINAL PLANTS

Apart from the nutritional value that medicinal plants offer, they have been recognised as an important source of therapeutic products (Lagnika et al., 2016) and people in developing countries depend on medicinal plants to manage their health due to their folkloric beliefs and easy accessibility of plants (Sigidi et al., 2017; Mthethwa et al., 2014). To date, the crisis of antibiotic or drug resistant microorganisms (Salehi et al., 2018) has led to an increase interest in exploring the potent phytochemical constituent in combating it globally; Particularly the multi/extreme drug-resistant tuberculosis (Pallant et al., 2012), an infectious disease that remains the ultimate killer in the world (Luo et al., 2011). Furthermore, the World Health Organisation (WHO) has suggested that medicinal plants should be systemically tested against HIV as they may yield effective and more affordable therapeutic agents (Salehi et al., 2018).

2.2 DESCRIPTION OF *T. ELEGANS*

2.2.1 ORIGIN OF *T. ELEGANS*

T. elegans belongs to the Apocynaceae family which comprises of about 3700 species and 424 genera; It is distributed throughout Africa, America and East Asia. The synonyms of *T. elegans* Stapf include *Conopharyngia elegans* (Stapf) Stapf and *Leptopharyngia elegans* (Stapf) Boiteau (Silveira et al., 2017). The specie was named after Jakob Theodor von Bergzabern, who was an herbalist in the 16th centuries.

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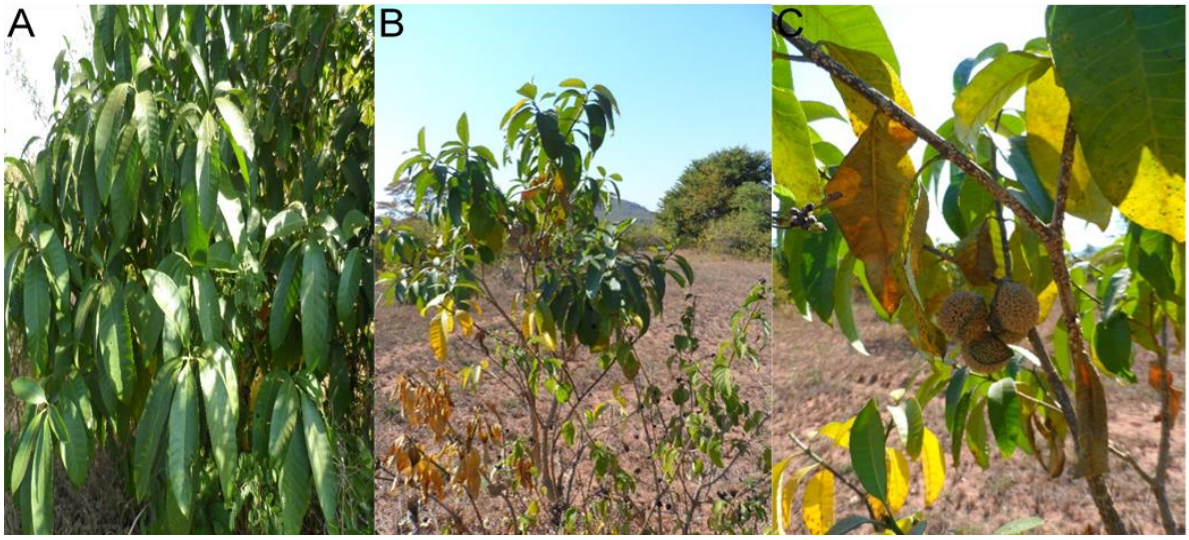

Figure 2.1: Leaves and fruits of *T. elegans* (Mashilo, 2018; unpublished)

2.2.2 BOTANICAL DESCRIPTION

T. elegans Stapf is known to be a beautiful bushveld tree distinguished by its leathery and dark glossy green large leaves (Figure 2.1A), fragrant white flowers and large fruits borne in pairs (Figure 2.1C). The fruits are green, edible with warty skin, resembling a toad, hence its common name “toad tree” (Figure 2.1C). Usually, the fruits split open when mature along one side while still on the tree, displaying the yellowish pulp inside. In Tshivenda language, it is called “Muhatu” which means stopping the pain. Amazingly, the fruits grow in pairs as depicted in the image below (Figure 2.1).

2.2.3 ETHNOMEDICINAL USES OF *T. ELEGANS*

In South Africa, the root decoction is used in the treatment of pulmonary diseases, chest pains, as well as for wound wash mostly by the Zulu and Vhavenda population. Additionally, the root decoction is also known to have aphrodisiac properties. The stem bark, root, and fruits are used for the treatment of cancer, malaria, tuberculosis and heart disease (Pallant et al., 2012). The leaves are used in the treatment of venereal diseases (De Wet et al., 2012).

2.2.4 ISOLATED COMPOUNDS FROM *T. ELEGANS*

Several phytochemical compounds such as Tabernaemontanine and Dregamine have reportedly been isolated from the roots (Paterna et al., 2017). Additionally, bisindole

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alkaloid: (3'R)-hydroxytabernaelegantine C was also isolated from the roots (Paterna et al., 2016); Tabernaemontanine, dregamine, 16-epidregamine, tabernaelegantine C, tabernaelegantinine B, voacangine and vobasine (Dey et al., 2017; Mansoor et al., 2013) were also obtained. Whereas, the following compounds such as monoterpene indole alkaloids, vobasine, tabernaemontanine and eleganine A were isolated from the leaves; figure 2.2 (Mansoor et al., 2009a, b). Fourteen indole alkaloids were isolated from a callus culture of *T. elegans* (Prakash Chaturvedula et al., 2003): (1) 3-oxo-isovoacangine, (2) isovoacangine, (3) 3-*R*/*S*-hydroxy-isovoacangine, (4) 3-*R*/*S*-hydroxy-coronaridine, (5) isositsirikine, (6) geissoschizol, (7) tabernaemontanine, (8) vobasine, (9) vobasinol, (10) apparicine, (11) 16-hydroxy-16,22-dihydro-apparicine, (12) tubotaiwine, (13) 3-*R*/*S*-hydroxy-conodurine and (14) monogagaine (Van der Heijden et al., 1986a).

Despite studies on the alkaloids in *T. elegans,* there are no available literature on the isolation of other classes of compounds. Moreover, there are limited literatures on *T. elegans* leaves as well as the compounds isolated from them.

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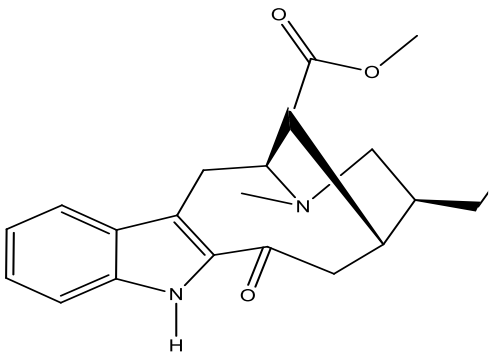
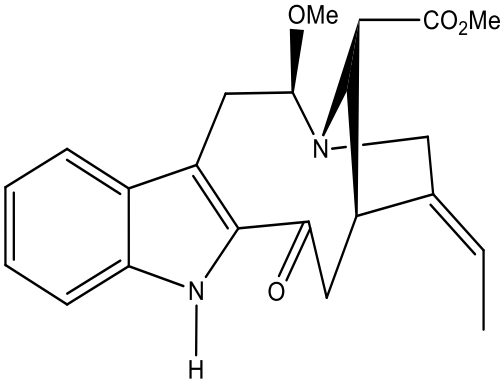
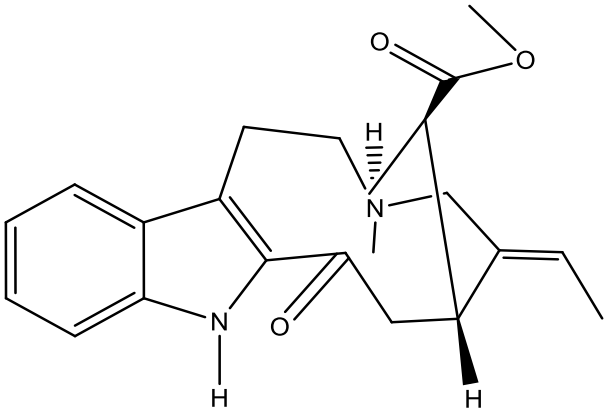


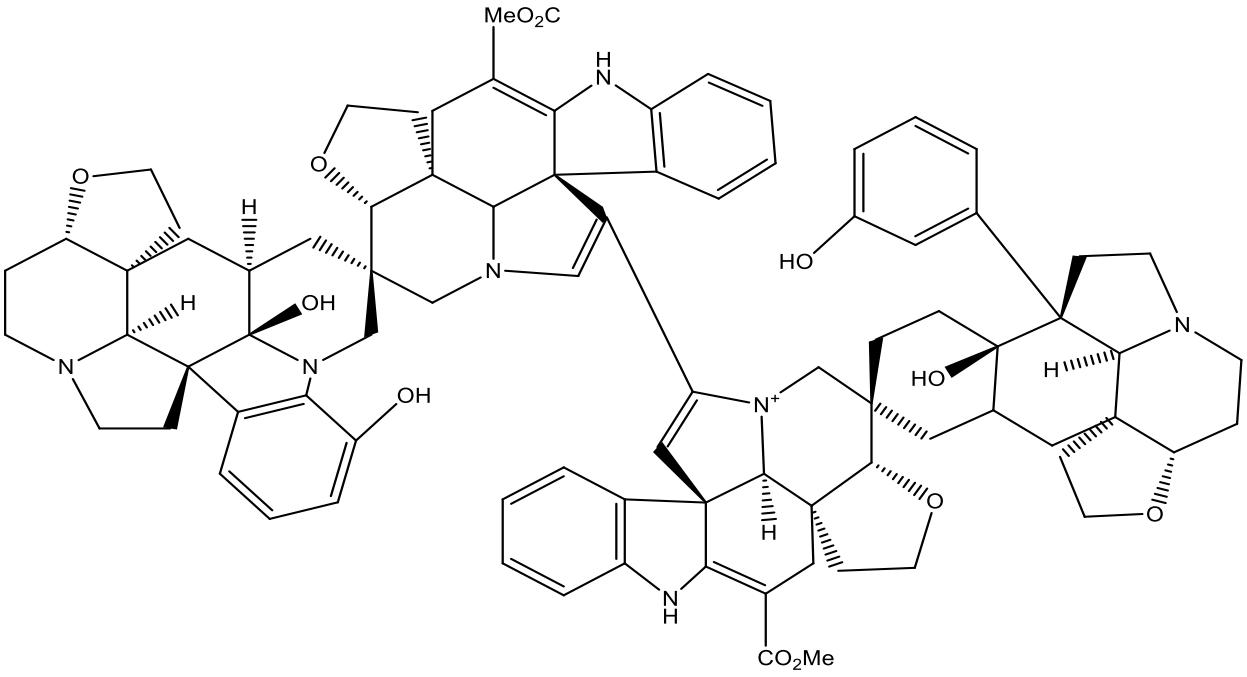
Tabernaemontanine (C21H26N2O3)



Eleganine A



Vobasine (C21H24N2O3)



Alasmontamine

Figure 2.2: Major alkaloids isolated from the *T. elegans* leaf extracts tabernaemontimine, eleganine A, vobasine and alasmontamine (Self drawn using ChemDraw Ultra12).

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2.2.5 PHARMACOLOGICALPROPERTIES OF *T. ELEGANS*

Antimicrobial activity

Multiple *in vitro* studies have shown that the roots of *T. elegans* crude extract, as well as some fractions, were active *against Bacillus subtilis* (ATCC 6633), *Enterococcus faecalis* (ATCC 29212) and *Staphylococcus aureus* (ATCC 12600), the *Mycobacterium tuberculosis* H37RV (ATCC 25177) and *Mycobacterium smegmatis* (ATCC 14468), and the Gram-negative bacteria *Escherichia coli* (Pallant et al., 2012), *Klebsiella pneumoniae* (ATCC 13883) and *Pseudomonas aeruginosa* (ATCC 9027) (Silveira et al., 2017). The root crude extract and alkaloidal fraction were also tested against a clinical isolate of methicillin-resistant *Staphylococcus aureus* (NHLS 363) and a clinical isolate of *Mycobacterium tuberculosis* displaying resistance to isoniazid (MRC 3366) and the MIC value was ≤128 µg/mL (Pallant et al., 2012).

Antifungal activities

The *T. elegans* aqueous root extract was found to have an activity against *Candida albicans*; four different clinical isolates and a referral strain with the MIC range of 0.4-3.30 mg/mL.

Anti-parasitic infections

The *T. elegans* bark extract has been tested for anti-plasmodium activity in *in vitro* testing on infected human erythrocytes. This test resulted in IC_{50} values of 0.3 and 0.8 µg/mL against chloroquine sensitive and resistant Plasmodium falciparum strains respectively (Cock et al., 2019).

Anti-cancer activities

Several *in vitro* studies have shown that *T. elegans* roots extracts, as well as various isolated alkaloids, were active against different types of cancerous cell lines such as resistant colon (HCT116, SW620) and liver (HepG2) cancer cells, HCT116 human colon carcinoma cells; normal human dermal fibroblasts, human lymphocytes, hepG2 hepatocytes (ATCC HB 8065) and THP1 macrophages (ATCC TIB 2.2) HuH-7 cancer cells; multidrug resistance in mouse lymphoma cell lines (Paterna et al., 2017; Paterna et al., 2016; Mansoor et al., 2013; Pallant et al., 2012; Mansoor et al., 2009a,b).

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2.3 ANTI-INFLAMMATORY ACTIVITY OF NATURAL PRODUCTS

Inflammation is a general non-specific innate immune response that protects the body from exogenous entities such as pathogens. This process is mediated by a variety of signalling molecules (Figure 2.3) synthesized by leukocytes and extravasation of plasmatic proteins in the zones of infection as well as the activation of these leukocytes and proteins to eliminate the infectious agents (Wikarta and Kim, 2016; Thao et al., 2013). Acute and controlled inflammation processes are part of an optimal immune response which is beneficial and that allows the body to fight and eliminates potentially pernicious agents effectively (Figure 2.3). However, uncontrolled inflammation is life-threatening and are characteristic of chronic infectious disease such as HIV (Figure 2.4). In HIV patients the literature reports that ART reduces the inflammation, but the inflammation persists if the viremia is reduced to undetectable levels (Delgado-Velez and Lasalde-Dominicci, 2018).

Furthermore, diseases like eczema, arthritis and asthma are inflammatory diseases that are treated with conventional medicine and yet due to the undesired side effects which result after pro-longed usage of conventional medicine, patients tend to use medicinal plants as an alternative. Hence the increase in plant research for the development of novel anti-inflammatory drugs with less or no adverse effects is highly observed. Diets with a high content of antioxidant polyphenols are related to low prevalence of cancer and cardiovascular.

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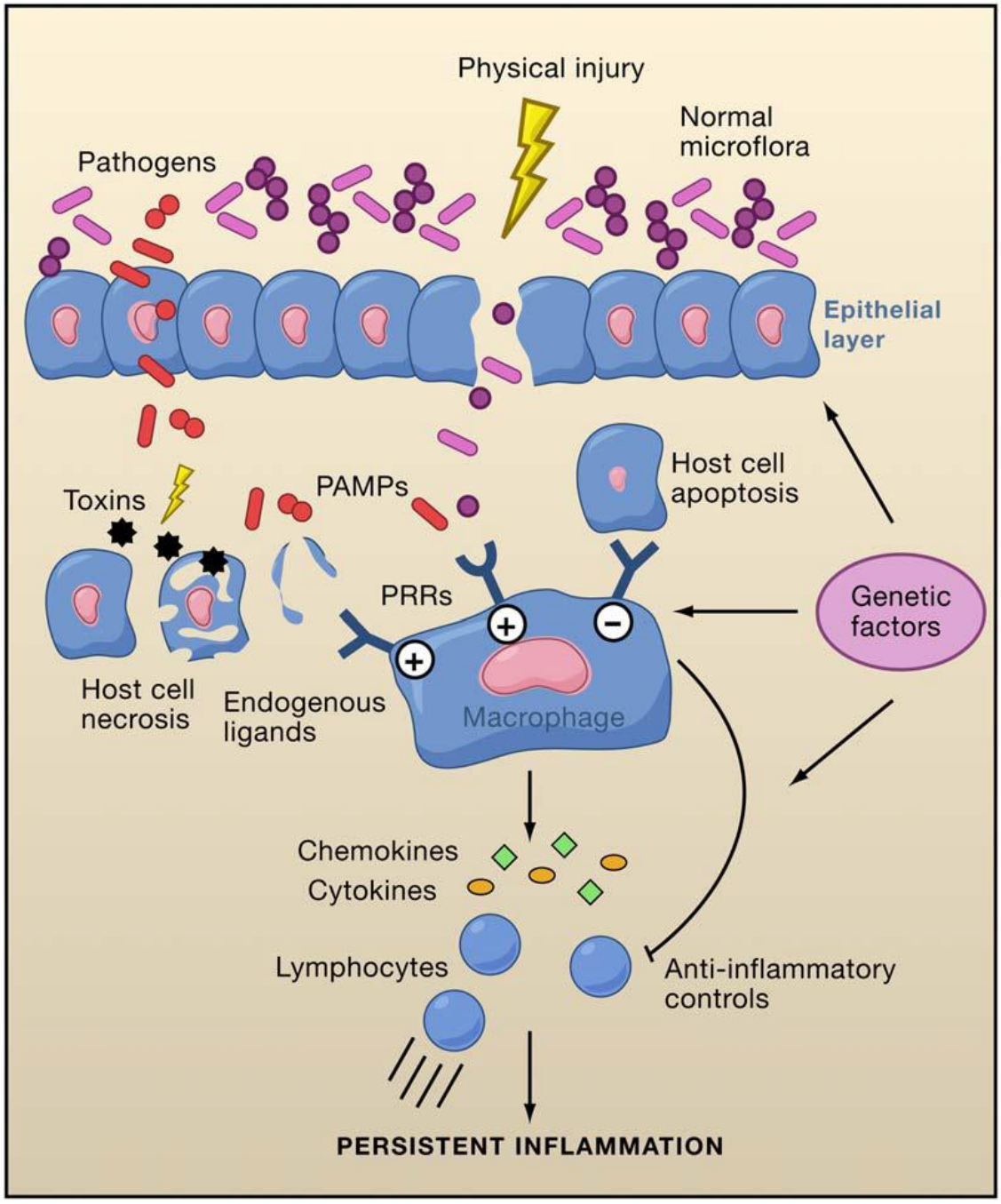

Figure 2.3: Factors which predispose humans' body to experience persistent inflammation (Karin et al., 2018).

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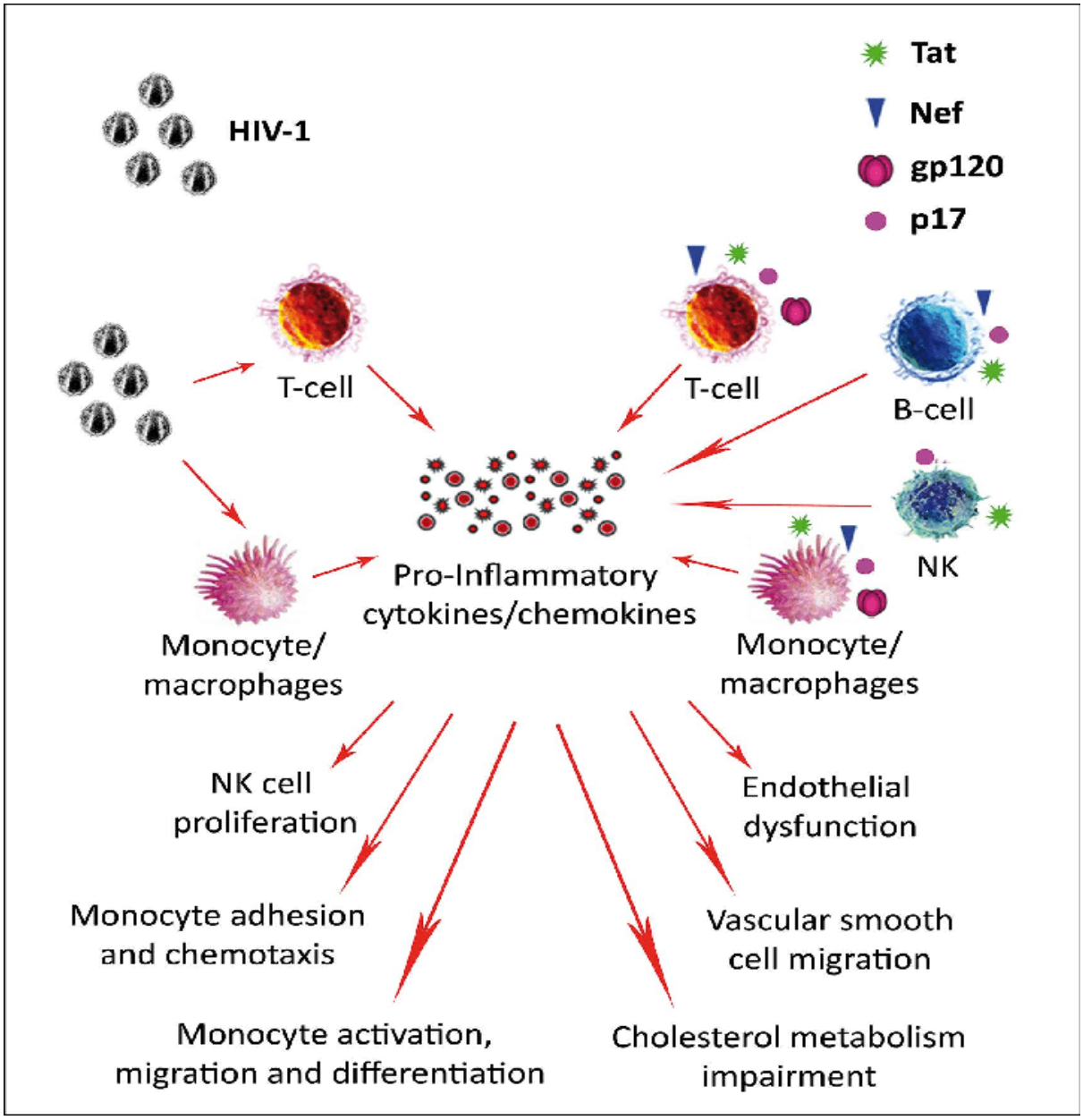

Figure 2.4: Role of HIV-1 proteins in promoting inflammation (Mazzuca et al., 2016).

2.4 ANTI-HUMAN IMMUNODEFICIENCY VIRUS (HIV) ACTIVITY

The World Health Organisation (WHO) estimates that over 75 million people worldwide live with the human immunodeficiency virus (HIV), wherein an approximately 26 million of these patients reside in Africa (Salehi et al., 2018).

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HIV is a retrovirus with the ability to integrate a viral DNA copy into the DNA of the host cells (Figure 2.4). This virus is distinguished by its crucial unique three enzymes required during its replication cycle: (i) reverse transcriptase, (ii) integrase and (iii) protease. The HIV therapies focus on inhibiting many of the stages of replication such as entry as well as the three crucial enzymes (Figure 2.5). Reverse transcriptase inhibitors form a part of the recommended initial regime for antiretroviral therapy (ART) for all adults with HIV (Muswe et al., 2017).

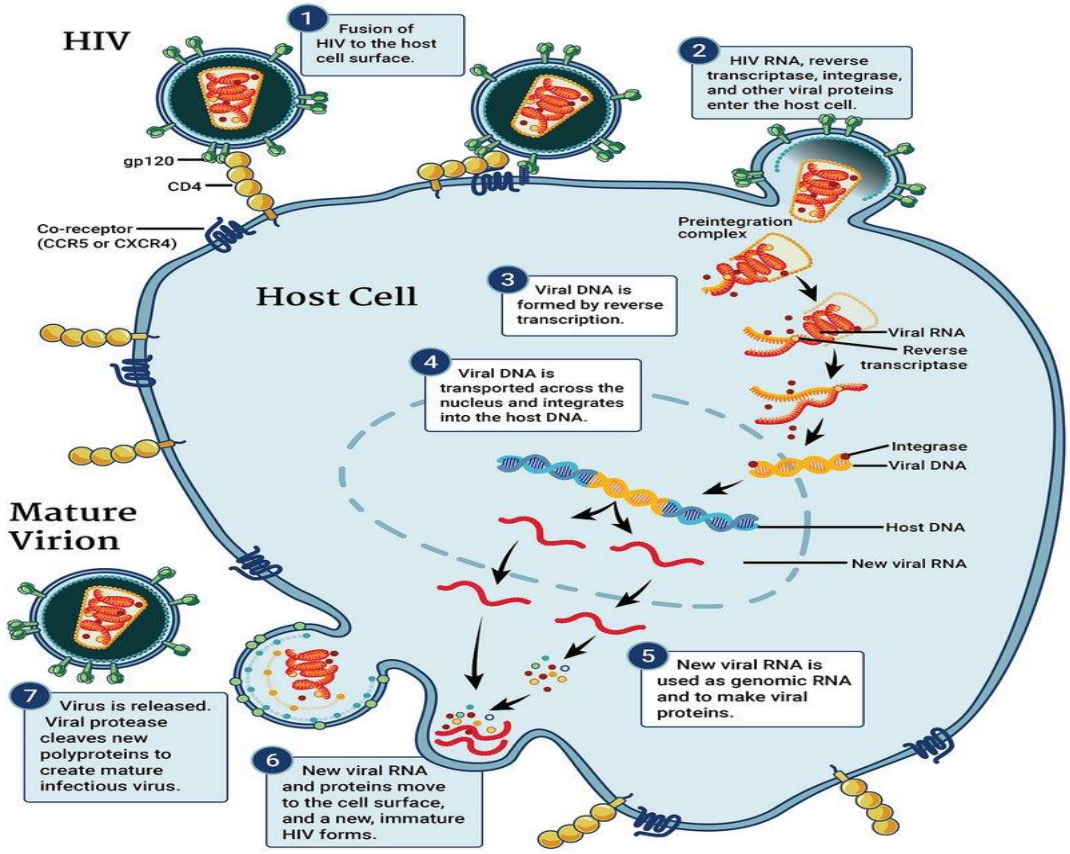

Figure 2.5: Seven stages of HIV replication cycle.

https://www.niaid.nih.gov/sites/default/files/hivReplicationCycle2.jpg

Human Immunodeficiency Virus has no cure thus far and there is a need for the search of new drugs for managing HIV. This is due to the adverse events which were observed in the patients undergoing long-term treatment with ART (Salehi et al., 2018). It has been suggested that natural products and ethnomedicines should be

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systemically tested against HIV (Salehi et al., 2018). Moreover, the exploration of natural products for discovering new anti-HIV drugs is due to the fear of side effects from highly active antiretroviral therapy (HAART) that drive people living with HIV/AIDS worldwide to drift towards medicinal plants (Boukandou et al., 2018; Muswe et al., 2017).

2.5 SELECTED OPPORTUNISTIC MICROBES TO BE USED IN THE STUDY

Opportunistic pathogens are a common public health concern, particularly in developing countries. They are known to initiate infections in the immunocompromised patients. In Africa, opportunistic infections are correlated with the increase in the number of immune-compromised patients in healthcare facilities (Steenkamp et al., 2007).

Opportunistic infections normally occur while the HIV patient is on antiretroviral therapy (ART), either due to the disguise of the pre-infection that occurs with the immune recovery or due to the immunosuppression (Akase et al., 2017) or also as the result of the treatment failure at any time of ART (Tanuma et al., 2016).

2.5.1 ESCHERICHIA COLI

It is a *Gram-negative* rod-shaped bacterium. It is estimated that 100 million of international travellers suffer from mild to severe diarrhoea out of 250 million people travelling each year (Black, 2008). The pathogenic strains of *E. coli* are the most common cause of traveller's diarrhoea. Enteroinvasive strains have a plasmid with a gene coding for K antigen, that enhances the attachment and invading of the mucosal cells. The enterotoxigenic strains have a plasmid, which enables them to synthesize enterotoxins. Their attachment to the mucosa is enhanced by pili or fimbriae. These strains cause multiple cases of infant diarrhoea (Black, 2008).

When comparing the two strains, enteroinvasive causes extreme dehydration than enterotoxigenic. The infection may result in loose stools passed out about four to five times a day. Enterohemorrhagic *E. coli* (0157: H7) is one of the most common causes

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of deadly outbreaks in bloody diarrhoea (Bauman, 2012; Black, 2008). This strain also produces the Shiga toxins, either 1 or 2. According to Black (2008), about 100 cells are required to initiate an infection with three or four days of incubation. The infection results in the non-bloody diarrhoea, which usually becomes bloody on either the second or third day. *E. coli* is an extremely versatile opportunistic pathogen, present in enormous bacteraemia cases and it can cause septicaemia.

It can also cause systemic infections, particularly in immunodeficient patients. The disease caused by this bacterium is known as traveller's diarrhoea. Furthermore, the translocation of *E. coli* could lead to the inflammation-associated disease in HIV patients as a result of a damaged mucosa of the gut (Deeks et al., 2013).

2.5.2 *PSEUDOMONAS AERUGINOSA*

Pseudomonas aeruginosa is a highly antibiotic resistant Gram-negative bacterial pathogen responsible for infectious diseases such as urinary tract, respiratory system, gastrointestinal and different types of systemic infections. It is one of the major pathogens of the healthcare-associated infections (HAI). The infections caused by *P. aeruginosa* have been challenging to health care providers considering their intrinsic resistance against a wide range of antimicrobials (Tsao et al., 2017). Ugurlu et al (2016) described *P. aeruginosa* as an opportunistic human pathogen that preferentially infects patients with cancer or HIV/AIDS patients immunocompromised by surgery, cytotoxic drugs, burn, wounds, people with fibric fibrosis or blood, skin, eye and genitourinary tract infections (Bosgelmez-Tinaz et al., 2005).

2.5.3 STAPHYLOCOCCUS AUREUS

Staphylococcus aureus is a Gram-positive bacterium, frequently present in the normal flora of the skin and the upper respiratory tract, whereas certain, strains of *S. aureus* causes toxic shock syndrome (TSS). Moreover, they commonly infect skin, wounds and may also cause pneumonia. Hence, the infections result from the transfer of staphylococci in the normal flora of an infected, asymptomatic individual, to a susceptible individual (Madigan et al., 2015). More other staphylococcal diseases include boils, impetigo, pimples, acne, meningitis, osteomyelitis, carditis and arthritis.

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Due to the increase of antibiotic or drug-resistant *S. aureus*, exploration of medicinal plants as an alternative medicine has gained momentum.

2.5.4 STREPTOCOCCUS PYOGENES

Streptococcus pyogenes, Gram-positive bacteria also known as Group A streptococci which are often isolated from the upper respiratory tract of the healthy adults. The utmost, clinical isolates *S. pyogenes* produce exotoxins that lyse red blood cell (Madigan et al., 2015). It is known to cause mild to life-threatening (systemic infections). *Streptococcus pyogenes* causes numerous infections in humans such as strep throat, pharyngitis, scarlet fever, rheumatic fever and many more.

2.6 SUMMARY OF THE LITERATURE

Opportunistic pathogens are a common public health concern, particularly in developing countries. They are known to initiate infections in immunocompromised patients (Marbou and Kuete 2017). In Africa, opportunistic infections are increasing with the increase in the number of immune-compromised patients in healthcare facilities (Steenkamp et al., 2007). HIV related opportunistic infections normally occur while HIV patient is on antiretroviral therapy (ART), either due to the disguise of the pre-infection that occurs with the immune recovery or due to the immunosuppression or as the result of the treatment failure at any time of ART (Tanuma et al., 2016). Even though the association between the HIV infection and the adverse effects of ART with respect to (CHD) is not clear, Muswe and co-workers (2017) reported that a high ratio of TNF-α/ IL-10 indicates the higher risk in ART-naive patients compared to the ART-exposed.

Despite, the usage of ART by HIV infected patients some of these patients suffer from a persistent chronic inflammation (Delgado-Velez and Lasalde-Dominicci, 2018). Hence, the need in exploring medicinal plants in discovering more effective and affordable therapeutic compounds. Moreover, it is clear that microbial infections can trigger inflammation (immune defence) as observed in figure 2.3 and 4 and if not controlled can be detrimental. Some microorganisms can be the etiologic agents of cancer which may affect different important organs in humans (Mongalo et al., 2016).

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There is an estimation that by 2030 there will be approximately 26 million new cancer cases and 17 million deaths (Thun et al., 2009). Additionally, Tanuma et al (2016) reported that cancers and cardiovascular disorders are becoming the leading causes of mortality in HIV infected individuals with improved prognosis. Hence, the study embarked on the quest to explore the effect of *T. elegans* leaves on the HeLa cells, ability to scavenge free radicals, NO inhibition, anti-bacterial, anti-HIV-1 RT activity and of course evaluating its safety on Vero cells.

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Chapter 3

MATERIALS AND METHODS

3.1 PLANT MATERIAL COLLECTION

T. elegans sample collection was carried out at Makonde village, which is situated in Limpopo, South Africa (Figure 3.1). The actual location of Makonde village is highlighted in the top red box (Figure 3.1-1D) 22.8064° S, 30.5826° E.

T. elegans leaves were collected in April 2018 at Makonde Venda (Limpopo, South Africa) and taken to the Laboratory at the University of Venda. The plant materials were identified by a botanist Prof P. Tshisikhawe and a voucher specimen no. LMUV001 was deposited at the Herbarium of the University of Venda. The leaves were dried through direct sunlight and then ground to a fine powder using a Retsch SM100 grinder (Retsch Gmbh; Haan; Germany).

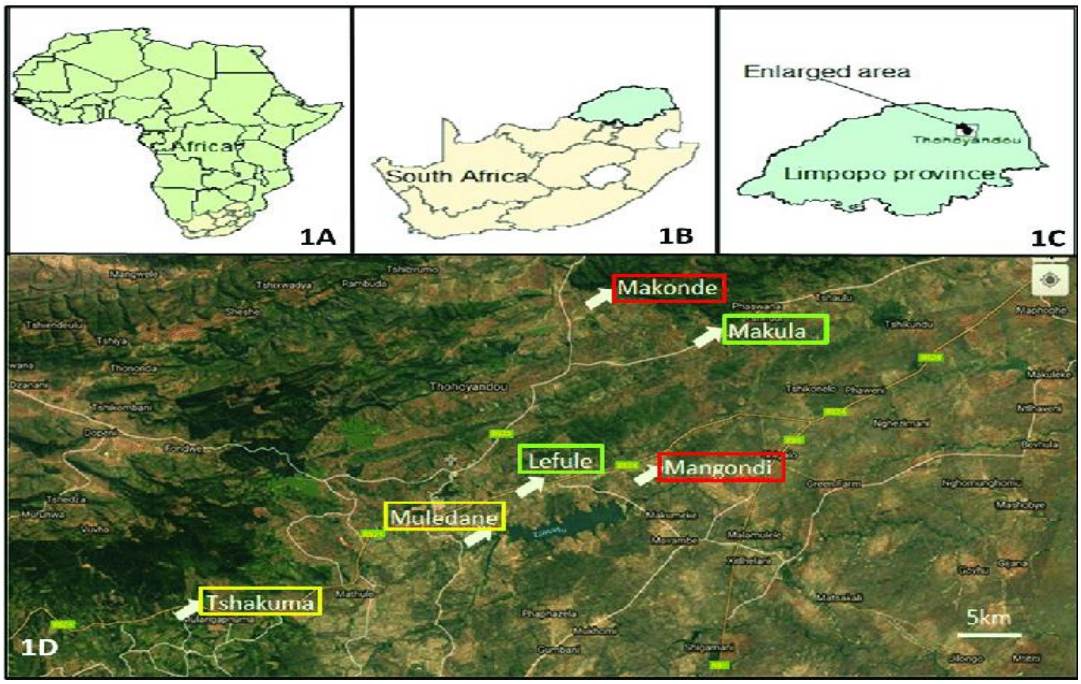

Figure 3.1: Map depicting the site of sample collection (1A) Africa (Continent). (1B) South Africa (Country). (1C) Limpopo (Province). (1D) Makonde; village along with its neighbouring villages. (https://www.google.co.za/search limpopo+map+with+makonde).

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3.2 CHEMICALS AND REAGENTS

The chemicals were obtained from Sigma-Aldrich (St Louis, MO, USA) such as quercetin; Folin-Ciocalteu; 2-2-diphenyl-1-picrylhydrazyl (DPPH); ascorbic acid; aluminium chloride; DMSO and sodium carbonate. Potassium ferricyanide was purchased from Rochelle chemicals (Johannesburg, Gauteng, RSA); trichloroacetic acid; di-sodium hydrogen phosphate anhydrous; sodium dihydrogen phosphate and ethanol were obtained from Merck chemicals (Darmstadt, Germany). Ferric chloride hexahydrate and ascorbic acid were bought from Associated Chemical Enterprise (Johannesburg, Gauteng, RSA).

3.3 EXTRACTION PROCEDURE

3.3.1 SEQUENTIAL SOLVENT EXTRACTION

A sequential solvent extraction as described by Mthethwa et al (2014), with slight modifications was used. Leaf samples were defatted with n-hexane (n-hex) before sequential extraction with dichloromethane (DCM) and methanol (MeOH) using a previously described method (Jeyaseelan et al., 2012). Briefly, the ground leaf sample (1249.95 g) was treated first with 3.5 L of n-hex for 48 h. The same volume of DCM was added to the defatted sample and left to stand for another 48 h. After the filtration of the DCM extract, the residue was treated with MeOH using the same conditions. The DCM and MeOH extracts were evaporated to dryness with a Rotavapor (IKA®; Staufen, Germany) at 40-45ºC under reduced pressure and then the extraction yield was quantified using a Sartorius Entris weighing balance (Sartorius, Göttingen, Germany). The extracts obtained were filtered using Whatman's filter paper no.1 (Sigma-Aldrich, St Louis, USA). The extracts were concentrated using a Buchi Rotavapor R-210 (Büchi Labortechnik AG; Flawil, Switzerland) with the water bath set at 40-45°C as mentioned above. The crude extracts were subjected to biological tests and both DCM and MeOH crude extracts were fractionated using silica gel column chromatography.

The formula used for calculating the percentage yield; % yield = (dried extract/ground weight) *100.

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3.4 ISOLATION OF ACTIVE COMPOUNDS

The dried powder of DCM and MeOH extracts were separately subjected to column chromatography in silica gel (60-120 mesh) glass column (Merck Chemicals, Darmstadt, Germany) respectively.

3.4.1 FRACTIONATION OF DICHLOROMETHANE AND METHANOL EXTRACT BY CHROMATO-GRAPHIC TECHNIQUES.

The dried DCM extract (30 g) was adsorbed on silica gel (150 g) and subjected to column chromatography (25 mm diameter and 450 mm height) using silica gel (360 g; 60-120 mesh). Isolation of compounds was carried out with gradient elution using n-hex and DCM. Fractions obtained were combined based on similarities of bands obtained from thin layer chromatography (TLC).

The dried MeOH extract (72.74 g) was adsorbed on silica gel (500.58 g) and subjected to column chromatography (the same size as the one mentioned above) using silica gel (500.58 g; 60-120 mesh). Isolation of compounds was carried out with gradient elution using n-hex and ethyl acetate. Fractions obtained were combined based on similarities of bands obtained from the TLC.

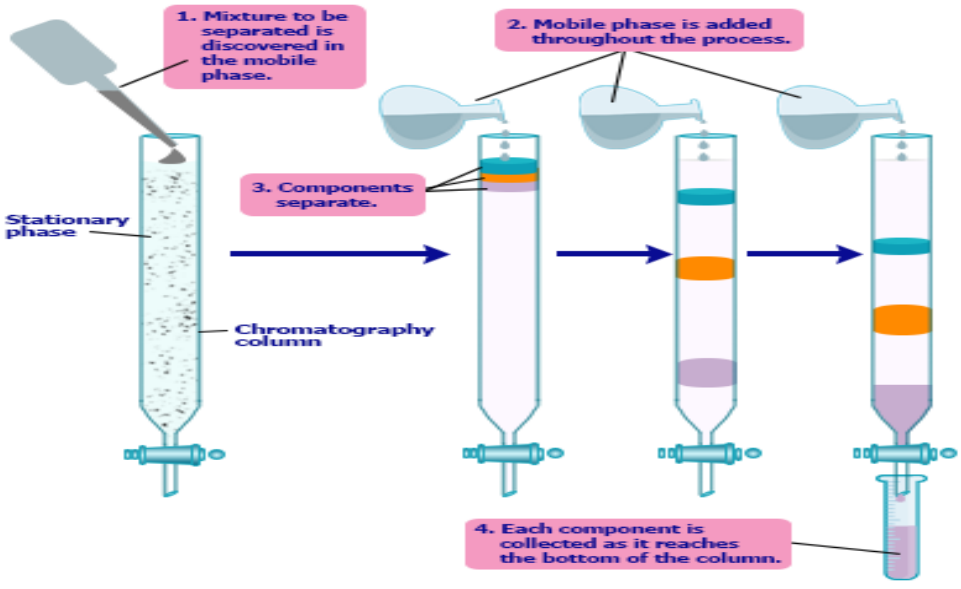

Figure 3.2: Column chromatography procedure (Sobolev and Dorner, 2002)

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The collected fractions were monitored by TLC as seen in Figure 3.3) wherein the similar fractions were combined, and the reading was done under both long wave (365 nm) by Spectroline® Model CM-10 Fluorescence Analysis cabinet (Saint Louis, MO, USA).

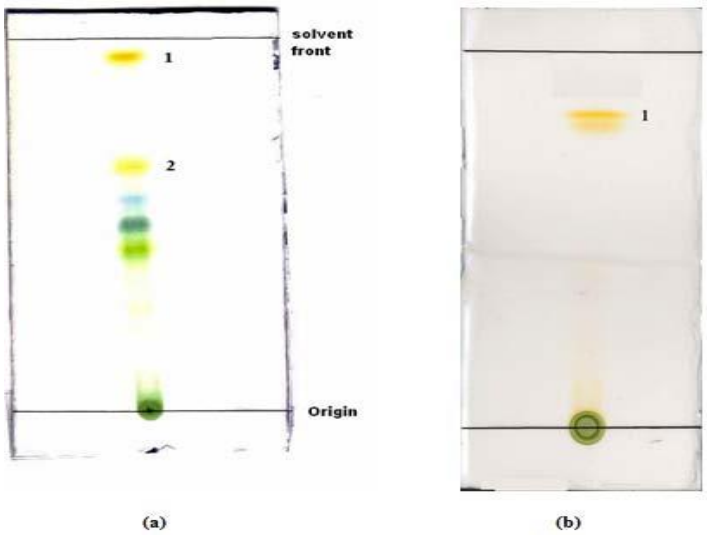

Figure 3.3: TLC plates showing the presence of cardiac glycosides in plant extract (Kreis and Müller, 2018).

3.5 QUALITATIVE PHYTOCHEMICAL SCREENING

3.5.1 THIN LAYER CHROMATOGRAPHY (TLC)

TLC plates (Merck, Darmstadt, Germany) silica gel 60 F_{254} aluminium sheet 20 x 20 cm was used qualitatively to separate the phytochemical constituents of *T. elegans* extracts and fractions as described by Bisi-Johnson et al (2017) with slight modifications. The crude extracts were reconstituted in acetone (Sigma-Aldrich, St Louis, MI; USA) (at a concentration of 10 mg/mL), then 10 µL of each extract was placed on the aluminum-backed TLC plates.

The development of the plate involved the usage of three different mobile solvent systems namely: Ethylacetate: methanol: water (EMW) (40:5.4:4) polar/neutral; Chloroform: ethylacetate: formic acid (CEF) (20:16:4) intermediate polarity/acidic and Benzene: ethanol: ammonium hydroxide (BEA) (36:4:0.4) non-polar/basic. An aliquot of 10 µL of extract (representing 1 mg/mL of the extract) was placed on a line measured 1 cm from the bottom of the plate. Subsequently, after the extract was dried, the plates were placed in the respective saturated chamber containing a solvent

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system. Fractions with similar profiles on the TLC were combined, resulting in fewer fractions for the DCM (four fractions FD 1-4) as shown in the appendix A1 and MeOH (three fractions FM1-3) extracts (Figure A2, Appendix). Thereafter, the samples were subsequently tested for biological activities.

3.5.2 PRELIMINARY PHYTOCHEMICAL SCREENING

The determination of the chemical components of *T. elegans* was done according to the method previously described (Ismail et al., 2016; Nandagoapalan et al., 2016; Ayoola et al., 2008).

Test for Saponins (Froth test)

To 1 mL of Crude ex or fractions was added 5 mL of distilled water in test tube separately and then shaken vigorously for 5 minutes. The formation of stable foam was taken as an indication for the presence of saponins (Satheesh et al., 2012).

Test for Steroids (Libermann Burchard test)

To 0,5 mL of the extracts, 2 mL of acetic anhydride was added followed by a 2 mL of concentrated H_2SO_4 along the sides of the tubes. The formation of green colour indicated the presence of steroids.

Test for Alkaloids (Wagner's test)

Wagner's reagent was prepared by the mixing of 2 g of iodine, 6 g of potassium iodide and 100 mL of distilled water. A few drops of the prepared Wagner's reagent were added to 1 mL of crude extracts. The formation of reddish-brown precipitate indicated the presence of alkaloids.

Test for Terpenoids (Salkowski test)

To 500 µL of extracts, 1 mL of chloroform and 1,5 mL of concentrated H_2SO_4 was added. Formation of yellow colour ring at the interface of the two liquids that turn reddish brown colour after 2 minutes, showed the presence of terpenoids.

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Test for Glycosides (Keller- Killani test)

To 1 mL of extracts, 1 mL of glacial acetic acid containing one drop of $FeCl_3$ solution and 1 mL of concentrated H_2SO_4. A brown ring at the interface indicated the presence of cardiac glycosides (Figure 3.4 ★).

Figure 3.4: Tubes showing presence of cardiac glycosides. The dark coloration indicates presence of the chemicals (★), the tubes with yellow coloration are the negative (■) controls for each extract.

Test for Flavonoids

In a test tube, a few drops of a 4 mg/mL sodium hydroxide (NaOH) solution were added to 1 mL of crude extract. This was followed by the addition of a few drops of 32% hydrochloric acid (HCl) solution. A yellowish colouration or a colourless mixture indicated the presence of flavonoids.

Tests for phenols and tannins

One (1) mL of crude extract was mixed with a few drops of 2 % ferric chloride ($FeCl_3$) in a glass tube. Any black, brown, green or dark blue colouration was taken as confirmation for the presence of both secondary metabolites (Figure 3.5).

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Figure 3.5: Tubes showing presence of tannins and phenols. The tubes with black coloration indicate presence of the chemicals (★), the tubes with yellow coloration are the negative (■) controls for each extract.

3.6 PHARMACOLOGICAL ASSAYS

3.4.1 QUALITATIVE ANTIOXIDANT ACTIVITY

Free radical scavenging assay

The 2, 2-diphenyl- 1- picryl- hydrazylhydrate (DPPH) free radical-scavenging activity of the extracts and fractions were assessed according to the method described by Masoko (2017). The DPPH free radical method is regarded to be one of the best, frequently utilized and accurate assays to evaluate antioxidant properties of natural products (Szabo et al., 2007). It is a stable free radical due to its spare electron delocalization over the whole molecule. It is based on the scavenging capacity of free radicals which are converted (Njoya et al., 2017) into a colourless or reduced DPPH product as depicted in the diagram below (Figure 3.6). When the solution of this DPPH is mixed with a substrate acting as a hydrogen atom donor, a stable non- radical form of a DPPH is obtained with simultaneous change of the violet colour to clear or yellow colour.

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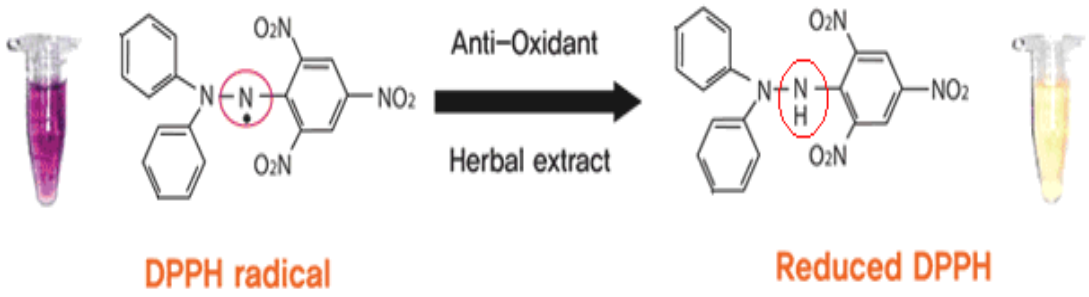

Figure 3.6: DPPH assay. The decolouration reaction brought by the radical scavenging molecule (active agent).http://radio.cuci.udg.mx/bch/EN/Forschung/DPPH.gif

DPPH assay was performed according to the published method of Masoko (2017) with few modifications: Briefly, TLC plates were used to separate the extracts and fractions. The chromatograms were sprayed with 0,2 % (w/v) DPPH in MeOH as an indicator. The clear or yellow zones against the purple background on chromatograms indicate the presence of scavenging potential of free radicals by compounds present in the extracts and or fractions. This was done in duplicates.

3.4.2 DETERMINATION OF MINIMUM INHIBITORY CONCENTRATION AND MINIMUM BACTERICIDAL CONCENTRATION.

The four microorganisms tested against in this study were purchased from ANTECH, MediMark, Europe and included: two Gram-positive (*S. aureus*; ATCC BAA- 2312 and *S. pyogenes*; ATCC 19615) and two Gram-negative (*E. coli*; ATCC 25922 and *P. aeruginosa*; ATCC BAA-1744) bacterial strains.

Minimum inhibitory concentration (MIC) determination

The minimal inhibitory concentration (MIC) for the investigated fractions and extracts were determined by the use of broth microdilution susceptibility assay adopted from Eloff (1999) with slight modifications. The test was performed in the Brain Heart Infusion (BHI) broth medium (Sigma-Aldrich, St, MO, USA). A volume of 100 µL of the BHI medium broth was added into each well of 96 microwell plate (NUNC, Rochester, USA) and 100 µL of the different fractions as well as the crude extracts with a starting concentration of 60 mg/mL was added in all wells of the first row; followed by two-fold serial dilution and resulted into six different concentrations: 20, 10, 5, 2.5, 1.25, and

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0.625 mg/mL. Then 100 µL of 0,5 McFarland inoculum was added in the plates. The plates were incubated overnight at 37 °C. After incubation 40 µL of 0,4 mg/mL of iodo – nitro – tetrazolium (INT) (Sigma-Aldrich, St, MO, USA) was added into each well and incubated for approximately 2 - 3 hrs at 37 °C. Following incubation, the colour change in the plates was observed and MIC was recorded as the lowest concentration of the extract that inhibited the visible bacterial growth.

The 10 mg/mL of Gentamicin solution (Sigma-Aldrich, St, MO, USA) and Amoxicillin purchased from Sigma – Aldrich, St, MO, USA (only tested against *S. pyogenes*) were used as a positive control, whereas distilled water was used as a negative control.

Minimum bactericidal concentration (MBC) determination

A total of 25 µL of the obtained MIC and all the wells where no bacterial growth was observed was inoculated on a sterile nutrient agar plate (Mueller Hinton media) and spread evenly with a glass spreader. The plates were then incubated overnight at 37 °C. The results were recorded, and the MBC was determined in the plates where there was a suppression of 99.9% of bacterial growth.

3.4.3 QUALITATIVE HIV-1 RT ASSAY

The experiment was aimed at determining the fractions which inhibited the viral replication of HIV-1 RT enzyme by using a HIV-1 RT colorimetric ELISA kit; ref-11468120910 (Sigma-Aldrich, St Louis, MO, USA). The assay was adopted from Mamba et al. (2016) with slight modifications. All plant material was weighed up to 1 mg and was dissolved in 1 mL DMSO to make a final concentration of 1 mg/mL stock solution. Subsequently three concentrations (50, 100 and 200 µg/mL) were prepared and all tests were carried in duplicates. In appropriate wells of microtiter plates, 20 µL of enzyme, 20 µL diluted extracts and 20 µL reaction mixture were added together. A 100 µg/mL of efavirenz (EFV) was used as a positive control; (1) lysis buffer was added with DMSO and (2) lysis buffer was added with no DMSO. The lysis and buffer and reaction mixture were added as a negative control. The plates were incubated at 37°C for one hr. The microtitre plates were washed five times with 150 µL of the washing buffer.

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One hundred and fifty microtitres of Anti-Dig-POD working solution was added in each well. Thereafter, the plates were incubated at 37°C for one hr with constant shaking. The microtitre plates were washed five times with 250 µL of the washing buffer. Two hundred microliters of ABTS substrate solution was added in each well and was allowed to stand for 30 minutes in the dark at room temperature (Figure 3.7). The substrate solution turns green if there is no activity. Thus, the intensity in colour of the substrate is inversely proportional to the activity of the test sample. In this study, the absorbance was not read due to technicality.

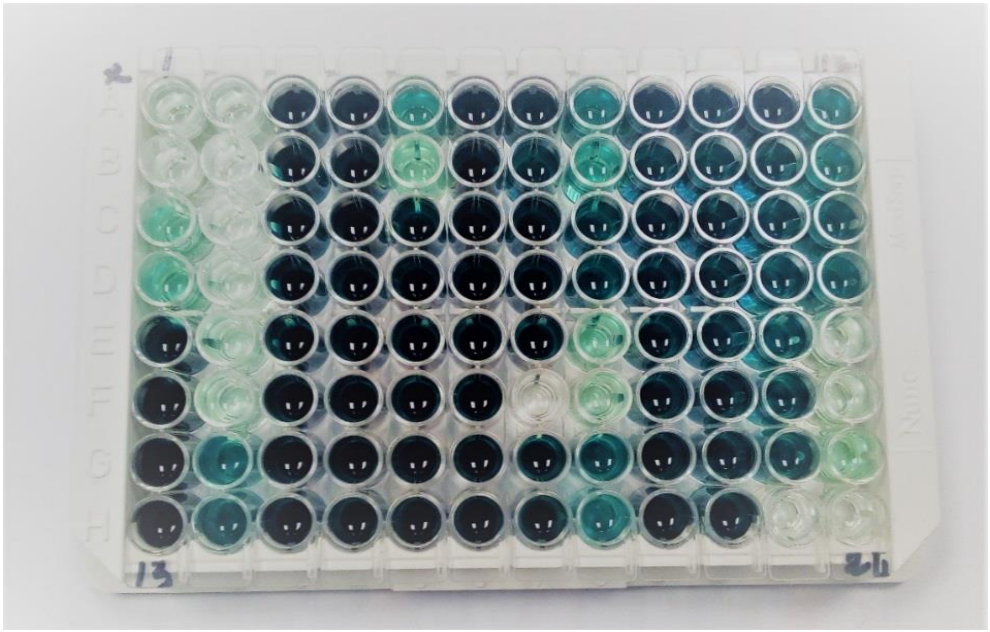

Figure 3.7: Expected plate for RT assay. The lighter the coloration, the more active the extract.

3.4.4 THE *IN VITRO* ANTI-INFLAMMATORY ASSAY

Inhibition of nitric oxide (NO) production

Mouse macrophage cell line, Raw 264.7 (ATCC® TIB-71™) purchased from the American Type Culture Collection (ATCC, Manassas, VA; USA). The cells were seeded into 96 well microtiter plates at a density of approximately $1.00x10^5$ cells per well in 96 well microtiter plates and allowed to attach overnight. The following day spent culture medium was removed and the positive control, silymarin (Sigma- Aldrich,

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Saint Louis, MO, USA), (diluted in DMEM complete medium) added to give final concentrations of 50 and 100 µM. To assess the anti-inflammatory activity, the cells were activated by addition of 50 µg/mL of Lipopolysaccharides (LPS), in the culture medium. LPS alone served as a negative control and treated simultaneously (Motlhatlego et al., 2018) with different concentration (25, 50, 100 and 200 µg/mL) of the test samples dissolved in dimethyl sulphoxide (DMSO). After 24 h of incubation, 100 µg/mL of supernatant from each well of the 96 well microtiter plates were transferred into new 96 well microtiter. For positive control, silymarin (St. Louis, MO, USA) was added at 50 and 100 µM.. The plates were further incubated for 18 hrs.

A volume of 50 µL spent culture medium was transferred into a new plate then followed by the addition of 50 µL of Griess reagent (Sigma – Aldrich, St Louis, MO, USA) for the quantification of nitrate. Changes in color were observed where there is anti-inflammatory activity in a period of 15-20 minutes. The absorbance of the mixture was read at 540 nm wavelength using ImageXpress Micro XLS Widefield Microscope (Molecular devices, Silicon Valley, California, USA). The cell viability was also assessed using the 3-(4,5-dimethylthiazol-2-yl)-2, 5-diphenyl tetra-zoliumbromide (MTT) assay to rule out toxicity as a contributory factor to NO production.

3.4.5 CYTOTOXICITY ASSAY

Reagents

Vero cells were purchased from Cellonex, South Africa. Dulbecco's Modified Eagle Media (DMEM), Rosewell Park Memorial Institute (RPMI) and Foetal Bovine Serum (FBS) were purchased from GE Healthcare Life Sciences (Logan, UT, USA). PBS with and without Ca^{2+} and Mg^{2+} and trypsin was purchased from Lonza (Wakersville, MD, USA). Bis-benzamide H 33342 trihydrochloride (Hoechst) and propidium iodide (PI) was purchased from Sigma (St. Louis, MO, USA).

Sample preparation

Test samples (crude and fractions) were reconstituted in DMSO to give a final concentration of 100 mg/mL. Samples were sonicated if solubility was a problem. Samples were stored at 4°C until required.

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Cytotoxicity using Vero cells

The African green monkey kidney cell line (Vero cells) was used for cytotoxicity screening. They were maintained at 37°C in a humidified incubator with 5% CO_2 in 10 cm culture dishes. Complete growth medium consisted of DMEM supplemented with 10% FBS and 10% penicillin-streptomycin. Cells were seeded into 96 well microtiter plates at a density of 3000 cells/well using a volume of 100 µL in each well. The microtiter plates were incubated at 37°C, 5% CO_2, and 100% relative humidity for 24 hrs prior to addition of test compounds to allow for cell attachment (Mfengwana et al., 2019)

Cells were treated with 50, 100 and 200 µg/mL of each extract and 10, 20 and 40 µM melphalan as the positive control, diluted in culture medium. One hundred microliter aliquots of the diluted extract in fresh medium was used to treat cells. Cells were incubated for a further 48 hrs.

Treatment medium was aspirated from all wells and replaced with 100 µL of Hoechst 33342 nuclear dye (5 µg/mL) and incubated for 20 minutes at room temperature. Thereafter, cells were stained with propidium iodide (PI) at 100 µg/mL in order to enumerate the proportion of dead cells within the population. Cells were imaged immediately after addition of PI using the ImageXpress Micro XLS Widefield Microscope (Molecular Devices, San Jose, CA; USA) with a 10x Plan Fluor objective and DAPI and Texas Red filter cubes. Nine image sites were acquired per well which is representative of roughly 75% of the surface area of the well.

Cytotoxicity using HeLa cells

The same treatment protocol was used as described for Vero cells. However, HeLa cells make use of RPMI (as used by the lab) growth medium supplemented with 10 % FBS and 10 % pen-strep.

Dual staining and fluorescence microscopy assay

The dual staining microscopy assay was performed to determine the cytotoxicity of the plant's crude extracts and fractions. Hoeschst 33342 and propidium iodide (PI) dye were used. Staining solution: Hoechst (5 µg/mL) in 5 mL. The PI solution (100 µg/mL):

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20 µL PI in 5 mL PBS (Ca + Mg). The medium was aspirated and treated. The 50 µL staining solution was added into each well and the results were acquired from the Image Xpress machine. Cell viability was expressed as nuclei per site of the cells treated with the extracts compared to the untreated control.

Data quantification

Quantification of live and dead cells for the screening assay was performed using the ImageXpress Micro XLS Widefield Microscope and acquired images analysed using the MetaXpress software and Multi-Wavelength Cell Scoring Application Module. Acquired data was transferred to an EXCEL spreadsheet and data was analysed.

An example of how data was interpreted is shown in Figures 3.8 to 3.11 (pictures obtained from the presentation given by Dr Koekemoer in 2019). A montage of images results after acquisition and depending on the settings, 9 sites in each well was analysed using MetaXpress software and required data such as total cell number, total "live" cells, total "dead" cells, percentage positively stained cells etc. was captured. This process was done independently for each dye/fluorescently labelled antibody in each experiment.

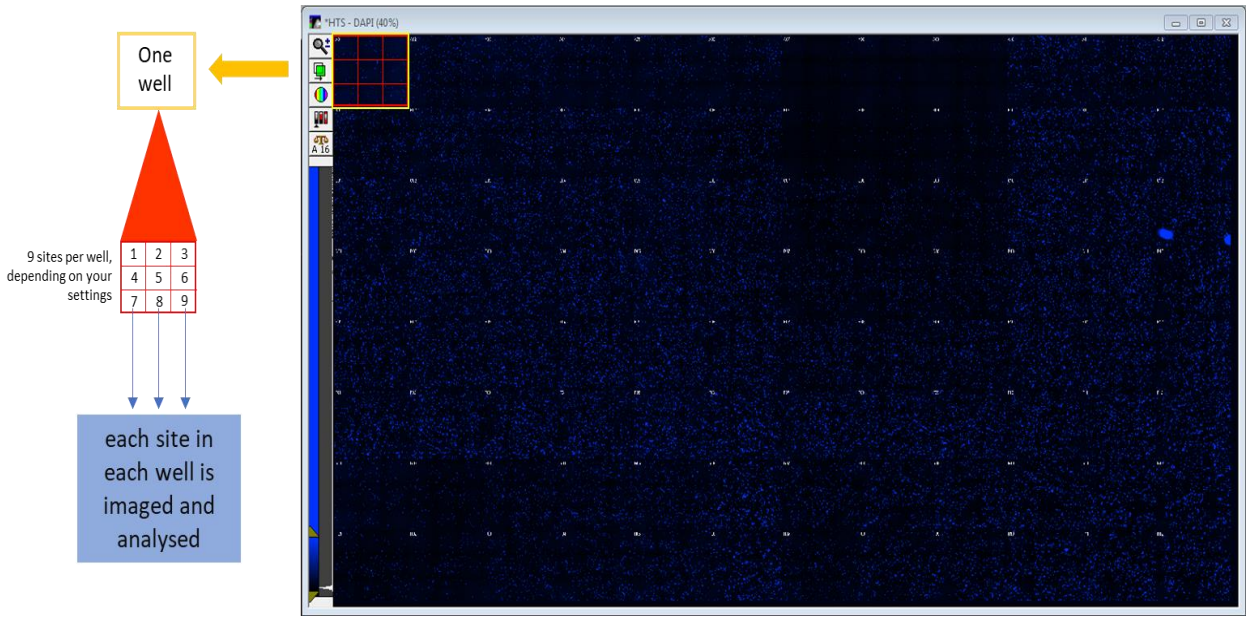

Figure 3.8: Determination of cytotoxic effect of extracts on cells using Hoechst 33342.

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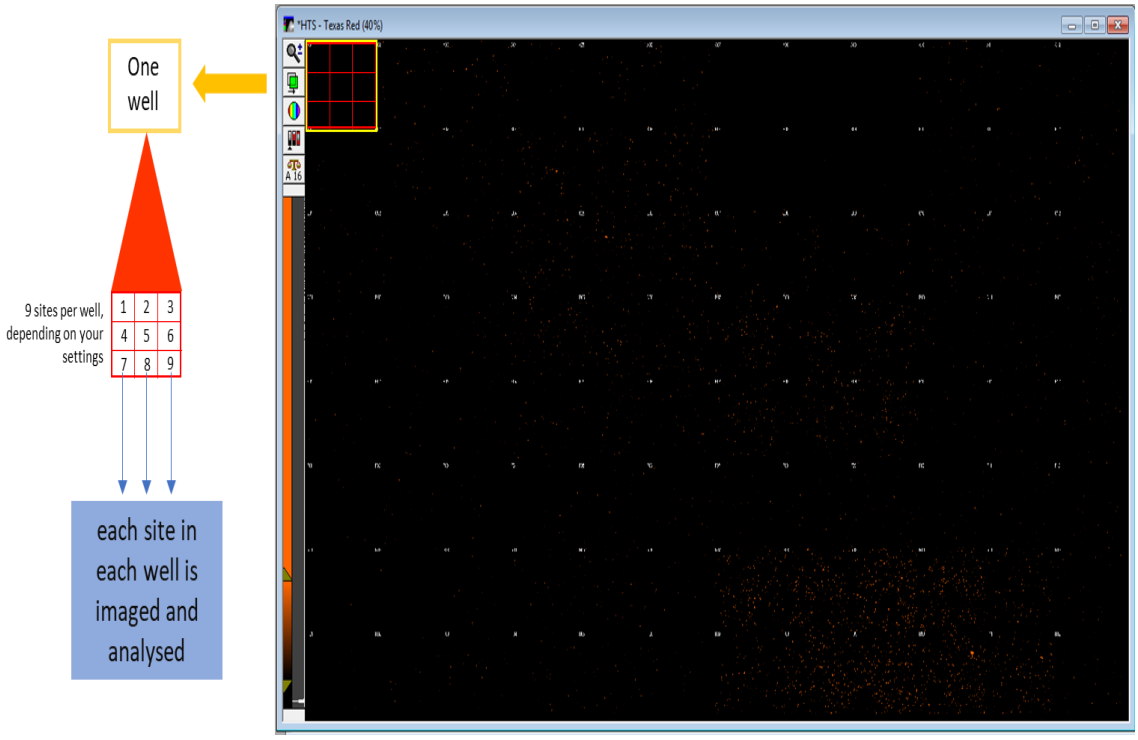

Figure 3.9: Analysis of cells stained with LysoTracker Red for the determination of the formation of lysosomes in response to extract treatment.

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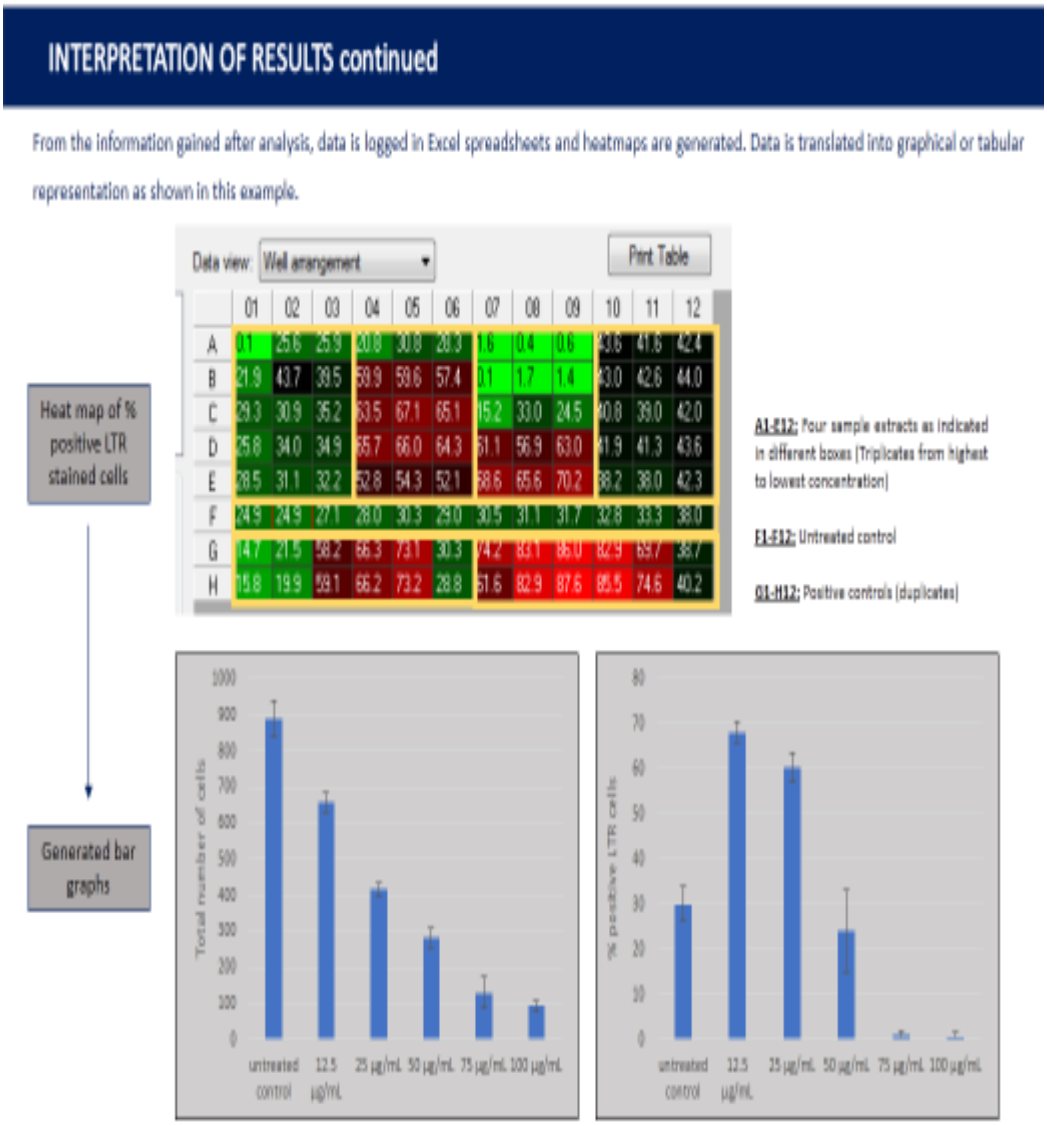

Figure 3.10: Effect of crude extracts and fractions on total cell number and lysosome formation.

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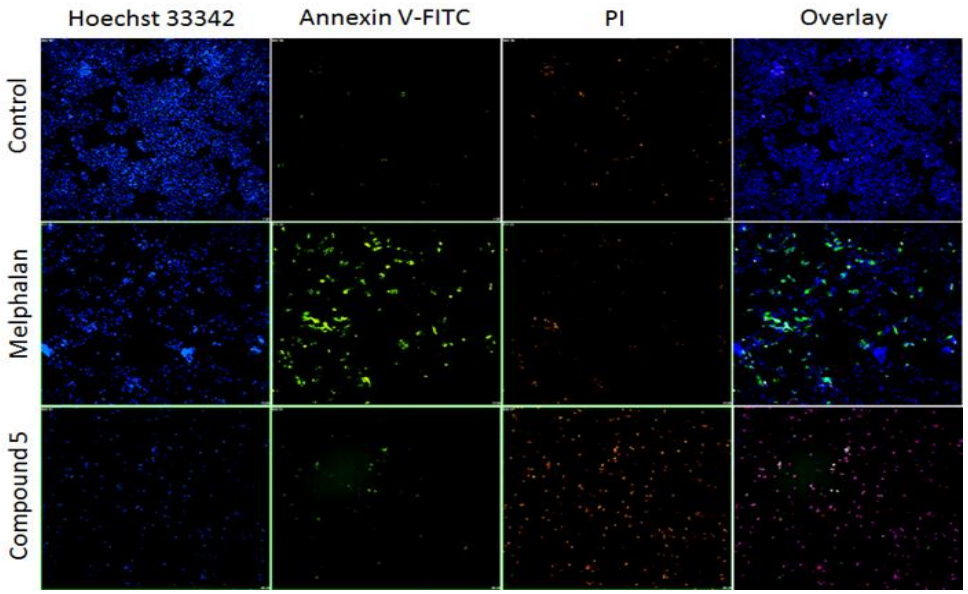

Figure 3.11: C3A hepatocytes were stained with Hoechst 33342 (blue), Annexin V-FITC (green) and propidium iodide (red).In this experiment Compound 5 induced necrotic cell death as opposed to apoptotic cell death as evident with melphalan treatment. This is evident with the high proportion of PI positive cells compared to Annexin V-FITC positive cells as with melphalan. Annexin V has a high affinity for phosphatidylserine on the extracellular surface of the cells, an early indicator of apoptosis.

3.5 DATA ANALYSIS

Data obtained was analysed using Microsoft Excel. The evaluated data were also presented in triplicates, unless stated and expressed as mean ± standard deviation. The mean between experimental conditions was compared by One-Way Analysis of Variance (ANOVA). The $p < 0.05$ value was considered significant.

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Chapter 4

RESULTS AND DISCUSSION

T. elegans is used extensively in Africa to treat various ailments, from simple wounds to cancer (Paterna et al., 2017; Paterna et al., 2016; Mansoor et al., 2013; Pallant et al.,2012). Overall, the results obtained in this study showed the presence of phytochemical constituents having good antioxidative, potent antibacterial, inhibition of Nitric oxide (NO) production, strong inhibition of HIV-1 RT enzyme activities5 and selective toxicity on cells. The detailed results are thoroughly described and discussed below.

4.1 SEQUENTIAL SOLVENT EXTRACTION

4.1.1 CRUDE EXTRACTS

The percentage yields of the n-hex, DCM and MeOH extracts are presented in Table 4.1. The extract with the highest yield was MeOH, followed by DCM and the least was n-hex.

Table 4.1: The percentage yield of *T. elegans* sequential crude extracts

Solvent for extraction	Mass obtained (g)	Yield (%)
n-Hex	3.12	0.25
DCM	34.9	2.79
MeOH	82.14	6.57

The extraction process is one of the first crucial step that determines the removal of secondary metabolites and depends on both the extraction method used and the solvent polarity (Wakeel et al., 2019; Sasidharan et al., 2011). Sequential extraction was carried out to avoid overlap of compounds in different extracts. The use of different solvents to extract from fresh batch of samples results in the same compounds extracted and found in different extracts. Sequential extraction reduces this overlap, making it easier to account for the compounds responsible for activities of the extracts. MeOH yielded the highest yield (6.57 %). This observation is supported by several

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studies (Masoko, 2017; 2005; Mpofu et al., 2014) that indicated that MeOH has the ability to extract compounds with a wide range of polarity

n-Hex extracted the least percentage yield, typically because of it extracts only non-polar constituents. Mpofu et al (2014) extracted the least percentage yield with hex among four selected solvents of two extracts obtained from different locations in Zimbabwe and Kwazulu Natal.

TLC was carried out on the three extracts using different solvent systems. The solvent system BEA separated multiple compounds shown by various bands followed by CEF with intermediate polarity and EMW, respectively (Figure 4.1). The phytochemical spectral fingerprints resulted in different color (Figure 4.1, Right) indicating the extracts and fractions contains different compounds. Most bioactive compounds travelled toward the solvent front, meaning that the separated compounds are non-polar (Figure 4.1).

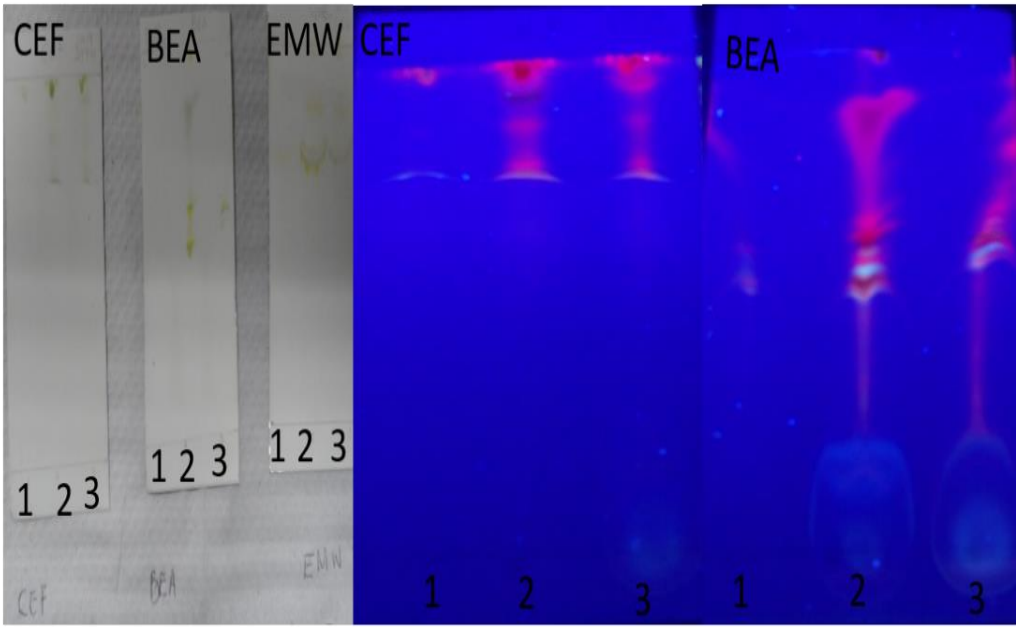

Figure 4.1: Chromatograms showing separated chemical constituents CEF, BEA, EMW as eluents. Prior to staining (Left) and under the UV light (a picture for EMW is not shown), 366nm (Right). 1- Hexane crude extract, 2- DCM crude extract and 3- MeOH crude extract.

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The DCM crude extract depicted multiple fluorescent compounds, whereas MeOH-ex portrayed a few fluorescent compounds (Figure 4.2, left).

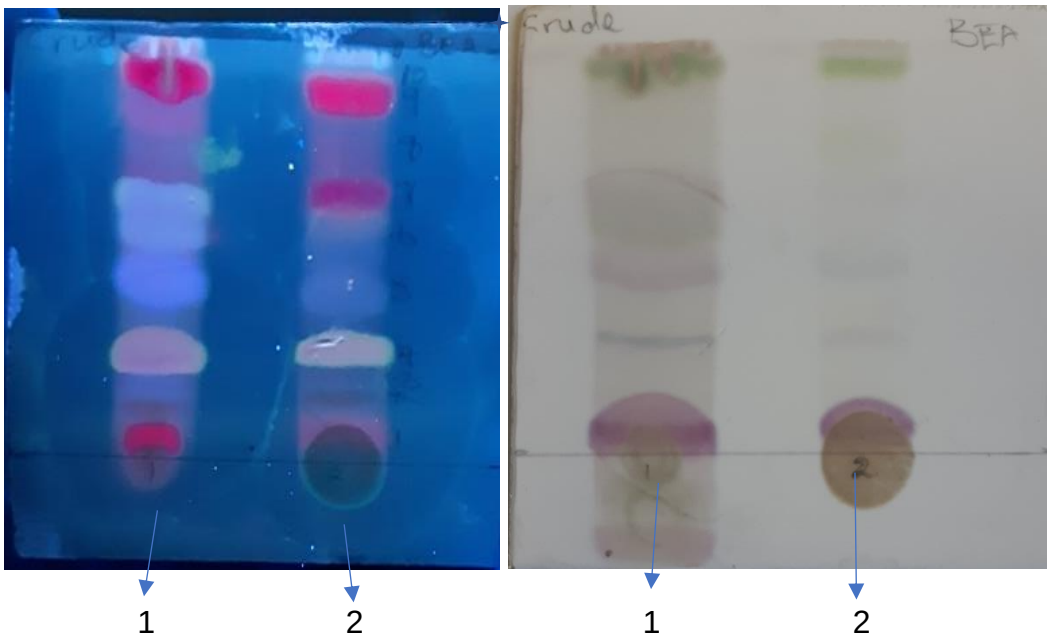

Figure 4.2: Chromatogram spotted with DCM (1) and MeOH (2) crude extracts, developed with BEA solvent system. Prior to staining under a longwave UV light, 366nm (Left) and 10% H_2SO_4 stained chromatogram, for the visualization of compounds (Right).

Based on the results different visible and colourful bands are observed on lane 1 (DCM extract) this could be due to the abundance of multiple non-polar soluble compounds whereas, lane 2 (MeOH extract) showed few and fainted non-polar bioactive compounds (Figure 4.2, right).

4.1.2 FRACTIONATION PROCESS

Column chromatography (Figure 4. 3) of DCM and MeOH extracts yielded 11 and 10 fractions, respectively. Fractions with similar spots on the TLC were combined, resulting in fewer fractions for the DCM (four fractions FD 1-4) as shown in appendix A, figure A1 and MeOH (three fractions FM1-3) extracts (Figure A2). The crude extracts and fractions were subsequently tested for biological activities.

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Figure 4.3: Column chromatography the second fraction collection of DCM crude extract F2 eluted by DCM 100%

4.1.3 FRACTIONS OBTAINED

The results of the percentage yield of *T. elegans* fractions of DCM and MeOH extracts are shown in Tables 4.2 & 4.3 respectively. It could be noticed that the first fractions showed a low percentage yield compared to the fractions collected last. The mass obtained from the DCM fractions was inversely proportional to the one obtained from the MeOH fractions (Table 4.2 and Table 4.3).

The mass obtained from the fractions ranged from 11.79 g to 0.36 g (Table 4.2). The highest yield (11.79 g) was obtained from 100% ethyl acetate eluted fraction (F4) followed by F5 (5.99 g) which was eluted by 90% ethyl acetate and the least yield (0.36 g) was obtained from 90 % MeOH (F11).

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Inverse to the results obtained after the DCM crude extract fractionation, the fractionation of MeOH crude extract resulted in the highest mass obtained of F9 (10.4 g), which showed that most compounds were soluble in 80% MeOH 20 % ethyl acetate.

Table 4.2: The percentage yield of *T. elegans* fractions from DCM extraction

Fraction eluted	Solvent for Elution	Mass obtained (g)	Final Fraction combination*	Mass combined (g)	Yield (%)
F1	n-HEX: DCM 50:50	1.15	FD1	1.15	3
F2	DCM 100%	2.44	FD2	4.55	13
F3	DCM: EA 50:50	2.11			
F4	EA 100%	11.79	FD3	19.74	57
F5	EA: MeOH 90:10	5.39			
F6	EA: MeOH 80:20	1.22			
F7	EA: MeOH 70:30	1.34			
F8	EA: MeOH 60:40	0.88	FD4	2.13	6
F9	EA: MeOH 50:50	0.52			
F10	MeOH: EA 90:10	0.37			
F11	MeOH: EA 80:20	0.36			

Hex- hexane, DCM- dichloromethane, EA- ethyl acetate, MeOH FD1- fraction 1 obtained from dichloromethane crude extract; **FD2**- fraction 2 obtained from dichloromethane crude extract; **FD3**- fraction 3 obtained from dichloromethane crude extract; **FD4**- fraction 4 obtained from dichloromethane crude extract; **FM1**- fraction 1 obtained from methanol crude extract; **FM2**- fraction 2 obtained from methanol crude extract; **FM3**- fraction 3 obtained from methanol

The mass obtained from the fractions range from 0.78 g to 10.4 g (Table 4.3). The highest yield (10.4 g) was obtained from 80% MeOH eluted fraction (F9) followed by F10 (4.83 g) which was eluted by 70% MeOH and the least yield (0.78) was obtained from 100% ethyl acetate (F2).

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Table 4.3: The percentage yield of *T. elegans* fractions from MeOH extraction

Fraction eluted	Solvent for Elution	Mass obtained (g)	Final Fraction combination*	Mass combined (g)	Yield (%)
F1	n-HEX: EA 50:50	0.83	FM1	1.61	2
F2	EA 100%	0.78			
F3	EA: MeOH 90:10	2.09	FM2	11.31	16
F4	EA: MeOH 80:20	1.45			
F5	EA: MeOH 70:30	2.19			
F6	EA: MeOH 60:40	2.3			
F7	EA: MeOH 50:50	3.28			
F8	MeOH: EA 90:10	1.75	FM3	16.98	23
F9	MeOH: EA 80:20	10.4			
F10	MeOH: EA 70:30	4.83			

Hex- hexane, DCM- dichloromethane, EA- ethyl acetate, MeOH FD1- fraction 1 obtained from dichloromethane crude extract; **FD2**- fraction 2 obtained from dichloromethane crude extract; **FD3**- fraction 3 obtained from dichloromethane crude extract; **FD4**- fraction 4 obtained from dichloromethane crude extract; **FM1**- fraction 1 obtained from methanol crude extract; **FM2**- fraction 2 obtained from methanol crude extract; **FM3**- fraction 3 obtained from methanol

In both cases of DCM and MeOH fractionation, the first eluted fraction (F1) resulted in the least percentage yield (3 and 2 respectively). This could be because a less polar/ nonpolar solvent system was used first and could only elute less polar compounds, this is similar to the observation by Braithwaite and Smith (2012) and Houghton and Raman (2012), who reported that in normal phase chromatography, when the stationary phase is polar, polar molecules will spend more time adsorbed on the stationary phase, while less polar ones will be carried quickly by the non-polar mobile phase. Therefore, it could be that the plant contained more polar compounds than non-polar compounds. The mass obtained of F2, showed that most compounds were soluble in 100% ethyl acetate. This observation is however, not in agreement with the study conducted by Anokwuru and co-workers (2017), where PaF3b fraction obtained from 70% ethyl acetate 30% MeOH yielded the highest. The same author further, elaborated that polarities of phytochemical components range from non-polar to polar

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for this reason our study agrees with him. Hence, one type of solvent may not be efficient in extracting all types of phytochemicals.

4.1.4 PHYTOCHEMICAL SCREENING

Phytochemical screening was carried out on the crude extracts and fractions (Table 4.4). Flavonoids were found to be present in all the tested samples (crude extracts and fractions). Hexane crude extract was also tested for phytochemical constituents since, some of the biological tests included the bioactivity of n-hex crude extract. Terpenoids were found in both crude extracts and only in 3 fractions. Terpenoids have been documented for their different therapeutic properties such as: inhibition of microorganisms (Barbieri et al., 2017; Koche et al., 2016; Saxena et al., 2013), neutralization of free radicals, anticarcinogenic, anti-ulcer and antimalarial (Saxena et al., 2013) activities.

Table 4.4: Qualitative phytochemical test

Phytochemical	FD1	FD2	FD3	FD4	FM1	FM2	FM3	DCM extract	MeOH extract	n-Hex extract
Tannins and phenols	-	-	-	-	-	-	-	-	-	-
Alkaloids	-	-	-	-	-	-	-	-	-	-
Saponins	-	+	+	+	-	+	+	+	+++	-
Flavonoids	+	++	+++	+	++	+	+	+++	+++	+
Terpenoids	+	-	-	+	-	-	-	+	+	+
Steroids	-	-	++	+++	+++	++	+	-	++	+
Glycosides	-	-	-	-	-	-	-	-	-	-

+; ++; +++ & - . **FD1**- fraction 1 obtained from dichloromethane crude extract; **FD2**- fraction 2 obtained from dichloromethane crude extract; **FD3**- fraction 3 obtained from dichloromethane crude extract; **FD4**- fraction 4 obtained from dichloromethane crude extract; **FM1**- fraction 1 obtained from methanol crude extract; **FM2**- fraction 2 obtained from methanol crude extract; **FM3**- fraction 3 obtained from methanol; **DCM**- dichloromethane crude extract; **MeOH**- methanol crude extract & **n-Hex**- n-hexane crude extract

Some of the phytochemical constituents have been reported to be responsible for the anti-inflammatory activity of plants; such as saponins (Aiyegoro and Okoh, 2010) and terpenoids as well as flavonoids (Nandagoapalan et al., 2016). It is worth mentioning that apart from flavonoids being anti-inflammatory, they are also reported for their ability to scavenge free radicals, treat allergies and ulcers as well as tumors and inhibit

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microorganisms including viruses (Nandagoapalan et al., 2016). Moreover, just like flavonoids, saponins are also good candidates for treating microbial infections (Nandagoapalan et al., 2016). Surprisingly, alkaloids tested negative for this test, which opposes to what was reported by Silveira et al. (2017). In addition Mansoor et al. (2009a,b) isolated alkaloids from the *T. elegans* methanol leaf extracts collected in Mozambique. Paterna et al. (2017; 2016) reported that some isolated alkaloids from the *T. elegans* root extract exhibited an activity against different cancer cell lines. Indeed, it was found that extracts from the root contained high concentration of alkaloids (Pallant et al., 2012). Nevertheless, in our study we strongly believe that there is a need of a more robust testing (HPLC) instead of the basic biochemical test used and or more different alkaloidal assay tests. Since, this plant is known to be an alkaloid rich plant moreover alkaloids such as alasmontamine (Hirasawa et al., 2009), vobasine, tabernaemontanine and eleganine A (Figure 2.2) have been isolated from the leaf parts of *T. elegans* (Mansoor et al., 2009a; Van der Heijden et al., 1986b).

4.2 BIOCHEMICAL TEST

4.2.1 ANTIOXIDANT POTENTIAL

The qualitative antioxidant activity of the leave fractions obtained separately from both DCM and MeOH crude extracts are depicted in Figures 4.4 and 4.5 respectively. The clear or yellow zones in the purple background are areas where the chemical constituents exhibit antioxidant effect after spraying with DPPH.

The constituents of both crude extracts are shown to exhibit free radical scavenging potential. The DCM extract exhibited the antioxidant potential together with the fractions particularly FD1, FD2 and FD3, Amazingly, FD2 exhibited a more pronounced antioxidant profile (Figure 4.4).

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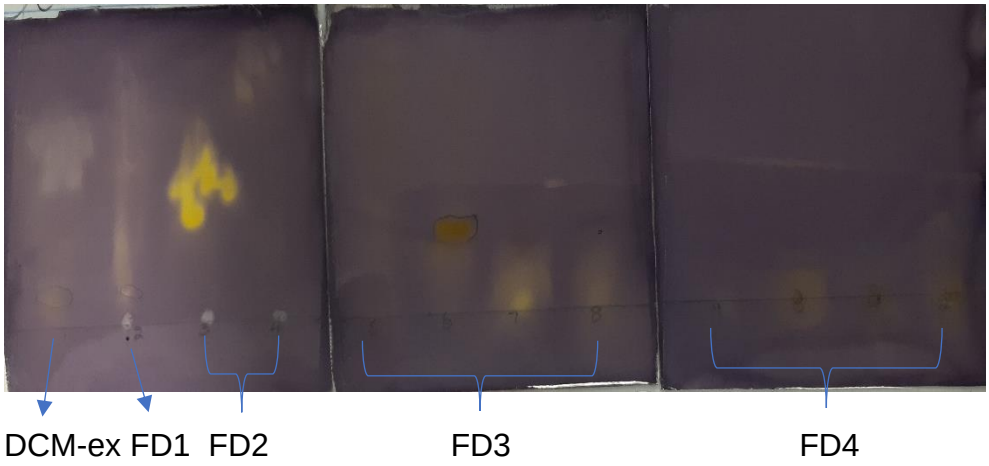

Figure 4.4: Chromatograms of *T. elegans* DCM extracts and its fractions developed in BEA solvent system. **FD1**- fraction 1 obtained from dichloromethane crude extract; **FD2**- fraction 2 obtained from dichloromethane crude extract; **FD3**- fraction 3 obtained from dichloromethane crude extract; **FD4**- fraction 4 obtained from dichloromethane crude extract; **DCM**- dichloromethane crude extract; & **n-Hex**- n-hexane crude extract

The MeOH extract also exhibited the antioxidant activity with FM3 containing the most active antioxidant compounds of different polarities followed by FM2.

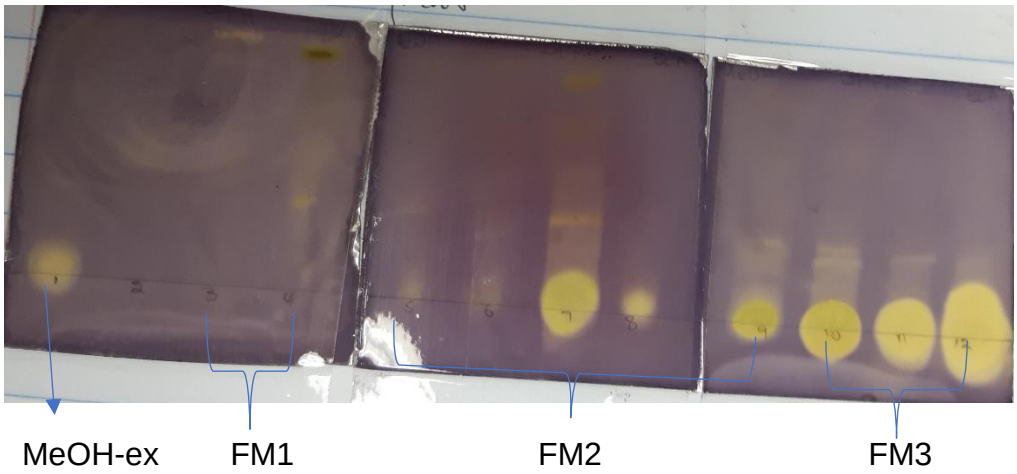

Figure 4.5: Chromatograms of *T. elegans* MeOH extracts and its fractions developed in BEA solvent system. **MeOH-ex**- methanol crude extract; **FM1**- fraction 1 obtained from methanol crude extract; **FM2**- fraction 2 obtained from methanol crude extract; **FM3**- fraction 3 obtained from methanol.

Singh et al. (2012) reported that flavonoids and phenols possess a potent scavenging ability of free radical and reactive oxygen species produced in mammals. Hence, *T. elegans* leaf can be studied further, wherein the compounds responsible for scavenging free radicals (Figures 4.4 and 4.5) could be isolated, purified and also characterized. The FD4 showed fainted yellow bands (Figure 4.4) due to the trace amount of flavonoids exhibited by qualitative test tube phytochemical analysis (Table

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4.1). These results concur with findings by Satheesh et al. (2012) and Ayoola et al. (2008) who defined flavonoids as the primary major group responsible for potent free radical scavenging potential. Additionally, more studies reported on the presence of flavonoids in plants associated with the free radical scavenging effect (Njoya et al., 2017; Aiyegoro and Okoh, 2010).

Of note, the results of the potent free radical scavenging activity suggest that *T. elegans* leaves contain compounds that are rather capable of donating hydrogen to free radicals, which will result in removing the odd electron responsible for radical's reactivity (Aiyegoro and Okoh, 2010).

4.3 ACTIVITIES AGAINST PATHOGENS

4.3.1 ANTI-BACTERIAL ACTIVITY

The antimicrobial activity of crude extracts and fractions of *T. elegans* were tested against four microorganisms namely, two opportunistic pathogens (*E. coli* and *S. pyogenes*) and two multidrug-resistant bacteria (*P. aeruginosa* and *S. aureus*). It was observed that all the tested crude extracts and fractions were active against the selected bacterial strains (Figure 4.6).

The MIC values of the crude extracts and obtained fractions against the tested bacteria are shown in Table 4.5. The anti-microbial activity of extracts is based on values defined by Lagnika et al (2016); as high activity if the MIC is ≤ 0.1 mg/mL, moderate if 0.1 <MIC ≤ 0. 625 mg/mL and weak if MIC > 0.625 mg/mL. In the present study, the MIC values varied between 0.625 and 20 mg/mL. Gram-positive bacteria were the most susceptible compared to Gram-negative bacteria. FD4 was found to be the most potent fraction which inhibited all tested bacterial stains with the MIC value of 0.625 mg/mL. Therefore, FD4 exhibited moderate bioactivity (0.625 mg/mL), whereas FD2 showed weak bioactivity (20 mg/mL) against all tested bacteria. *T. elegans* displayed a broad-spectrum antibacterial activity.

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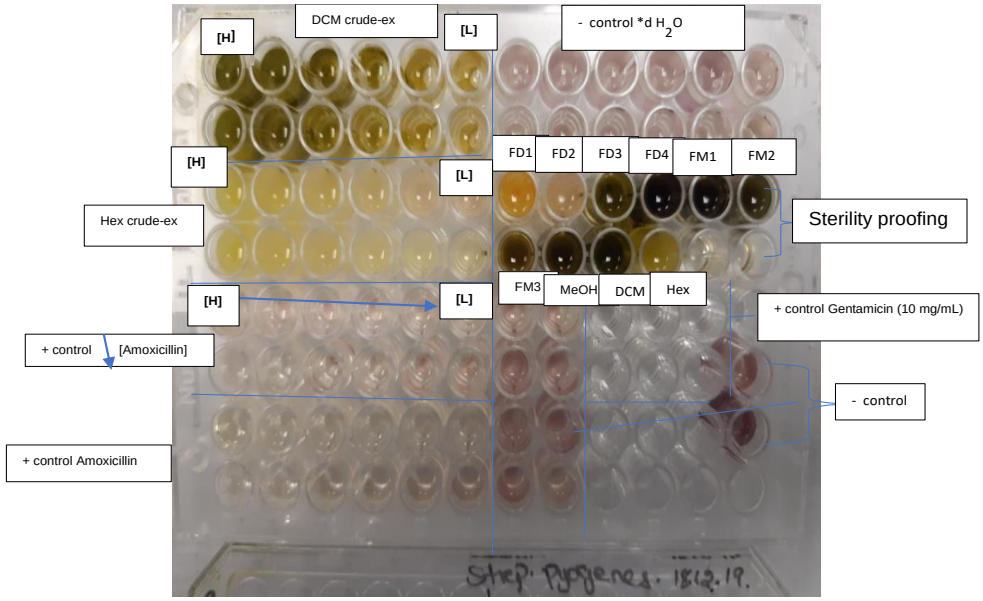

Figure 4.6: A 96 microwell plate depicting results of microdilution. **FD1**- DCM fraction 1; **FD2**- DCM fraction 2; **FD3**- DCM fraction 3; **FD4**- DCM fraction 4; **FM1**- MeOH fraction 1; **FM2**- MeOH fraction 2; **FM3**- MeOH fraction 3; **MeOH**- methanol crude extract; **DCM**- dichloromethane crude extract; **n-Hex**- n-hexane crude extract; +control- positive control; -control - negative control; dH2O- distilled water, [H]- high concentration, [L]- low concentration.

Table 4.5: Antibacterial activity of *T. elegans* crude extract and fractions

Sample	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. pyogenes*
FD1	20 mg/mL	20 mg/mL	5 mg/mL	2.5 mg/mL
FD2	20 mg/mL	20 mg/mL	20 mg/mL	20 mg/mL
FD3	5 mg/mL	5 mg/mL	1.25 mg/mL	0.625 mg/mL
FD4	0.625 mg/mL	0.625 mg/mL	0.625 mg/mL	0.625 mg/mL
FM1	2.5 mg/mL	5 mg/mL	1.25 mg/mL	0.625 mg/mL
FM2	10 mg/mL	10 mg/ML	2.5 mg/mL	0.625 mg/mL
FM3	10 mg/mL	10 mg/mL	2.5 mg/mL	0.625 mg/mL
MeOH	2.5 mg/mL	10 mg/mL	2.5 mg/mL	0.625 mg/mL
DCM	5 mg/mL	5 mg/mL	1.25 mg/mL	0.625 mg/mL
Hex	20 mg/mL	20 mg/mL	20 mg/mL	0.625 mg/mL

FD1- DCM fraction 1; **FD2**- DCM fraction 2; **FD3**- DCM fraction 3; **FD4**- DCM fraction 4; **FM1**- MeOH fraction 1; **FM2**- MeOH fraction 2; **FM3**- MeOH fraction 3; **MeOH**- methanol crude extract; **DCM**- dichloromethane crude extract; **n-Hex**- n-hexane crude extract.

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Table 4.6: Activities of antibiotic standards

Antibiotics	*E. coli*	*P. aeruginosa*	*S. aureus*	*S. pyogenes*
Gentamycin (10 mg/mL)	S	S	R	S
Amoxicillin (1 mg/mL)	N/A	N/A	N/A	R

S- Susceptible; R- Resistant; N/A-Not applicable

The antibacterial activity ranged from moderate (≤ 0.625 mg/mL) to weak activity (20 mg/mL). It is worth mentioning that some extracts and fractions showed potent activity against the tested bacteria and they were limited by the plate design, as well as the concentration of interest in initiating this assay (20 mg/mL). It is considered too high and a much lower starting concentration (1 mg/mL) will be used for the manuscript in preparation. Hence, the antibacterial results obtained in this study are considered as preliminary. However, it is worth mentioning that the extracts and fractions exhibited a broad-spectrum activity looking at the three factors: Gram staining, pathogenicity (opportunistic pathogens) and susceptibility (multidrug resistance). Despite the pronounced antioxidant activity exhibited by FD2, in our study this fraction possessed a weak antibacterial potential in all the tested bacterial strains (Table 4.5). Nevertheless, it might be due to the lack of terpenoids that were present in the FD4 (the most potent fraction against all tested bacterial strains) as presented in Table 4.5.

The antibacterial compounds are found in DCM extracts wherein FD4 displayed a potent antibacterial activity (Table 4.5). FD4 fraction showed an antibacterial effect against all the tested diverse bacteria, suggesting the presence of broad-spectrum antibacterial phytoconstituents. The antibacterial activity of *T. elegans* is corroborated by previous studies; where an ethanolic extract of the root part was reported to have antibacterial properties against *B. subtilis* and *S. aureus* (Pallant et al., 2012) and the ethyl acetate extract of the leave part displayed an activity against three Mycobacterium spp (*M. bovis* BCG, *M. tuberculosis* H37Ra and H37Rv) (Luo et al., 2011).

Based on the results in our study FD4 showed to be the most active fraction therefore, further isolation is required to identify the active compounds. Even though they tested negative in this study, it cannot be concluded that the *T. elegans* leaves do not contain alkaloids so the type of the test method used in chapter 3 could have played a role,

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that is at least 2 or more additional test methods should have been performed. Additionally, alkaloids have been isolated from the *T. elegans* leaf extracts (Hirasawa et al., 2009; Mansoor et al., 2009a,b; Van der Heijden et al., 1986b).

In the current study, *T. elegans* showed an activity against Gram positive bacteria and this is in agreement with the results obtained from testing some of the Tabernaemontana species (*T. chippi* and *T. dichotoma*). Antibacterial activity has been reported in other Tabernaemontana species such as *T. catharinensis*, *T. pachysiphon* and *T. citrifolia* (Silveira et al., 2017).

Most importantly, all the extracts and fractions inhibited the *S. aureus,* unlike Gentamicin which failed to inhibit this bacterial strain. Furthermore, the activity against the tested Gram-negative bacteria is supported by the ethnomedicinal usage of this plant. Indeed, it has been mentioned to have the ability to treat gonorrheal infections (De Wet et al.,2012). Of course, keeping in mind that *Neisseria gonorrheoae*; a Gram-negative bacterium is an etiology of gonorrheal infection.

The MBC was defined as the concentration of the extract and or fraction that resulted in 99.9% killing of the bacterium (Pallant et al., 2012). All extracts and fractions were within two serial dilutions of the respective MICs obtained for the bacterial strains. Hence, it was concluded that the *T. elegans* leaf extracts and fractions are bactericidal. It is worth mentioning that this plant did not only exhibit anti-bacterial activity against both Gram-negative and Gram-positive, but also against the two multidrug resistant bacteria (*S. aureus*; ATCC BAA- 2312 and *P. aeruginosa*; ATCC BAA-1744). It is evident that the potent antibacterial bioactive compounds are contained in the FD4 fraction.

4.3.2 ANTI-HIV-1 RT ACTIVITY

The HIV-1 reverse transcriptase inhibition assay was performed with two crude extracts (MeOH and DCM extract) and 7 fractions (FD1, FD2, FD3, FD4, FM1, FM2 & FM3). For the current study, only qualitative description of the respective assay was achieved (Figure 4.7). All extracts showed potential anti-HIV-1 RT activity. The FM2 fraction shows a fainted green color, suggesting a partial inhibition of RT enzyme. Even though the anti-HIV-1 RT inhibition was limited to the qualitative assay, the inhibition of RT enzyme seemed to be comparable to the positive control (Figure 4.7).

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The leave extracts of *T. elegans* possess anti-HIV-1 effects, this could be due to various phytochemicals which tested positive (Table 4.4).

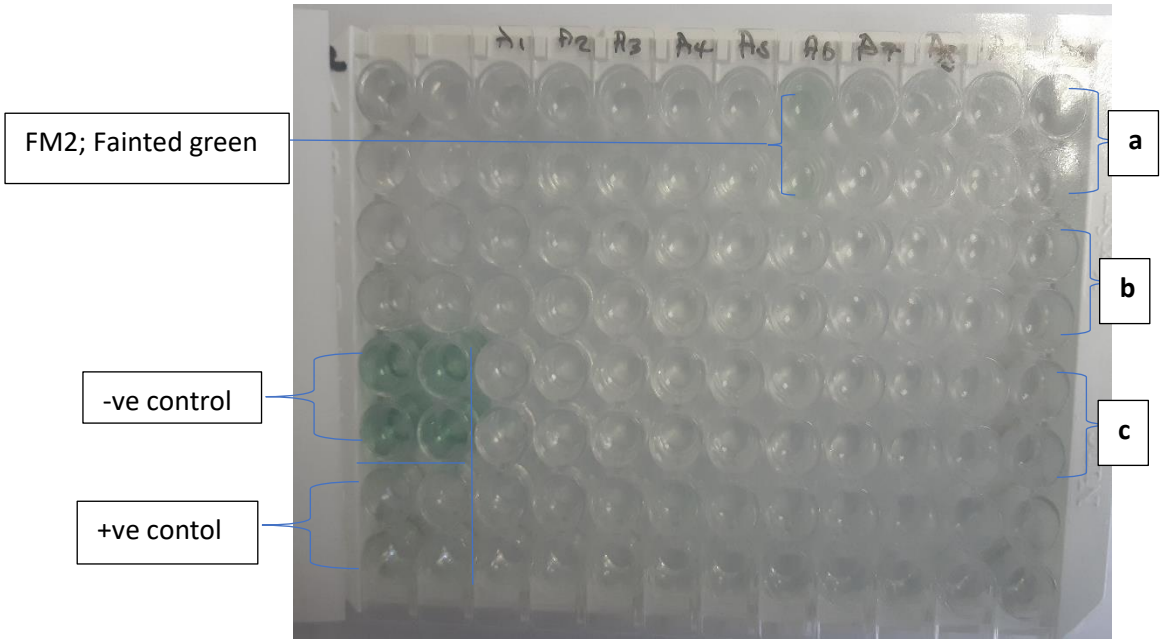

Figure 4.7: The effect of *T. elegans* crude extracts and fractions on the HIV-1 reverse transcriptase. Positive control used was EFV (100 µg/mL). The decolouration reaction brought by the active agent (A1-A10). A1: FD1; A2: FD2; A3: FD3; A4: FD4; A5: FM1; A6: FM2; A7: FM3; A8: methanol crude extract; A9: dichloromethane crude extract; and A10: hexane crude extract. Letter **a, b, c,** represents the duplicates of three concentrations (50 µg/mL, 100 µg/mL and 200 µg/mL) respectively.

The anti-HIV-1 RT test was conducted due to the curiosity behind the ethnomedicinal usage of *T. elegans*. De Wet and co-workers (2012) listed this plant as one of the plants used in treatment of various sexually transmitted infections. Recently, the search for efficient and nontoxic drugs to manage HIV and to overcome drug resistance, researchers are considering plant-based medicine which had proven its potential in managing many diseases. Several studies demonstrated the usefulness of plants as reservoirs for anti-HIV agents (Rege et al., 2015; Leteane et al., 2012).

The capacity of plants in the management of HIV is reported to be caused by the chemical compounds they contain such as flavonoids; coumarins; alkaloids; terpenoids; phenolics; saponins and tannins (Selehi et al., 2018). These phytochemicals have shown anti-HIV properties through various mechanisms targeting crucial steps of HIV replication cycle including inhibition of key enzymes (RT,

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protease, entry and fusion and integrase) activities (Salehi et al., 2018; Kapewangolo et al., 2017; Silprasit et al., 2011).

4.4 *IN VITRO* ASSAYS

4.4.1 ANTI-INFLAMMATORY ACTIVITY

The inhibitory activity of the 2 crude extracts and 7 fractions on NO production by induced RAW 264.7 macrophage cell lines is shown in Figures 4.8 - 4.11.

Some extracts and fractions showed a strong NO inhibition, while the other ones presented a weak NO inhibition, and few revealed no meaningful anti-inflammatory potential at varying toxicity ranging from mild to severe. Sample FD3, FM2, C2 and C3 show strong anti-inflammatory activity potential, however mild cytotoxicity challenges the accuracy of determining the anti-inflammatory activity, particularly at higher concentrations. FD4 has strong anti-inflammatory potential, however severe cytotoxicity may contribute significantly to the anti-inflammatory activity. Sample FM3 presented a weak anti-inflammatory potential.

C2 (DCM extract) and C3 (MeOH extract), FD3 as well as FM2 strongly inhibited the NO production, where the cell viability was above 60% (Figure 4.8, 4.10 and 4.11 respectively). Moreover, FM2 exhibited a certain anti-inflammatory potential with a cell viability > 66%. However, at 200 µg/mL could be argued that it was due to the reduction of cell viability which was approximately 42%. FD1, FD2 and FM1 showed no meaningful anti-inflammatory potential. However, at 25 µg/mL FM1 seemed to induce NO production ($p < 0.05$, compared to untreated) together with the cell proliferation. FD3 showed a potent NO inhibition in all tested concentrations; at 25 µg/mL the NO production was inhibited highly significant ($p < 0.0001$, compared to untreated) and at 50, 100 & 200 µg/mL a significant NO inhibition was observed ($p < 0.05$, compared to untreated), where above 66% of cells remained viable.

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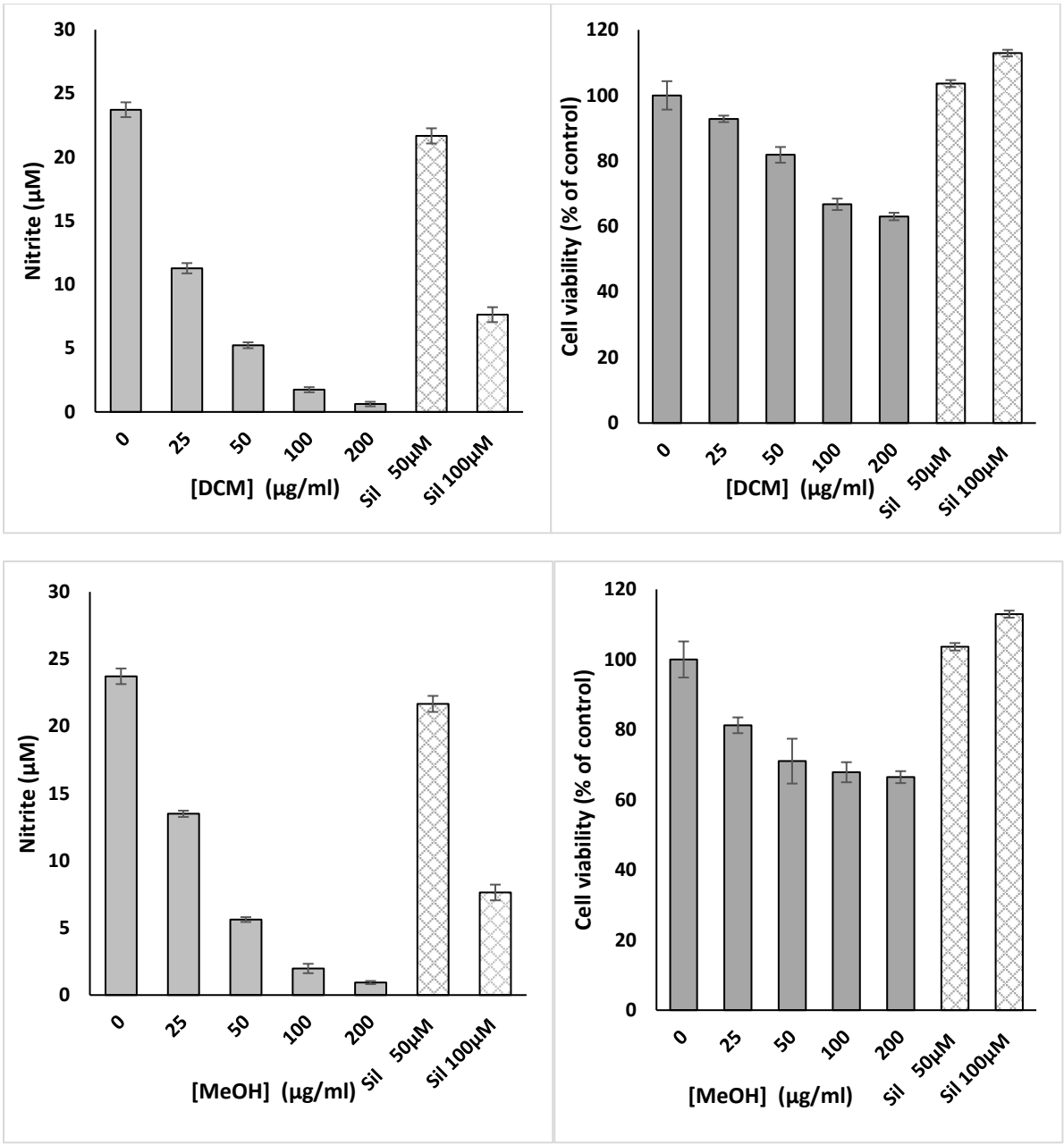

Figure 4.8: Inhibition of NO production in LPS induced RAW 264.6 cells by the crude extracts and corresponding cell viability.

FD1 at 25 and 50 µg/mL and FD2 at 25, 50 and 100 µg/mL the fraction seemed to have promoted NO production, but at 200 µg/mL FD2 inhibited NO production significantly ($p < 0.01$, compared to untreated) surprisingly with an induction of cell viability (Figure 4.9).

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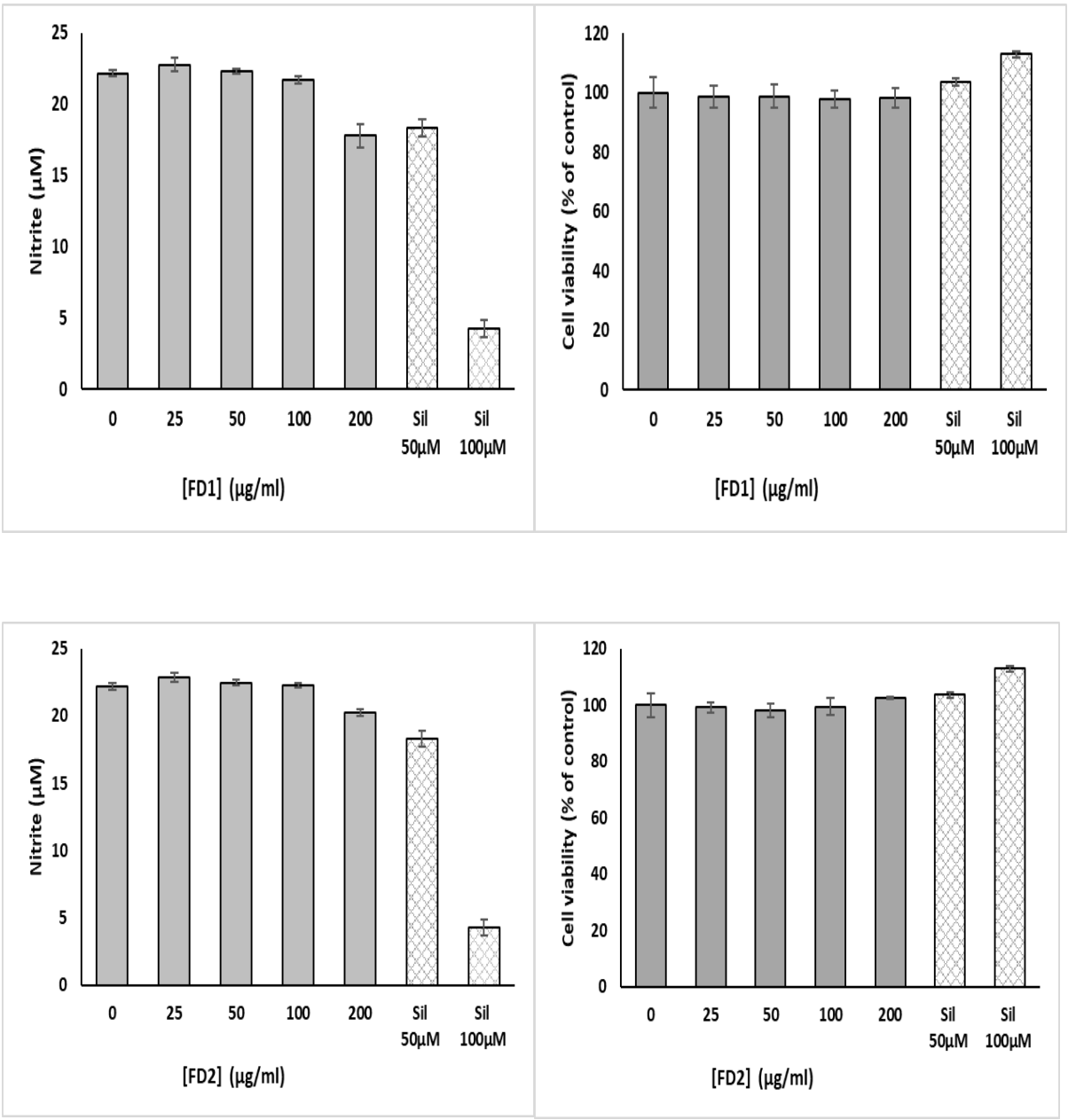

Figure 4.9: Inhibition of NO production in LPS induced RAW 264.6 cells by FD1 and FD2 fractions obtained from the DCM crude extract and corresponding cell viability.

FD3 strongly inhibited NO production in a concentration-dependent manner, where cell viability was > 66%. Conversely, FD4 at 50, 100 and 200 µg/mL strongly inhibited the NO production, however as depicted in Figure 4.10 the cell viability reduced significantly to 21, 5 and 4% respectively.

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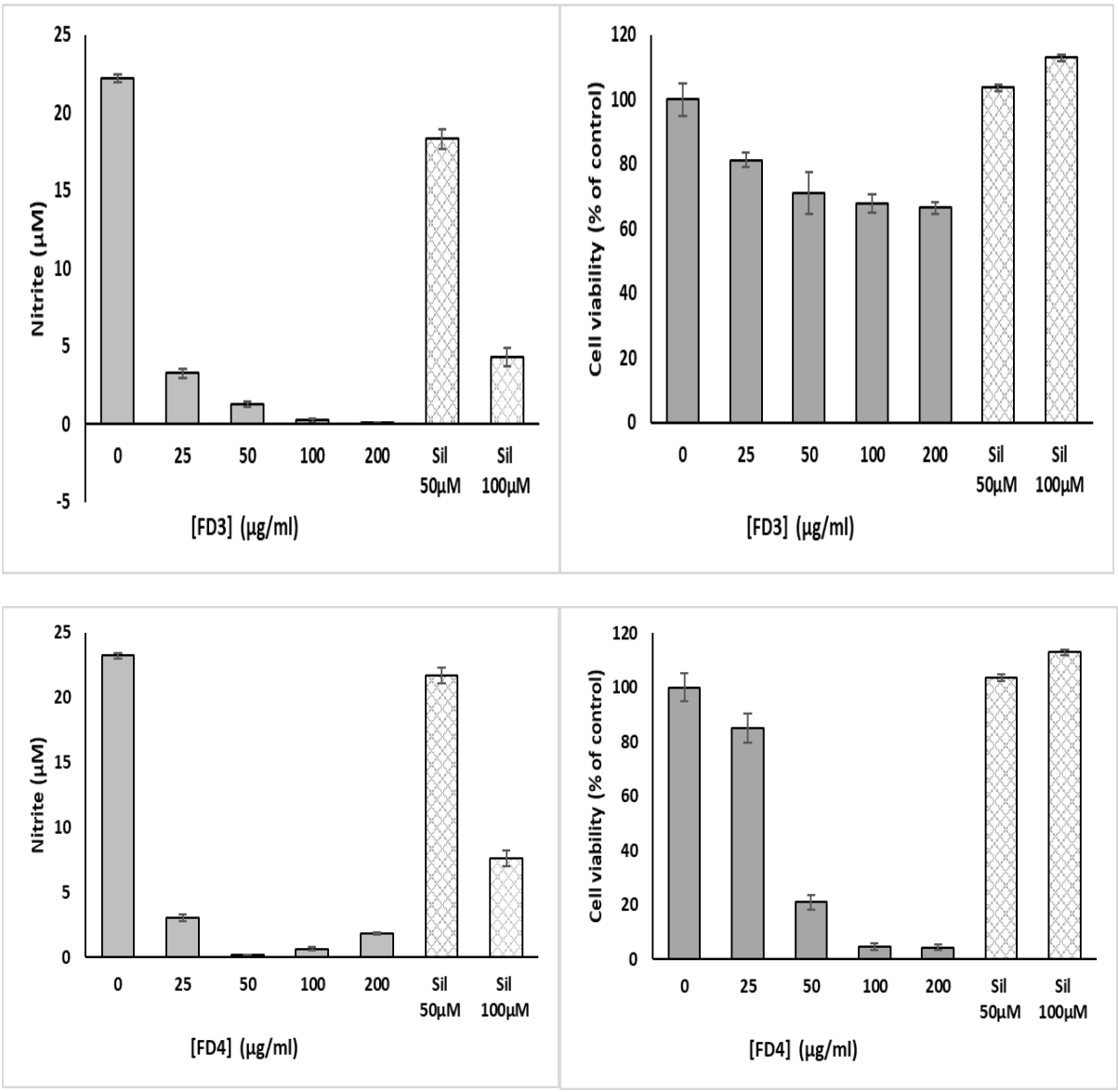

Figure 4.10: Inhibition of NO production in LPS induced RAW 264.6 cells by FD3 and FD4 fractions obtained from the DCM crude extract and corresponding cell viability.

FM1 at 25 µg/mL seemed to might have lightly promoted both the NO production and the cell viability and FM2 at 25, 50 and 100 µg/mL strongly inhibited NO production, where cell viability was > 66% but at 200 µg/mL the cell viability decreased to < 50%. FM3 present a weak NO inhibition, where cell viability was > 95% and at 200 µg/mL amount of cell viability exceeded the viable cells of the untreated.

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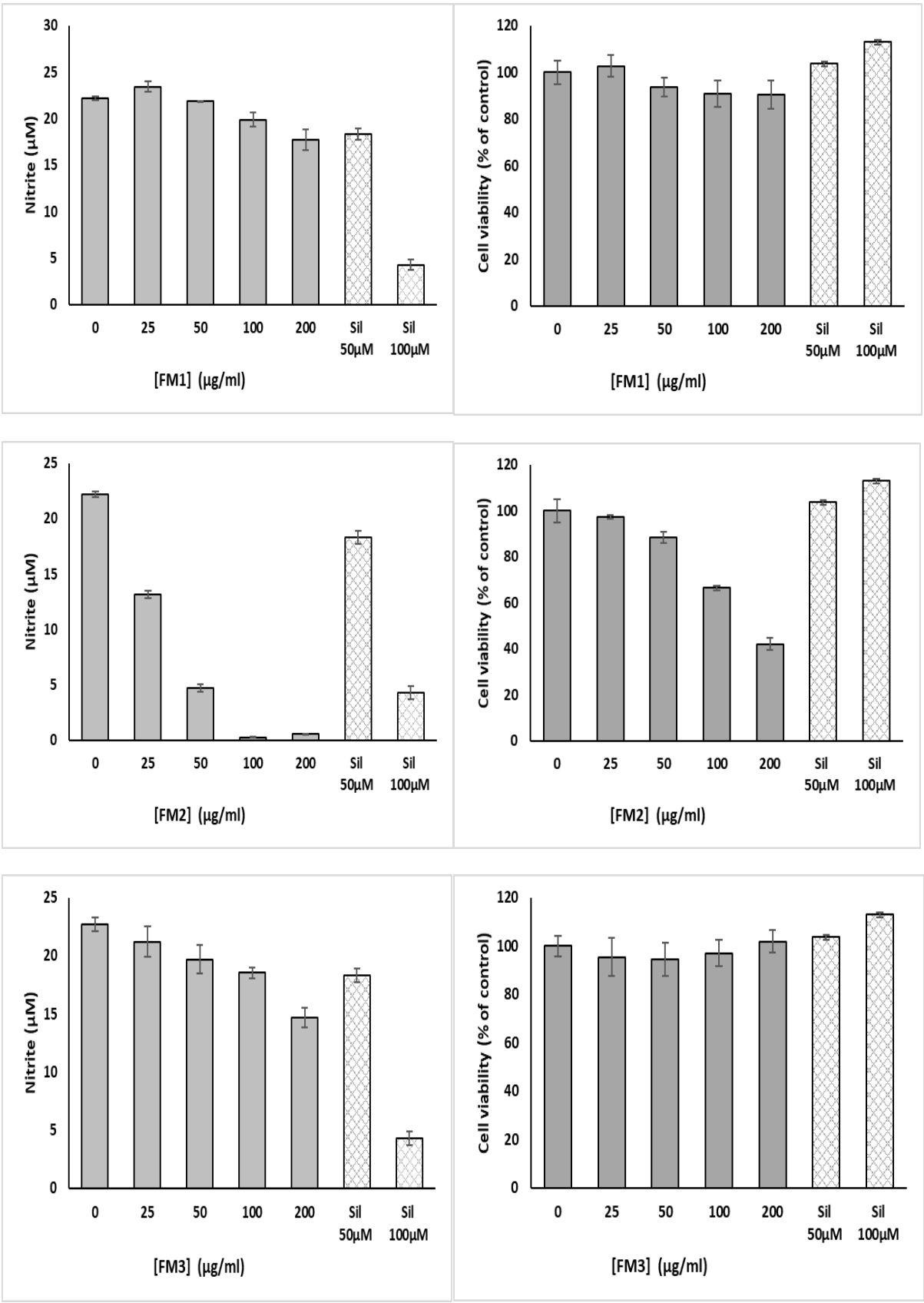

Figure 4.11: Inhibition of NO production in LPS induced RAW 264.6 cells by FM1-3 fractions obtained from the MeOH crude extract and corresponding cell viability.

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The regulation of inflammation is crucial as a defense mechanism against various entities in bringing homeostasis in the human body (Förstermann and Sessa, 2012; Scoditti et al., 2012). However, the over production of some of these pro-inflammatory molecules such as NO could be fatal (Njoya et al., 2017; Delgado-Velez and Lasalde-Dominicci, 2018; Adebayo et al., 2015; Dzoyem and Eloff, 2015; Förstermann and Sessa, 2012).

Our results demonstrated that *T. elegans* leaf extracts and fractions inhibit the inflammatory response in LPS-stimulated macrophages by suppressing the overproduction of NO, while on the other hand the severe cytotoxicity exhibited by some fractions might have contributed significantly to the anti-inflammatory activity measured. FM3 showed a weak NO inhibition where cell viability was > 94% and at 200 µg/mL it showed a weak NO inhibition with an approximate increase in cell viability of 2%. In all crude extracts and 2 fractions which exhibited a potent anti-inflammatory activity, this could be due to the presence of the flavonoids, saponins (Aarland et al., 2017). Flavonoids are well documented for their anti-inflammatory benefits.

It is worth mentioning that for an extract or fraction to be classified as a good inhibitor of NO production, it has to have a low cytotoxicity (Adebayo et al., 2015). Therefore, FD3, FM2, FM3, C2 and C3 might be used to control inflammation caused by the overproduction of NO because the inhibition correlated to a low cytotoxic effect.

4.4.2 CYTOTOXICITY

Toxicity results

To investigate the cytotoxicity of *T. elegans* leaf extracts and fractions, we used the dual staining procedure with nuclear dye, Hoechst 33342 and propidium iodide (PI) on two cell types (HeLa cancer cell and Vero cell lines). All live cells would stain positive with Hoechst 33342, whereas dying or dead cells would be stained positive with PI (Figure 4.12 and 4.13). In overall, the toxicity of extracts observed was concentration dependent.

Figure 4.12 clearly shows that most fractions (FD1, FD2, FM1, FM3) encouraged cell proliferation at different concentrations, since the number of live cells was increased in comparison to the untreated. Conversely, FD1 (only at the higher concentration), FD3, FD4, FM2, C2 and C3 induced the death of HeLa cell lines. FD1 was only toxic at the highest concentration (200 µg/mL). FD2, FM1 (only at 50 and 100 µg/mL) and

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FM3 including FD1 but only at the lower concentrations (50 and 100 µg/mL) were not cytotoxic. FD3, FD4 and FM2 exhibited a potent antiproliferative effect against the HeLa cancer cells at 100 and 200 µg/mL, compared to the positive control (Melphalan).

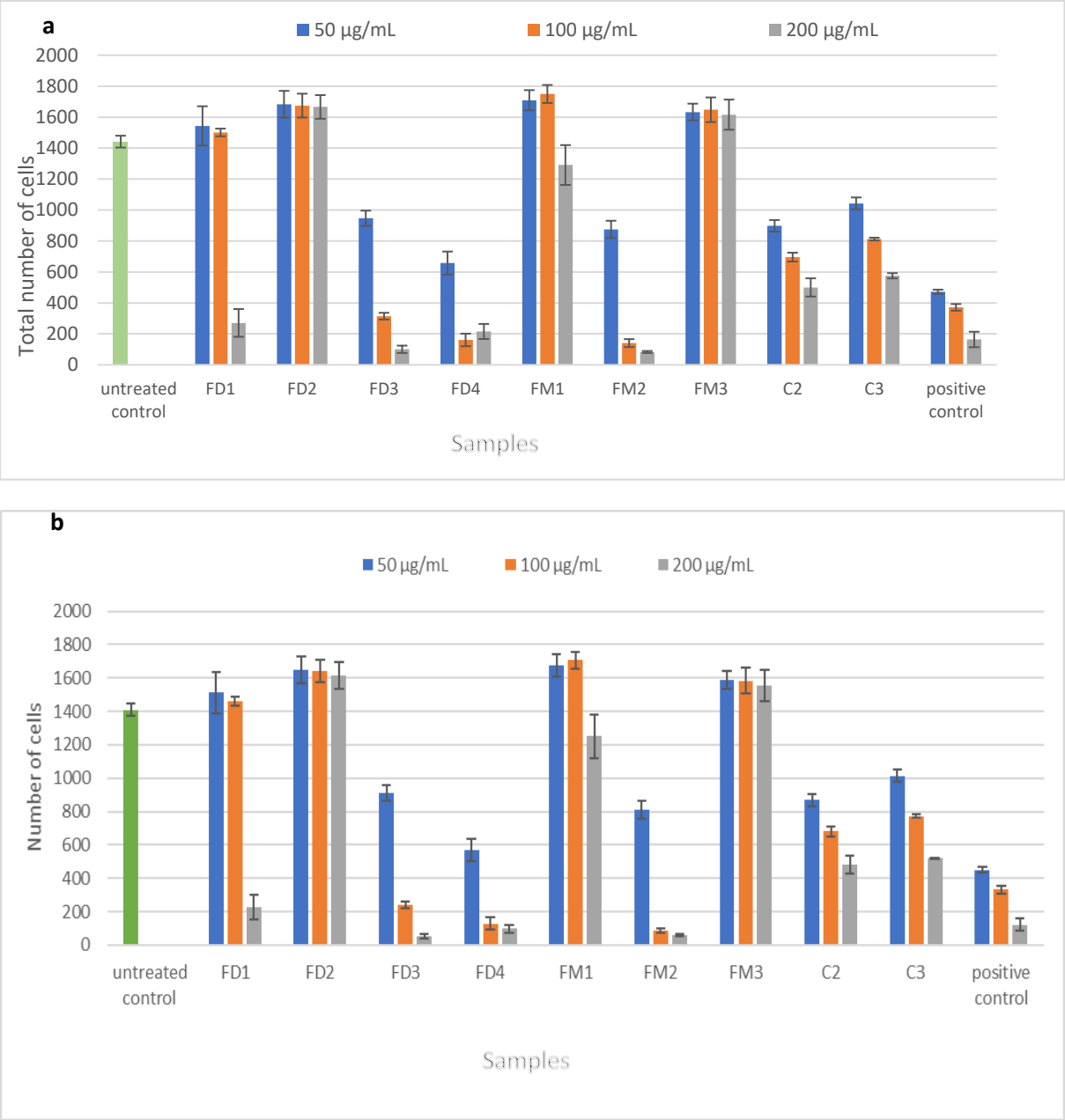

Figure 4.12: Toxicity effects of *T. elegans* crude extract and fractions against HeLa cancer cell lines. **FD1**- DCM fraction 1; **FD2**- DCM fraction 2; **FD3**- DCM fraction 3; **FD4**- DCM fraction 4; **FM1**- MeOH fraction 1; **FM2**- MeOH fraction 2; **FM3**- MeOH fraction 3; **C2**- dichloromethane crude extract & **C3**- methanol crude extract

Toxicity effects of the tested crude and its various fractions on Vero cells are illustrated in Figure 4.13. Just like in Figure 4.12, the toxicity observed below (Figure 4.13) is

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concentration dependent. Toxicity effects was observed on Vero cells by FD3, FD4, FM2, C2 and C3 reduced the total cell number and live cells significantly at 50 µg/mL ($p < 0.001$, compared to untreated). While FD1 (only at a lower concentration), FD2, FM1 and FM3 promoted a slight cell proliferation of Vero cells. FD2 and FM1, both promoted a significant increase of viable cells ($p < 0.05$ at 50 µg/mL, compared to untreated).

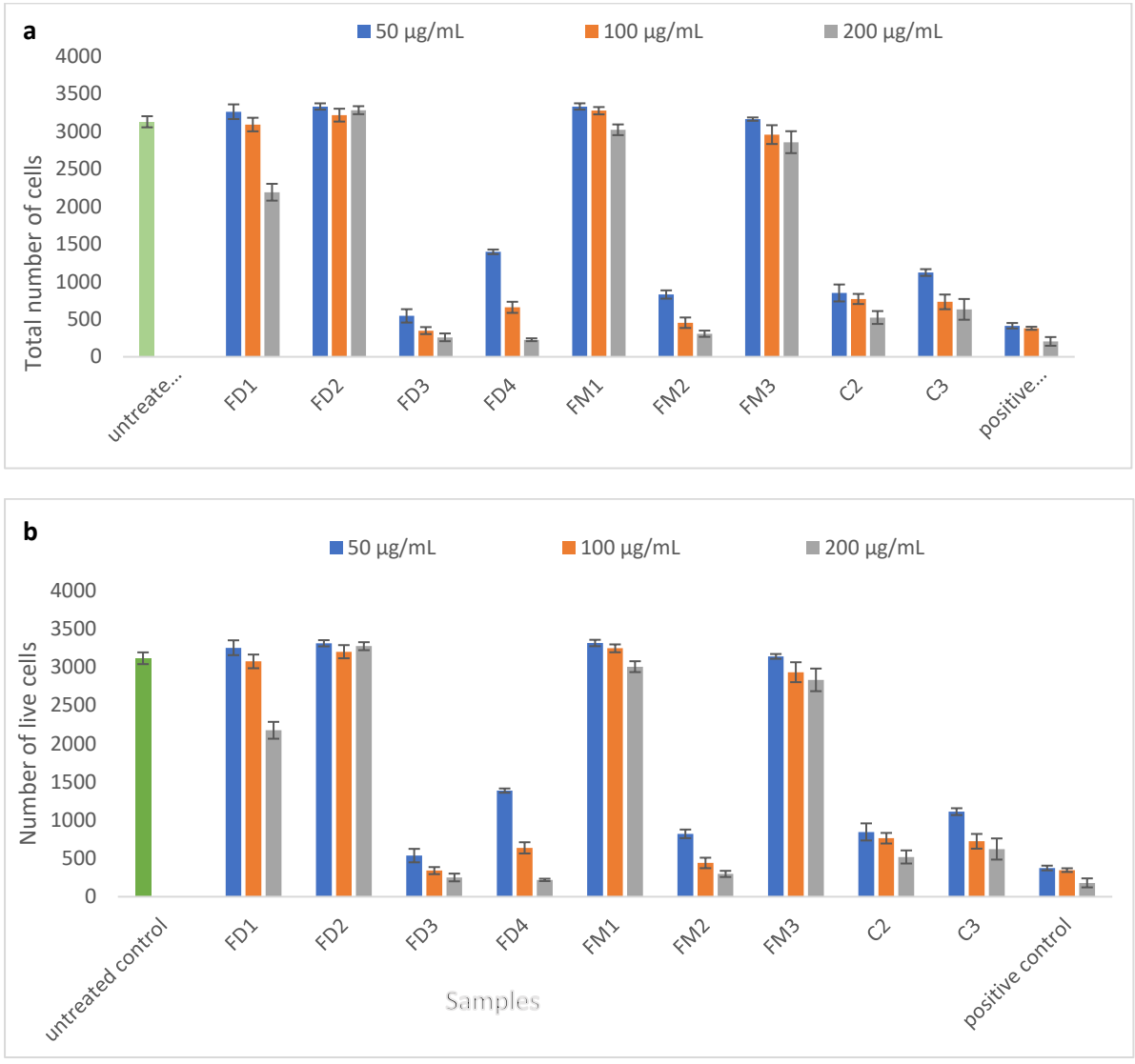

Figure 4.13: Toxicity effects of *T. elegans* crude extracts and fractions against Vero cell lines. **FD1**- DCM fraction 1; **FD2**- DCM fraction 2; **FD3**- DCM fraction 3; **FD4**- DCM fraction 4; **FM1**- MeOH fraction 1; **FM2**- MeOH fraction 2; **FM3**- MeOH fraction 3; **C2**- dichloromethane crude extract & **C3**- methanol crude extract

The isolates and characterized phytochemical constituents of *T. elegans* root extracts have been reported to induce apoptosis and antiproliferative effect against several cancer cell lines including a multidrug resistance (MDR) cancer cell line (Paterna et

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al., 2017; Paterna et al., 2016; Mansoor et al., 2013; Pallant et al., 2012), but little is known about the phytochemical constituents of *T. elegans* leaf extracts together with its pharmacological value. Moreover, as much as some phytochemicals are beneficial or useful for the pharmaceutical purposes, some are detrimental (Jaradat et al., 2017; Demma et al., 2009).

Therefore, it is necessary to evaluate the toxicity or whether a plant used for medicinal purpose is safe for human consumption or not. This assay was carried out in order to determine the inhibitory effects of extracts together with their different fractions on the proliferation of a cancerous cell line (HeLa) and also to compare their safety toward normal Vero cells. With this objective in mind, a selective toxicity, which is a basic principle of cancer chemotherapy, should be observed (Njoya et al., 2017). With that being mentioned, some of the fractions and extracts exhibited toxic effects against the tested human cervix cancer cell line (HeLa) whereas moderate toxicity towards the normal Vero cell lines was observed. This trend was also observed in a study conducted by Njoya et al. (2017).

It is not surprising to see the antiproliferation activity of the current tested samples since *T. elegans* belongs to the family, Apocynaceae: characterized by the presence of indole alkaloids and *Tabernaemontana spp* highly valued for their composition (Silveira et al., 2017). Hence, we took the interest in evaluating the pharmacological potential of *T. elegans*. Furthermore, these alkaloids have been isolated from root extracts and were found to be antiproliferative against various cancer cell lines. Moreover, the MeOH *T. elegans* leaf extract has been found to induce apoptosis in human hepatoma cells (Rizo et al., 2013). Conversely, FD2 induced the proliferation of HeLa cancer cells and this could be due to a certain novel phytochemical constituent.

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Visualization of the effects of tested extracts and fraction on Vero and HeLa cells

It is worth mentioning that the results were interpreted in two ways namely: * whether the sample was cytotoxic or not and ** whether the sample was necrotic. The necrosis determination was based on the % of PI staining relative to the amount of live and dead cells. Figures 4.14 to 4.16 show the density of cells after treatment with crude extracts and the fractions of *T. elegans* (Tables 4.7 & 4.8).

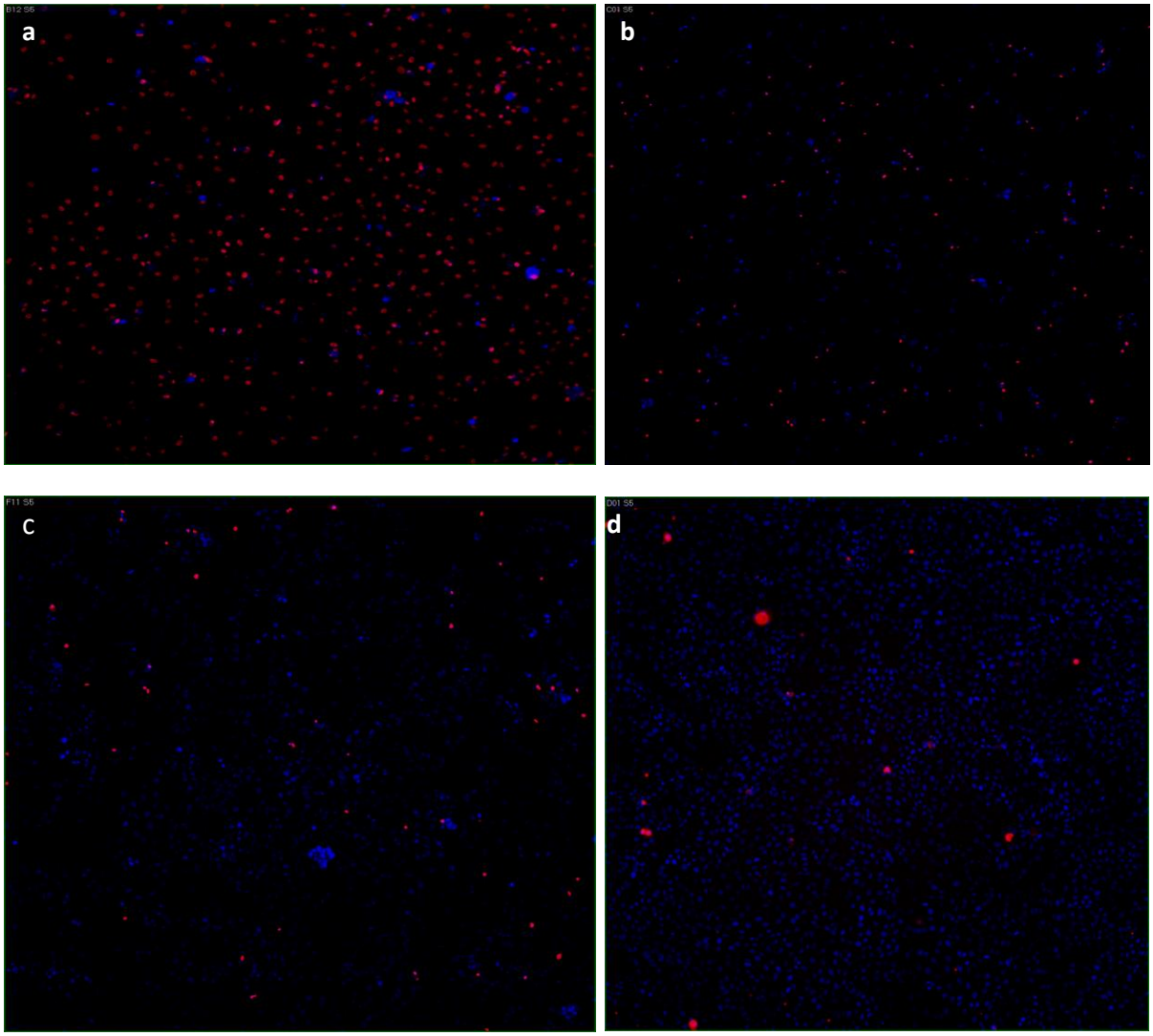

Figure 4.14: Evaluation of *T. elegans* crude and fractions effect on Vero cell line by fluorescence microscopy of Hoechst (to stain the live cells, indicative by blue dots) stained nuclei as well as the PI (to stain the damaged or dead cells, indicative by red dots). (a) Depicts a highly cytotoxic and necrotic extract**. (b) Depicts C2 (DCM extract) is cytotoxic* but not necrotic (c) Untreated neither cytotoxic nor necrotic; (d) Just like the untreated, FD1 at 50 µg/mL was neither cytotoxic nor necrotic. The cells were examined using a confocal microscope.

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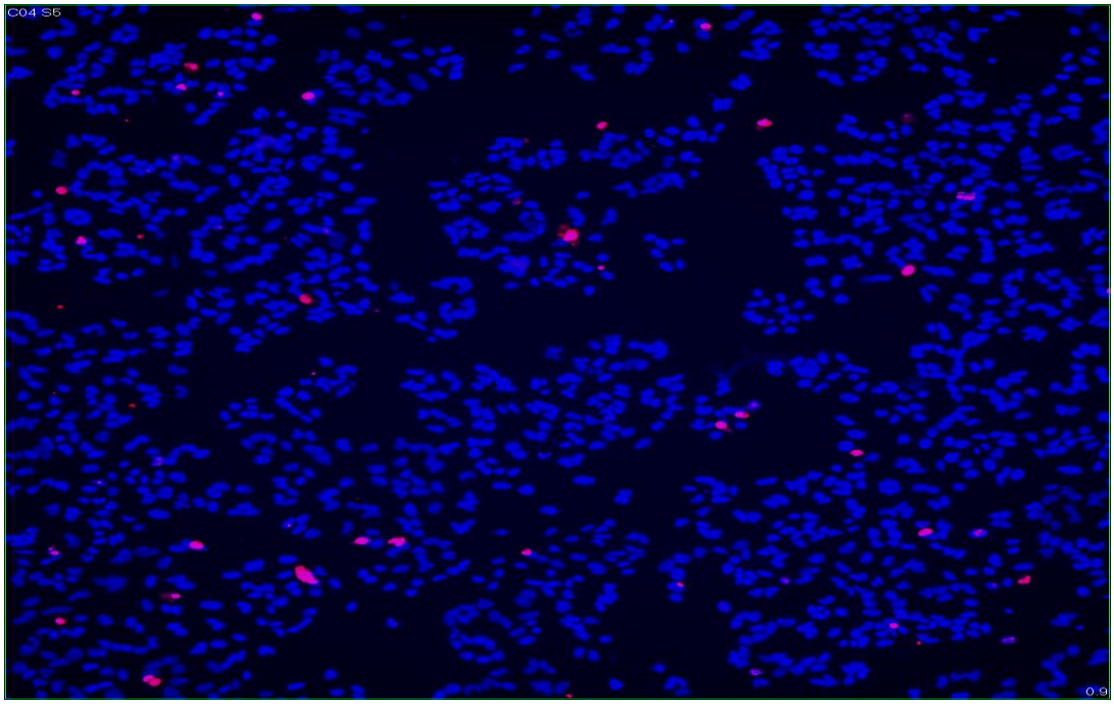

Figure 4.15: FD2 promoted cell proliferation of human cervix cancer cell line (HeLa).

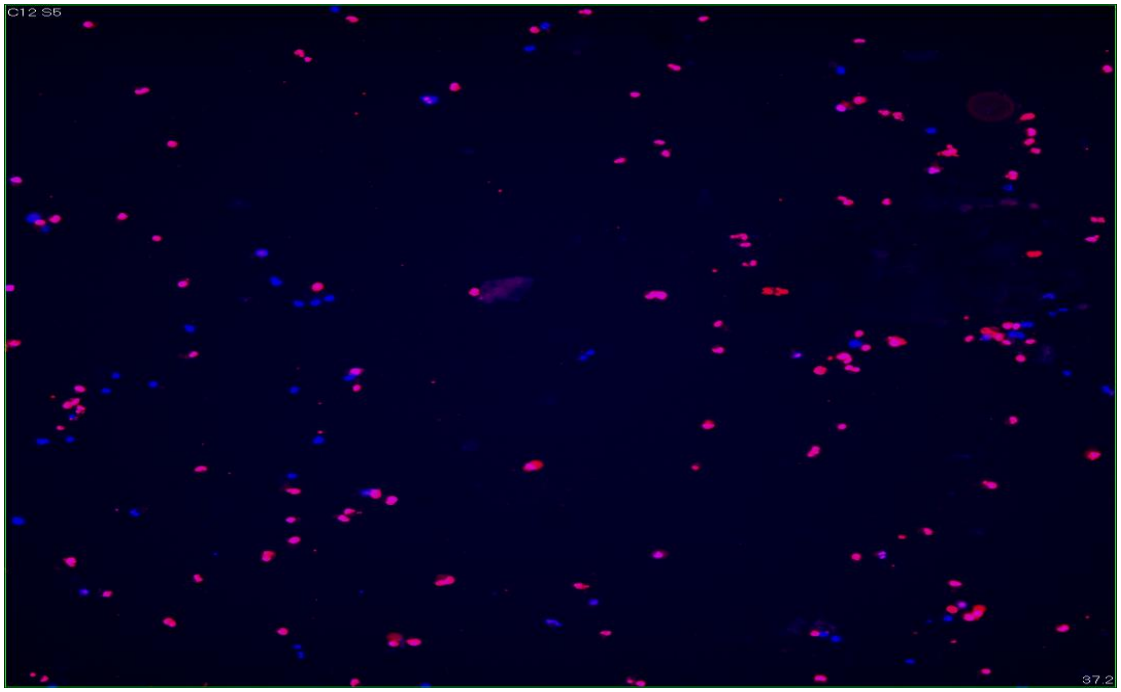

Figure 4.16: FD4 at 200 highly potent against human cervix cancer cell line (HeLa), making it a good anti-HeLa agent.

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Jaradat et al. (2017) reported that the high consumption of some phytochemicals such as oxalic acid and nitrate contained in *Portulaca olercea* to be harmful for patients suffering from renal failure. They further mentioned that in literature there are plants which are classified as harmful and cannot be used for the treatment of diseases. Additionally, some plants have been reported to be capable of inducing genotoxic and mutagenic effect in *in vitro* studies (Demma et al., 2009).

The effect observed in Figure 4.16 could be attributed to the presence of phytochemical constituents contained in Fraction 4 of the DCM extract (FD4). These observations are in agreement with the findings from a study conducted by Paterna et al. (2016) who reported that the DCM soluble alkaloid fraction displayed potent cytotoxic effects against two colons (HCT116 and SW620) and liver (HepG2) cancer cell lines. Additionally, alkaloidal derivatives were found to be potent against drug resistant cancer cells (Paterna et al., 2017). It is worth highlighting the fact that literature has shown that *T. elegans* root extract is a rich source of indole alkaloids which are believed to be a potent antiproliferative agent against several human cancer cell lines (Paterna et al., 2017; Paterna et al., 2016; Mansoor et al., 2013; Pallant et al., 2012).

Additionally, the presence of alkaloids in *T. elegans* root extract was supported by one of our work wherein alkaloids tested positive in the ethanol, ethanol + water and chloroform extract (data not yet published). Luo et al. (2011) reported that a novel monoterpene alkaloid was isolated from the *T. elegans* leaves. Nevertheless, in the present study, alkaloids were not found yet the plant still showed a potent antiproliferative activity against HeLa cell line. This could be due to the terpenoids which tested positive by the qualitative screening as illustrated above.

It is worth mentioning that despite terpenoids being able to induce inhibitory effect on microorganism (antimicrobial agent), they are also called detoxifying agents (Koche et al., 2016). Hence, it is no surprise that FD1 only at 200 µg/mL, FD3, FD4 and FM2 exhibited significant antiproliferative activity against the HeLa cancer cell line and have less or no toxicity against Vero cell line compared to melphalan. Conversely, FD2 at all tested concentrations (50, 100 and 200 µg/mL), FM1 at 50 and 100 µg/mL and FM3 at all tested concentrations (50, 100 and 200 µg/mL) induced cell proliferation of HeLa cells and this could be due to different phytochemicals harbored by *T. elegans* leaf fractions. This observation agrees with Saxena et al. (2013) who defined

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phytochemicals as molecules that can detoxify carcinogens or substances that promotes cancer. This behavior is observed in Figures 4.12 and 4.13.

Based on our results it is better to work with fractions than crude extracts. Since a fraction (FD1 at 200 µg/mL) contained different compounds and some appeared to have compounds which displayed a better selective toxicity unlike the crude extracts which showed moderate toxicity toward the Vero cells. Moreover, this is because crude extracts contained a mixture of different compounds of which some were cytotoxic to both HeLa and Vero cells.

Summary of results obtained for Cytotoxicity

The tables below show the summative effects of the two crude extracts and 7 fractions of *T. elegans* on HeLa (Table 4.7) and Vero cells (Table 4.8).

Table 4.7: Summary results of the sample effect against the HeLa cell line.

Samples	cytotoxic*
FD1	Y
FD2	N
FD3	Y
FD4	Y
FM1	N
FM2	Y
FM3	N
C2	Y
C3	Y

Y- Yes; **N-** No. **FD1**- DCM fraction 1; **FD2**- DCM fraction 2; **FD3**- DCM fraction 3; **FD4**- DCM fraction 4; **FM1**- MeOH fraction 1; **FM2**- MeOH fraction 2; **FM3**- MeOH fraction 3; **C2**- dichloromethane crude extract & **C3**- methanol crude extract

Table 4.8: Summary results of the sample effect against the Vero cell line.

Samples	cytotoxic*	necrotic**
FD1	N	N
FD2	N	N
FD3	Y	N
FD4	Y	N
FM1	N	N
FM2	Y	N
FM3	N	N
C2	Y	N
C3	Y	N

Y- Yes; **N-** No. **FD1**- DCM fraction 1; **FD2**- DCM fraction 2; **FD3**- DCM fraction 3; **FD4**- DCM fraction 4; **FM1**- MeOH fraction 1; **FM2**- MeOH fraction 2; **FM3**- MeOH fraction 3; **C2**- dichloromethane crude extract & **C3**- methanol crude extract

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Chapter 5

CONCLUSION AND RECOMMENDATIONS

5.1 CONCLUSION

The overall objective of this study was to evaluate the cytotoxic, anti-inflammatory, antibacterial and anti-HIV RT effects of *T. elegans* leave crude extracts and fractions. This was achieved by setting up a set of secondary objectives as follows:

Objective 1 was to obtain fractions from *T. elegans* leave crude extract using column chromatography; DCM and MeOH extracts yielded 4 and 3 fractions respectively. FD3 (EA and MeOH) and FM3 (MeOH and EA) resulted in the highest yield of 57 and 23% respectively.

Objective 2 was to determine the antibacterial activity of the fractions together with the crude extracts using the Minimum Inhibitory Concentration assay (MIC) and the Minimum Bactericidal Concentration (MBC). The result indicated that all microorganisms tested against in this study were susceptible to *T. elegans* fractions. Most importantly, FD4 exhibited a potent antibacterial activity against all tested bacterial strains with an MIC and MBC of 0.625 mg/mL.

Objective 3 was to evaluate the HIV-1 reverse transcriptase effect of the crude extracts and fractions using RT ELISA kit. The result indicated that the tested fractions together with the crude extracts showed activity against HIV-1 RT.

Objective 4 was to determine the anti-inflammatory activity of the fractions by measuring the nitric oxide (NO) inhibition in tissue culture; *T. elegans* exhibited a potent NO inhibition with a low toxicity making it a potential promising anti-inflammatory agent. Since, FD3, FM2, C2 and C3 exhibited strong NO inhibition with the corresponding mild toxicity and FM3 resulted in a weak NO inhibition with a corresponding weak toxicity.

Objective 5 was to evaluate the cytotoxicity effects of the plant crude extracts and fractions towards Vero and HeLa cells. FD3, FD4, FM2, C2 and C3 showed a strong toxicity against the cancer cell line (HeLa), however these fractions showed moderate toxicity toward the non-cancer cell line (Vero). Amazingly, FD1 displayed a strong toxicity against HeLa and none toward the Vero cells. *T. elegans* leaves displayed a

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selective toxicity that is being more toxic to the human cervix cancer cell line and less or non-toxic toward the Vero cell line. It is worth mentioning that most anti-HeLa compounds were obtained from the DCM extract (FD1, FD2 and FD4), whereas only one fraction (FM2) acquired from MeOH extract was found to be potent against HeLa cells.

Lastly, the study aimed at evaluating the qualitative antioxidant potential of the extracts and fractions by DPPH assay. The results revealed that FM3 exhibited the most pronounced free radical scavenging activity.

In conclusion, the study showed that *T. elegans* leaves revealed various phytochemical constituents which attributed to all its pharmacological activities shown in this study. Moreover, fractions appeared to be better than working with the whole crude extract. Since: firstly, the crude extracts tested in this study failed to show a better selective toxicity principle between cancerous (HeLa) and non-cancerous cell (Vero), whereas the FD1 at the highest concentration managed to achieve this principle; Secondly, FM3 showed a more pronounced antioxidant activity compared to the extracts; Thirdly, FD4 displayed a potent antibacterial activity against all tested bacterial strains. Hence, making this plant an effective candidate of multiple novel drug development which can be used in prevention of free radical disorders. However, despite DCM fractions being effective in all tested assays some showed to be toxic against macrophages and Vero cells. Therefore, the identification of the compound responsible for this activity is of high value. Nonetheless, with all that has been mentioned we accept the hypothesis that *T. elegans* leaves and fractions are effective against the selected opportunistic microorganisms, have anti-inflammatory and anti-HIV properties and have selective toxicity to cells.

Therefore, the null hypothesis (Ho) of the study is rejected.

5.2 SHORTCOMINGS OF THE STUDY

5.2.1 ANTIBACTERIAL ASSAY

Working with high concentrations. The initial working concentration was too high resulting in the lowest concentration in this assay to be 0.625 mg/mL of which most of

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the fractions which exhibited the MIC value of 0.625 mg/mL we anticipate that they might have had exceeded this lowest MIC concentration. Hence, much lower starting concentration is considered for the manuscript in preparation.

5.2.2 ANTI-HIV-1 RT

The assay was based only on qualitative aspect due to technicality. For accurate purposes a quantitative evaluation of HIV-1 RT is of paramount. Therefore, quantitative evaluation of this assay will be repeated in preparation for the manuscript.

5.3 RECOMMENDATIONS

In vitro studies bear little resemblance to the actual diseased tissue environment, which is a complex combination of chemicals, regulating signals generated by the living, injured and dying cells. The current study was successful in justifying the toxicity, anti-inflammatory, antioxidant, antibacterial and anti-HIV-RT activities of the plant compounds. The following recommendations for further studies and research are necessary:

- ✓ A thorough fractionation and characterization of *T. elegans* leave extract.
- ✓ Identification of individual compounds that should be tested in pharmacological assays.
- ✓ Also, a correlation between the phytochemicals and the pharmacological activities needs to be established.
- ✓ As much as it was important to evaluate *T. elegans* leave for cytotoxicity, it is also important to screen it for genotoxicity.
- ✓ *In vivo* studies and more clinical trials are of high value since a body is composed of a complex physiological process. Therefore, there is a need of understanding the mechanism of action bioactive compounds in the body.

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APPENDIX-A

The column was eluted with Hex (100%) followed by the increasing gradient of eluting (Figure 3.2):

DCM-ex

F1 Hex (50%): DCM (50%)

F2 DCM 100%

F3 DCM (50): EA (50%)

F4 EA 100%

F5 EA (90%): MeOH (10%)

F6 EA (80%): MeOH (20%)

F7 EA (70%): MeOH (30%)

F8 EA (60%): MeOH (40%)

F9 EA (50%): MeOH (50%)

F10 EA (10%): MeOH (9650%)

F11 EA (20%): MeOH (80%)

F12 EA (30%): MeOH (70%)

F13 MeOH 100%

MeOH ex

F1 Hex (50%): EA (50%)

F2 EA 100%

F3 EA (90%): MeOH (10%)

F4 EA (80%): MeOH (20%)

F5 EA (70%): MeOH (30%)

F6 EA (60%): MeOH (40%)

F7 EA (50%): MeOH (50%)

F8 EA (10%): MeOH (90%)

F9 EA (20%): MeOH (80%)

F1 0 EA (30%): MeOH (70%)

F11 MeOH 100%

Figure A 1: The detailed increasing polarity elution pattern which followed the 100% n-Hex elusion.

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The TLC chromatogram of the *T. elegans* leaf extracts and its fractions stained with 10% sulphuric acid produced a multiple number of colourful bands namely: pink, yellow, green, lemon, light brown and dark brown (Figure 4.4). Lane 5 exhibited an interesting band. Lane 6, 7 and 8 exhibited similar pronounced pink bands and 9, 10, 11 and 12 also exhibited the similar pink bands, however on lane 12 the band is faint. All fractions with similar compounds were combined.

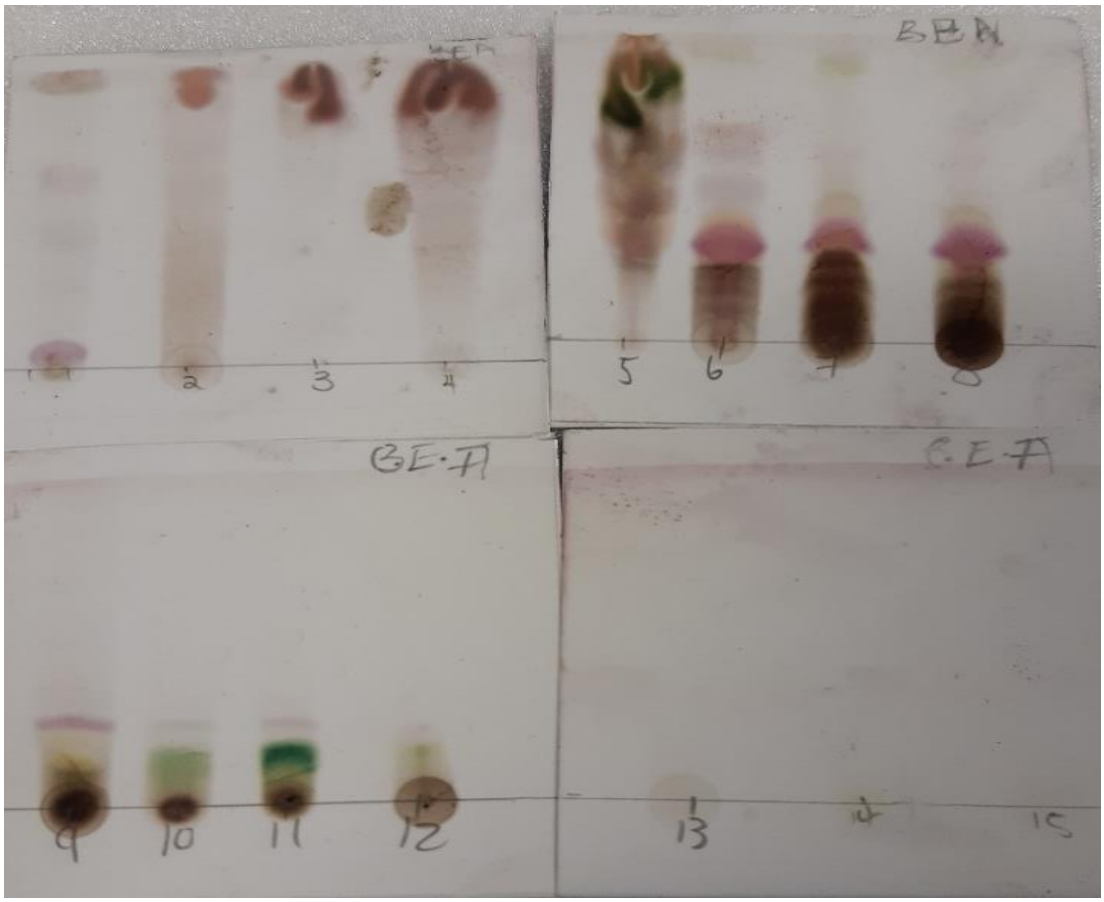

Figure A 2: DCM crude extract and fractions spotted on TLC plates sprayed with 10% sulphuric stain, to visualize different bioactive compounds.

All plates were developed with BEA solvent system. 1- DCM extract, 2-12 represent fraction 1 to 11 respectively.

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